

THE ROLE OF NON-STARTER BACTERIAL CONSORTIA IN MOULD-RIPENED CHEESE

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ABSTRACT

Stichelton is a blue-veined raw milk cheese which is made following the Stilton cheese making process. In a previous study, a preliminary examination of its microflora during production was examined by traditional culture methods and initial PCR DGGE profiling. The aim of this study was to complete the profiling of Stichelton cheese and examine the contribution of its microbiota components to product characteristics.

Stored samples of cheese production isolates were sequenced and in addition whole population DNA was extracted directly from a fully ripened Stichelton cheese (12 weeks) and from bulk cell suspensions collected on various media. The V3, V4V5 and V6V8 regions of 16S rDNA were amplified by PCR and separated by DGGE using 20 – 80 % urea formamide denaturing gradient. While *Lactobacillus casei/paracasei*, *Staphylococcus equorum*, *Bacillus* sp., *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were only found by the molecular method, traditional culture detected a large number of potentially raw milk microbiota.

Lactococcus lactis was detected in the raw milk sample and along the process by both methods. The *L. lactis* subsp. *lactis* which was detected in the core of matured Stichelton was shown by PFGE to be from the raw milk and not the starter culture used in Stichelton production.

S. equorum was found in the crust of cheese pre-piercing and in all parts of the cheese post-piercing by the molecular approach only. This suggested this organism was introduced originally via handling. Five *S. equorum* isolated from Stilton, Danish Blue and Reblochon could grow up to 10% salt but did not tolerate low pH levels suggesting *S. equorum* in Stichelton must have been introduced by

handling before or during ripening as if it was present in the early fermentation then it would die as fermentation progressed due to pH sensitivity.

A model cheese system made with commercial UHT milk was used to examine the interaction between mixed *Lc. lactis*, *P. roqueforti* and *S. equorum* B2 isolated from the Stilton crust. *S. equorum* was either added 1.5 h after the addition of *L. lactis* or it was smeared on the surface of the cheese immediately after un-moulding. The viable counts and pH were analysed throughout the process, while texture, water activity and flavour volatiles using GCMS SPME were determined for one month ripened cheeses only. The results showed *S. equorum* survived in the cheese following either method of introduction and that in cheeses without *P. roqueforti* addition, the presence of incorporated and surface-spread *S. equorum* could inhibit the surface growth of a contaminant *Penicillium*. It also slowed the growth of starter *P. roqueforti* in cheeses made with this mould. A paler coloured crust, firmer textured cheese and a low amount of alcohols were shown in the model cheeses made with surface-smear *S. equorum*. Conversely, addition of *S. equorum* in the initial process made the cheese core softer and produced low amounts of acids. Ethanol, 3-methyl-1-butanol and 2-pentanone were the main flavour compounds in the model cheeses examined.

The antifungal activity of the isolate was confirmed in laboratory media. Its ability to prevent *Penicillium* surface growth could be beneficial for white cheeses where this is an undesirable flaw. The results showed that the sporulation inhibitory effect on *P. roqueforti* was because of an antifungal agent produced by *S. equorum*, but it was not acid, bacteriocin or H₂O₂. Further study is needed to detect the antifungal agent. Overall, the study has expanded the understanding of the role non-starter bacteria may have in contributing to cheese ripening.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BLUE CHEESES

Blue cheeses are cheeses characterized by the growth of *Penicillium roqueforti* giving them their typical appearance and flavour (Cantor *et al.*, 2004). Cabrales from Spain, Danablu (Danish Blue) from Denmark, Gorgonzola from Italy, Roquefort from France are a few examples of blue cheeses with protected designation of origin (PDO)/protected geographical indication (PGI).

In the UK, the most strictly prescribed blue-veined cheese is Stilton. It is a PDO product of Derbyshire, Leicestershire and Nottinghamshire. It is also a blue-veined cheese in which milk is pasteurised at the first stage. There are six dairies licensed to produce Stilton Cheese which are Colston Bassett Dairy, Cropwell Bishop, Hartington Creamery, Long Clawson Dairy, Tuxford and Tebbutt Creamery and Websters (http://www.stiltoncheese.co.uk/the_stilton_producers). Over 400 tons of Stilton are produced each year per producer (personal communication with Randolph Hodgson, Neil's Yard Dairy). However, texture and flavour of the cheeses can vary among the producers or among different batches of the same producer.

1.2 STICHELTON: A BLUE-VEINED RAW MILK CHEESE

Stichelton cheese has been introduced by Joe Schneider and Randolph Hodgson of Neal's Yard Dairy since March 1990. It is a blue-veined raw milk cheese made by the same process as Stilton. This cheese is produced at a rate of about 60 tons a year (personal communication with Randolph Hodgson).

Like Stilton, fully matured Stichelton cheese has the same texture and appearance which has a creamy white curd, open texture with blue-green well-distributed veins, rough brown crust and typical blue cheese flavours (Robinson and Wilbey, 1998). Another similarity of these two cheeses is the lactic cheese starter cultures which are composed of mixed strains of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *P. roqueforti*.

1.3 STILTON/STICHELTON STARTER CULTURES

1.3.1. A MIXED OF *LACTOCOCCUS LACTIS*

Lactococcus lactis is a common lactic acid bacterium (LAB) in milk and dairy products. It has been chosen as a starter culture for its role in influencing final organoleptic properties by sugar fermentation, protein hydrolysis, flavour compounds and texturing component synthesis, as well as production of inhibitory components (Mäyrä-Mäkinen and Bigret, 1998). The growth of *Lc. lactis* also creates optimal biochemical conditions for cheese ripening (Smit *et al.*, 2005).

In 1919, Orla-Jensen differentiated *Lc. lactis* subsp. *cremoris* based on its characteristic of forming longer chains of coccus than the subspecies *lactis*. Schleifer *et al.* (1985) then distinguished *Lc. lactis* subsp. *cremoris* by its inability to grow at 40°C, in 4% NaCl, hydrolyze arginine and ferment ribose. Nomura *et al.* (1999) also studied novel characteristics for distinguishing these two subspecies, and the results showed that *Lc. lactis* subsp. *lactis* is able to produce γ -aminobutyric acid, whereas *Lc. lactis* subsp. *cremoris* does not. Indeed, *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* is distinguished by the ability to produce diacetyl (buttery flavour) and acetoin (fruity flavour).

According to Marshall (1987), the biovar. *diacetylactis* produces D-lactate and it is heterofermentative, while subsp. *lactis* and subsp. *cremoris* produce L-lactate and they are homofermentative so that when used as cheese starters, these produce different flavour compounds. Figure 1.1 shows the biochemical reactions during cheese ripening. Figure 1.2 shows more detail of pyruvate catabolism and the pathways to create lactate or diacetyl and acetoin. Lactate metabolism in homofermentative and heterofermentative pathways can be observed in Figure 1.3.

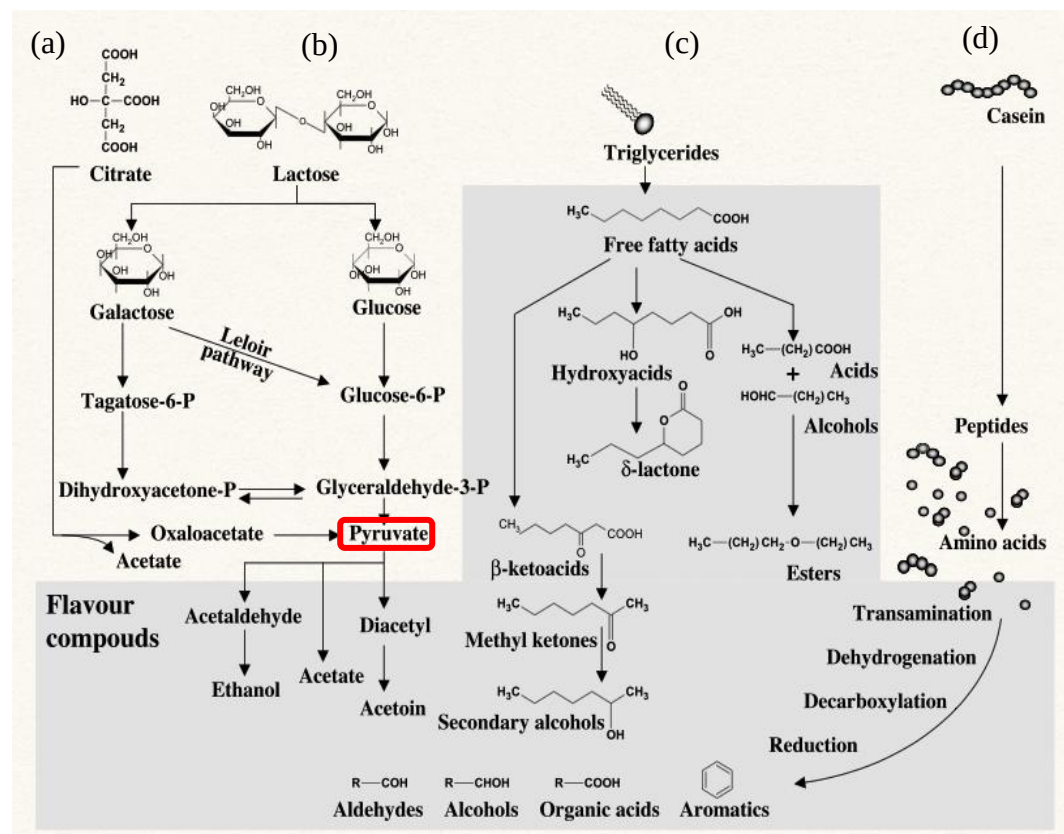


Figure 1.1. Biochemical reactions during cheese ripening: (a) catabolism of citrate, (b) catabolism of lactose (glycolysis), (c) catabolism of free fatty acids (lipolysis) and (d) catabolism of amino acids (proteolysis) (Button and Dutton, 2012).

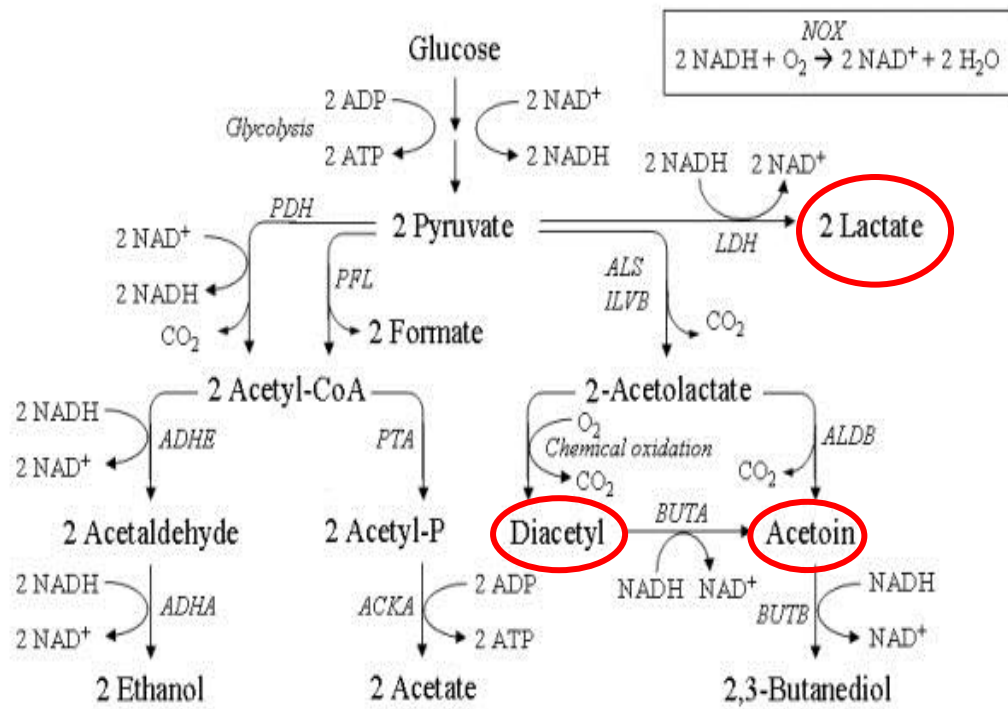


Figure 1.2. The pyruvate catabolism of *Lactococcus lactis* (Oliveira *et al.*, 2005).

LDH, lactate dehydrogenase;

PDH, pyruvate dehydrogenase complex;

PFL, pyruvate formate-lyase;

ADHE, acetaldehyde dehydrogenase;

ADHA, alcohol dehydrogenase;

PTA, phosphotransacetylase;

ACKA, acetate kinase;

ALS/ILVB, catabolic and anabolic 2-acetolactate synthase;

ALDB, acetolactate decarboxylase;

BUTA, diacetyl reductase;

BUTB, acetoin reductase;

ATP, adenosine triphosphate;

ADP, adenosine diphosphate;

NAD⁺, nicotinamide adenine dinucleotide;

NADH, nicotinamide adenine dinucleotide plus hydrogen;

NOX, NADH oxidase.

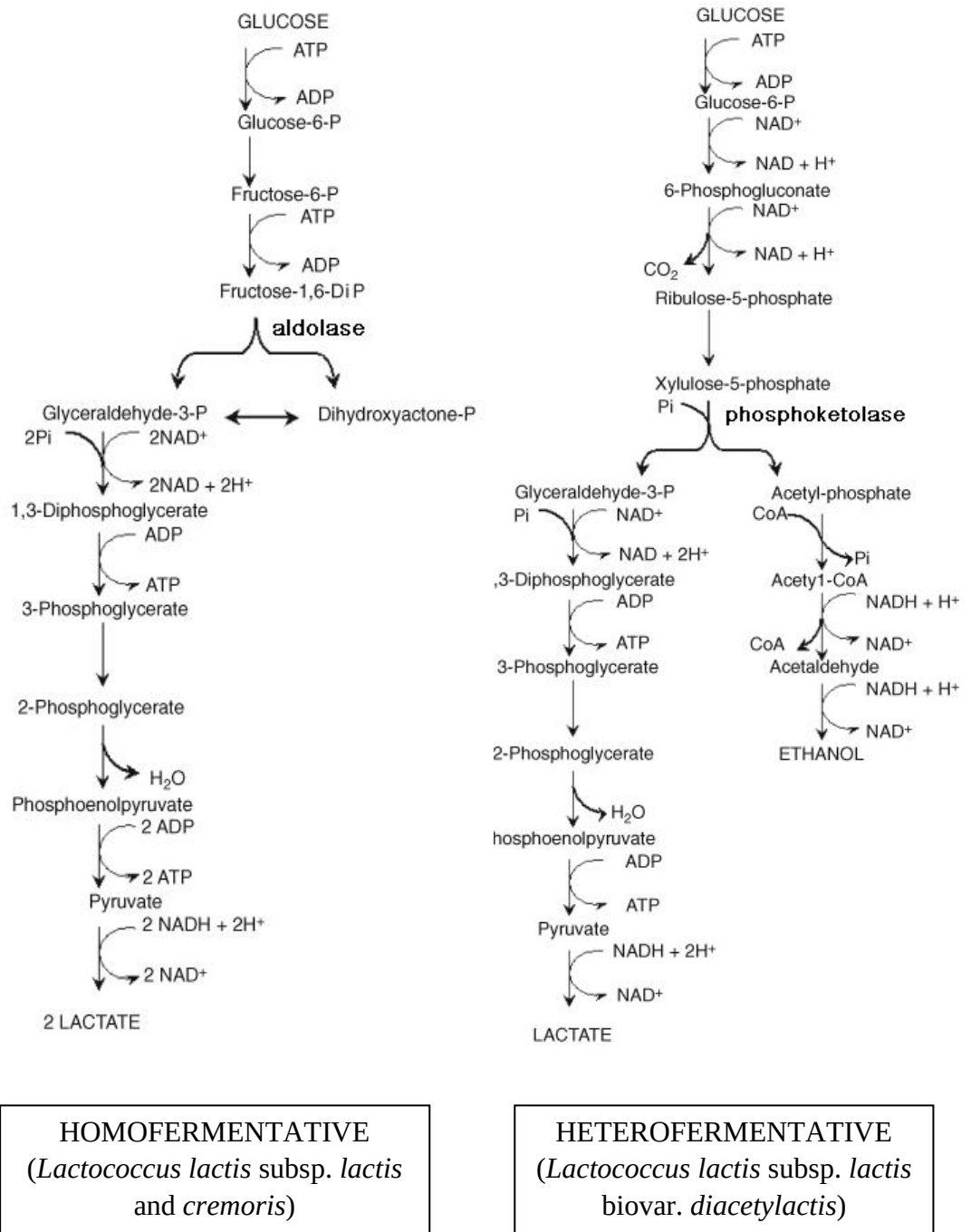


Figure 1.3. Lactate metabolism in homofermentative and heterofermentative pathways (Todar, 2012).

Besides its contribution to flavour, lactic acid produced by *Lc. lactis* also has an important role in reducing pH and then coagulating the milk protein casein, which is done in combination with rennet. These impact on cheese texture. According to Todar (2012), *Lc. lactis* subsp. *cremoris* strains LM0230 and MG1363 are usually used in hard cheese production whereas *Lc. lactis* subsp. *lactis* strain IL1403 is preferred for soft cheese production. This is because the *cremoris* strains yield a slower pH drop and are easier to control both with temperature and salt. Less acid defects (too much lactate) are a result. Also, the lower populations as well as perhaps proteinase level and specificity tend to result in less bitterness. Therefore, *cremoris* strains are a better choice for cheeses that will be ripened (typically hard cheeses). On the other hand, if that is not a concern, the *lactis* strains are preferred (Personal communication with Dr. Kenneth Todar and Prof. James L. Steele from Department of Food Sciences at University of Wisconsin-Madison, USA).

1.3.2. *PENICILLIUM ROQUEFORTI*

Penicillium roqueforti grows in the presence of 25% carbon dioxide and 0.3 – 21% oxygen. The growth of *P. roqueforti* was increased by the presence of *D. hansenii* when incubated at 25% carbon dioxide and 0.3% oxygen. *D. hansenii* caused a weak inhibition of *P. roqueforti* growth at 21% oxygen, but it did not affect the *P. roqueforti* sporulation. *Geotrichum candidum* can cause inhibition of growth and sporulation of *P. roqueforti* at 25% carbon dioxide while *P. caseifulvum* caused an inhibitory effect of the growth of *P. roqueforti* at 21% oxygen (van den Tempel and Nielsen, 2000).

In 1997, Hansen and Jakobsen investigated the interaction between starter lactic acid bacteria and *P. roqueforti*. The result showed that the lactic acid

bacteria stimulate *P. roqueforti* growth by releasing amino acids like arginine and leucine. It was important to note that interactions were highly affected by medium used and positive interactions increased with increasing NaCl concentrations (up to 10%).

Interaction between the yeasts *Candida famata*, *C. sphaerica*, *C. kefir*, *C. lipolytica* and *P. roqueforti* was examined by Juszczuk *et al.* (2005). The results showed that interactions were strain dependent and affected by a_w . Most of the yeasts slowed down the rate of vegetative growth of *P. roqueforti* strains, delayed the process of sporulation (by 1 – 3 days) and lowered the conidia number/petridish (by 1 – 10 %). However, only strains of *C. lipolytica* completely inhibited the growth and sporulation of *P. roqueforti* at a_w 0.995.

1.4 STAPHYLOCOCCUS EQUORUM AS A STILTON/ STICHELTON NON-STARTER CULTURE

Staphylococcus equorum is a part of the coagulase-negative staphylococci (CNS) group which are facultative anaerobes (Blaiotta *et al.*, 2004; Beresford and Williams, 2004). There are two subspecies of this bacterium, *equorum* and *linens*. *S. equorum* subsp. *equorum* has been isolated from horses, goats, mastitic-cow milk, human clinical materials and French smear-ripened cheese (Schleifer *et al.*, 1985; Meugnier *et al.*, 1996; Bockelmann, 2002; Alcaráz *et al.*, 2003). *S. equorum* subsp. *linens* was isolated from Swiss smear-ripened cheese (Place *et al.*, 2003). *S. equorum* was also identified from Stilton cheese (Ercolini *et al.*, 2003).

S. equorum is a well-known starter culture in traditional sausage and ham production (Leroy *et al.*, 2009; Landeta *et al.*, 2011). According to Carnio *et al.*, (2000), it could inhibit *Listeria monocytogenes*. This bacterium has also important

roles in nitrate reduction which produces the red colour of cured and fermented meats and peroxide decomposition preventing rancidity (Liepe, 1983).

In Tilsit cheese, *S. equorum* is an important bacterium for the growth of smear bacteria at early ripening. The combination of *S. equorum* and *Debaryomyces hansenii* stimulates the growth of salt-acid-tolerant bacteria by growing faster and increasing the pH (Figure 1.4) which could inhibit moulds on the surface of semi-soft smear cheese. The brines are the source of staphylococci (Bockelmann, 2002; Jaeger *et al.*, 2002).

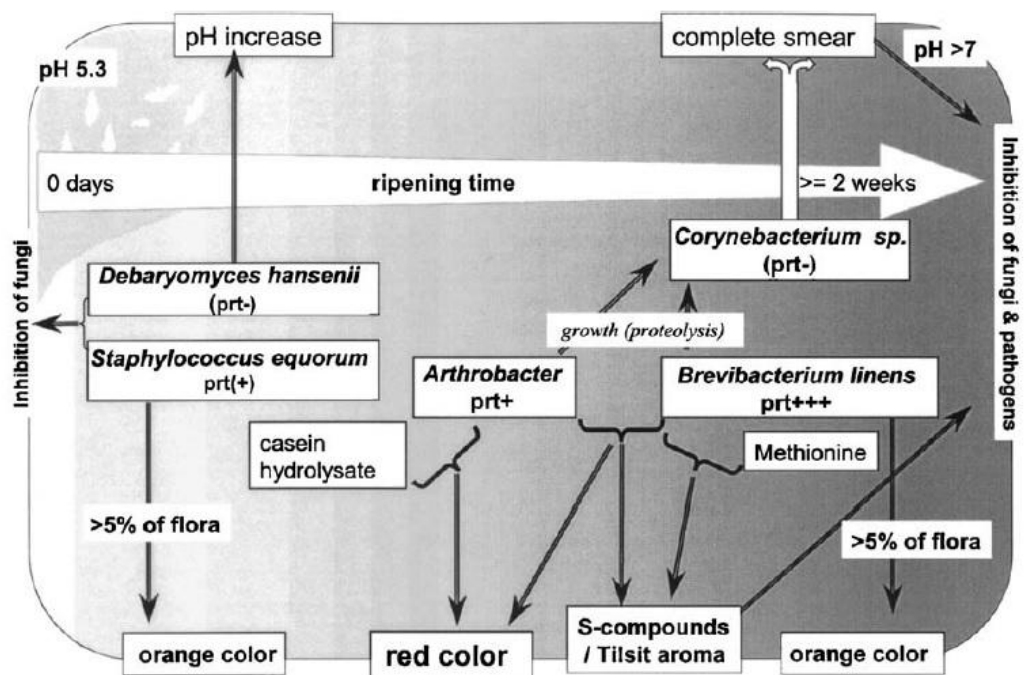


Figure 1.4. Surface ripening of smear-ripened Tilsit cheese (Bockelmann, 2002).

S. equorum subsp. *equorum* contributes to aroma compound production. The genome sequence of *S. equorum* subsp. *equorum* Mu2 has been examined (Irlinger *et al.*, 2012). Based on the sequence (accession number CAJL01000001 to CAJL01000030), it does not encode any pathogenicity genes such as are found in *Staphylococcus aureus*. It does not have any genes to degrade galactose by the

tagatose-6-phosphate pathway, but it has genes encoding the enzymes of the Leloir pathway (Figure 1.5) which make this species different from most staphylococci such as *S. saprophyticus* and *S. intermedius*.

S. equorum subsp. *linens* was firstly described by Place *et al.* (2003). It indicated a very close similarity with the subsp. *equorum*. The use of this bacterium in the production of semi-hard surface-ripened Küssnachter cheese could inhibit *Escherichia coli*, *Listeria monocytogenes* and *S. aureus*.

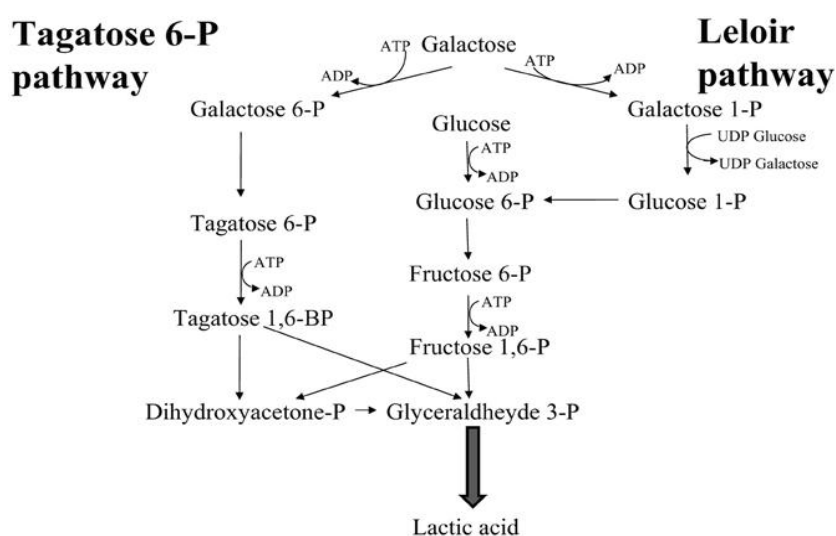


Figure 1.5. Galactose metabolism in LAB (Pessione, 2012).

1.5 ANTIMICROBIAL AGENTS

In microbial interactions, organic acids, fatty acids, cyclic dipeptides, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antibiotic or bacteriocin are produced as antimicrobial agents. Antibiotics or antibacterials are a type of antimicrobial which is active against bacterial infections (European Centre for Disease Prevention and Control (ECDC), 2015). Antibiotics exert their activity in different ways which are: i) damaging the cell wall; ii) destabilizing the cytoplasmic membrane; iii) changing the nucleic acid structure; iv) inhibiting

enzymatic action; v) antimetabolic action; and vi) affecting nucleic acid synthesis (Pelczar *et al.*, 1980).

Bacteriocins are proteins produced by bacteria that exert a lethal effect on other bacteria (Farlex, 2012). According to de Souza *et al.* (2005), bacteriocins have been characterized as molecules of high antimicrobial property even at low concentrations. Generally, these molecules present characteristics of stability to heat, low pH, refrigeration and freezing and resistance to weak organics solvents, salts and enzymes. On the other hand, they are very sensitive to proteolytic enzymes action.

Bacteriocins can be grouped into Lantibiotics (class I) which are small peptides (<5 kDa), low-molecular-weight antibiotic-like substances (class II) and high-molecular-weight (100 to 500 kDa) bacteriocins (classes III and IV) (Klaenhammer, 1993). Class III bacteriocins are defined as large heat labile proteins, while class IV bacteriocins have a complex structure and contain lipid or carbohydrate moieties (Fan and Song, 2013). Different characteristics between antibiotics and bacteriocins can be observed in Table 1.1.

A wide variety of bacteriocins has been isolated from LAB including *Lb. plantarum* (van Reenen *et al.*, 1998), *Lb. helveticus* (Bonade *et al.*, 2001), *Lb. delbrueckii* subsp. *lactis* (Boris *et al.*, 2001) and *Lc. lactis* (Bromberg *et al.*, 2004). The bacteriocin nisin produced by *Lc. lactis* has a bactericidal effect on Gram-positive bacteria including *Clostridium* and *Bacillus* spp. (Kojic *et al.*, 1991). Otaka and Kaji reported antibiotic micrococcin P₁ from *S. equorum* WS 2733 in 1974. *P. roqueforti* also produces the mycotoxins roquefortine C, patulin, penicillic acid and mycophenolic acid (Erdogan *et al.*, 2003).

Table 1.1. Characteristic aspects of bacteriocins and antibiotics.

Characteristics	Bacteriocins	Antibiotics
Application	Foods	Clinical
Synthesis	Ribosomally	Secondary metabolism
Activity	Limited spectrum	Wide spectrum
Presence of immune cells in the host	Present	Absent
Mode of action	Mostly through channel formation in the cell cytoplasmic membrane	Specific target
Toxicity/other effects in eukaryotic cells	Absent	Present

Source: de Souza *et al.*, (2005).

1.6 PREVIOUS WORK ON STILTON CHEESE

The correlation between the microflora and the quality of Stilton cheese was studied by Whitley (2002) using culture-based techniques. *Lb. plantarum* and *Lb. curvatus* were dominant species in good quality cheese, whereas *Lb. brevis* was associated with poor quality cheese, indicated by the presence of fruity off flavours. In a study of yeasts, *Candida sphaerica* was the dominant yeast in good quality cheeses, *C. famata* and *C. lipolytica* were dominant yeasts in poor quality cheeses, whereas *C. catenulata* was present in both good and poor quality cheeses.

Ercolini *et al.* (2003) used combined molecular and culture-based approaches to examine the bacterial community structure and location in Stilton cheese. The viable counts showed that the numbers of lactococci, LAB, staphylococci and psychrophilic microflora appeared to be quite similar, which was around 8 log₁₀ CFU/g except for the *Lactobacillus* counts on Rogosa agar which were 1 log CFU/g lower. The bacterial species found by 16S rDNA

polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis in Stilton cheese can be seen in Table 1.2.

Table 1.2. Bacterial species found in Stilton cheese using 16S rDNA PCR-DGGE analysis using sets of V3F-V3R and V4F-V5R primers.

Origin	Closest Relatives (% Identity)	
	V3	V4V5
Stilton finger print	<i>Lc. lactis</i> (99)	<i>Lc. lactis</i> (99)
	<i>E. faecalis</i> (96)	<i>Staphylococcus</i> sp. (99)
	<i>Lactobacillus curvatus</i> (100)	<i>Leuconostoc mesenteroides</i> (99)
	<i>S. equorum</i> (99)	<i>S. equorum</i> (100)
	<i>Lactobacillus plantarum</i> (100)	
Bulk cells on M17 agar (dilution, 10^{-7})	<i>E. faecalis</i> (96)	<i>E. faecalis</i> (100)
	<i>Staphylococcus</i> sp. (99)	<i>Staphylococcus</i> sp. (99)
	<i>S. equorum</i> (99)	<i>S. equorum</i> (100)
		<i>Leuconostoc mesenteroides</i> (99)
Bulk cells on MRS agar (dilution, 10^{-7})	<i>E. faecalis</i> (98)	<i>Leuconostoc mesenteroides</i> (100)
	<i>Staphylococcus</i> sp. (99)	<i>Staphylococcus</i> sp. (99)
	<i>S. equorum</i> (100)	<i>S. equorum</i> (100)
	<i>Lactobacillus plantarum</i> (100)	
Bulk cells on anaerobic MRS agar (dilution, 10^{-7})	<i>S. equorum</i> (100)	<i>Leuconostoc mesenteroides</i> (100)
	<i>Lactobacillus plantarum</i> (100)	
Bulk cells on Rogosa agar (dilution, 10^{-6})	<i>Lactobacillus plantarum</i> (100)	<i>Lactobacillus plantarum</i> (100)
	<i>Lactobacillus curvatus</i> (100)	
Bulk cells on MSA agar (dilution, 10^{-6})	<i>S. equorum</i> (100)	<i>S. equorum</i> (100)
	<i>E. faecalis</i> (98)	
Bulk cells on NA agar (dilution, 10^{-6})	<i>S. equorum</i> (100)	<i>S. equorum</i> (100)
	<i>E. faecalis</i> (98)	<i>Leuconostoc mesenteroides</i> (100)

Source: Ercolini *et al.*, (2003).

Fluorescence *in situ* hybridisation (FISH) was used to identify the distribution of the bacteria within the cheese. The results revealed that different locations in Stilton cheese had a different bacterial community. In the blue veins, mixed colonies of *Lactococcus lactis*, *Lactobacillus plantarum* and *Leuconostoc*

mesenteroides were found whereas single colonies of *Lactococcus lactis* were found in the core. Also, *Lactobacillus plantarum* was detected only underneath the surface. In contrast, *Leuconostoc* was identified in all the cheese parts (Ercolini *et al.*, 2003).

A study on diversity and activities of yeasts from different parts of a Stilton cheese has also been published (Gkatzionis *et al.*, 2014). In this study, the crust had *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Candida catenulata*; the white core consisted of *P. roqueforti*, *D. hansenii* and *Kluyveromyces lactis*; and blue veins were composed of *P. roqueforti* and *Kluyveromyces lactis*.

From flavour studies on the ripening of Stilton cheese, free fatty acids, carbonyl compounds, methyl ketones, free amino acids, peptones and amino acid nitrogen are produced during cheese ripening (Madkor *et al.*, 1987a; Madkor *et al.*, 1987b). Gkatzionis *et al.* (2009) examined the aroma profiles of three different parts of Stilton cheese (blue veins, white core and outer crust) by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS), solvent extraction gas chromatography-mass spectrometry (GC-MS) and solid phase micro extraction with GC-MS (SPME GC-MS). The results showed that while the white core contained higher amounts of alcohols and aldehydes, the blue veins and the outer crust contained higher amounts of ketones. A recent study was on aroma production of Stilton yeast isolates in milk models (Gkatzionis *et al.*, 2014). The results revealed that the combination between yeast *Yarrowia lipolytica* and mould *P. roqueforti* produced higher amounts of associated alcohols and ketones. These productions were not related to the presence of *Lc. lactis*. A higher amount of branched alcohols was produced by other yeast isolates (*Kluyveromyces lactis*, *D. hansenii* and *Trichosporon ovoides*).

1.7 PREVIOUS WORK ON STICHELTON CHEESE

The fact that Stichelton is made from raw milk is likely to affect the flora that may be present in the final product and could potentially lead to more variability in quality characteristics of the cheese. From a preliminary study of Stichelton (Yunita, 2010), nine samples from different stages of Stichelton production namely milk from the tanks, starter cultures, samples from pre-milling, post-milling and salting, pre-piercing core and veins, pre-piercing crust (6 weeks), post-piercing core and veins and post-piercing crust (9 weeks) were collected. The final cheese (12 weeks) could not be evaluated due to a time constraint. Also, three samples of Stilton cheese made by three different producers were purchased at different local markets and used for comparative analysis. All samples were examined for total viable counting on nine different agar media (nutrient, brain heart infusion, M17, MRS, Rogosa, Baird Parker, mannitol salt, KF Streptococcus and Rose Bengal chloramphenicol). The isolates were examined for Gram reaction and 16S rDNA PCR-DGGE analysis. However, reamplification, purification and sequencing was done only for the starter culture isolates, the PCR-DGGE analysis of the samples was done only for starter culture, post-piercing core as well as crust and the flavour study of Stichelton cheese has not been characterised.

From the results of the culture-based technique, it was concluded that 9 weeks old Stichelton cheese is quite different from samples of mature Stilton cheese. Firstly, the total viable count of *Lactococcus* sp. in pre-matured Stichelton was higher than that of commercial Stilton. In the crust, pre-matured Stichelton contained $11 \log_{10}$ CFU/g *Lactococcus* sp., while the three commercial Stiltons contained *Lactococcus* sp. in the range 7-9 $\log_{10}$ CFU/g. In the core, while pre-matured Stichelton contained 8 $\log_{10}$ CFU/g *Lactococcus* sp., the *Lactococcus* sp.

counts in the commercial Stilton were lower by 1 log CFU/g. Secondly, the pH of pre-matured Stichelton cheese ranged between 6.70 in the white core and 6.32 in the outer crust. On the other hand, the pH of Stilton cheese in the crust was higher (6.50 – 7.10) than the pH of Stilton cheese in the white core (6.0 – 6.70) because of yeast and mould growth in the crust. Interestingly, although Stichelton cheese making employs mixed *Lactococcus lactis* strains, which was also supported by sequencing results, *Lactobacillus* sp. was the dominant flora during the production. *Lactococcus lactis* become a dominant microorganism in the samples taken three weeks before ripening.

1.8 PRODUCTION OF STICHELTON

There are several steps in the production of Stichelton cheese (personal communication with Joe Schneider, give address if not given before). The first step is pouring the raw cows' milk into the tank and adding starter cultures (1 ml of starter cultures (Danisco MM100) per 100 litres milk or 1.2 ml of starter cultures (Danisco MT36) per 100 litres milk) and the spores of the mould *Penicillium roqueforti*. The enzyme rennet (15 ml/100 litres) is also added. The mixture is incubated for 90 min later at 28°C. This step is known as coagulation.

After 6 h, the curds form and are cut by hand into small cubes (1 cm x 1 cm). This allows whey to drain out from the curds. The soft curds are then transferred manually by ladles into the second tank and left overnight.

The next stage is milling, a step where the curd is diced into smaller particles. Salt is then added at a rate of 2.5-3% by weight of curd. Then, the salted curd is loaded into plastic cylindrical hoops without pressing and placed on boards in the hastener (21°C) and is turned daily for five days to allow natural whey drainage. The effect of not pressing is that the cheese texture is not solid. This

gives the cheese its characteristic crumbly texture and allows the mould to spread through the whole inner core during growth.

On the fifth day, the outer surface of the cheese is smoothed by using knives; this process is known as 'rubbing up'. The aim of this stage is to seal the surface which allows a crust to form and also prevents entry of the air so the mould does not grow; this also allows the aerotolerant anaerobic microorganisms to start growing.

Cheeses are then kept at controlled temperature (18-21°C) and humidity (95% RH) for 4 days and are turned once a day. The cheeses are then moved to the ripening rooms (at around 10-12°C and 90% RH) for 3-5 weeks and this is where the outer crust fully develops through the growth of a succession of microorganisms. Fresh Stichelton is pierced twice by stainless steel needles between 6 and 7 weeks of age. This allows air into the cheese mass and causes the mould to grow and sporulate turning bluish and forming the characteristic blue veins. Finally, the cheese takes about 3-5 weeks to be fully matured. The flow diagram of the Stichelton cheese production can be observed in Figure 1.6.

1.9 IMPORTANCE OF THIS STUDY

The microorganisms of raw milk cheeses vary batch-to-batch because of various sources of contamination: the cow, handlers and milking environment. Preliminary studies showed that 9-weeks old Stichelton has a quite different microflora and pH from Stilton. However fully ripened cheeses were never examined and the full dynamics of the microflora changes were not investigated.



Figure 1.6. The flow diagram of Stichelton cheese production. Image by: (a) Schneider and Hodgson, (2015); (b) Swinscoe (2013); (c) Wright (2012); and (d) Kimbell (2011).

Therefore, in the current study, Stichelton cheese would be further examined by collecting the fully ripened cheese (12 weeks old). This sample was assessed using a traditional culture approach together with 16S rDNA PCR-DGGE analysis. In this way, the microorganisms which contribute to the way the cheese ripens and produce the characteristics of the product could be identified. The previous six samples were also analysed using the molecular technique only as the traditional culture approach was done in the preliminary study. This would allow cheese makers to predict what microorganisms are important along the process and this is necessary to determine its quality characteristics in order to obtain a more consistent product because colour defect, off flavours and soft curd are problems in blue cheese making.

Moreover, from the previous study on identification of microorganisms in Stichelton cheese production, *Lactococcus* sp. was detected in the milk and was present as the dominant bacterium in the white core and blue veins of the cheese as well as the outer crust during processing. However, the added starter cultures consist of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*. So, it is a question whether the *Lc. lactis* isolates surviving in a mature Stichelton are from the raw milk or the added starter cultures. If the surviving *Lc. lactis* is from the raw milk, this isolate could also be used as a starter culture. Therefore, survival of *Lc. lactis* isolates in a mature Stichelton was examined by sub-species differentiation of the isolates using pulsed-field gel electrophoresis (PFGE) and genomic plasmid profiling.

The study then focused on *Staphylococcus equorum* as it is a well-known starter culture in fermented meat products where it has important roles in colour and flavour formation (Landeta *et al.*, 2011). The presence of *S. equorum* in mature cheeses is also known (Meugnier *et al.*, 1996; Place *et al.*, 2003) although

it is not often an added starter culture. Based on the PCR DGGE study, it was detected in the outer crust of the cheese pre-piercing and in all parts of the cheese post-piercing. However, the role of this bacterium in Stichelton cheese or other blue cheeses was unknown. Therefore, *S. equorum* would be first isolated from Stichelton as well as Stilton, Danish Blue and Reblochon cheeses for comparative analysis. The isolates then were examined by PFGE to detect the genotypic diversity followed by analyzing the response of *S. equorum* isolates to acid and salt.

Finally, the interaction between a mixed starter *Lc. lactis* and *Penicillium roqueforti* and non-starter *S. equorum* isolated from the crust of Stilton cheese was investigated in cheese models to determine if *S. equorum* survives early fermentation or whether its presence was due to contamination during ripening as suggested by the PCR-DGGE study. The potential role of the latter in contribution to the microbial and physical characteristics as well as flavour profiles of the cheese models was also analysed.

1.10 OBJECTIVE

The main objectives in this study were to complete the microbial profiling of Stichelton cheese and to determine the role of its microbiota components in general, and that of the non-starter *S. equorum* in particular, to blue cheese characteristics.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEESE COLLECTION

The fully matured Stichelton cheese (12 weeks ripening) was collected from Welbeck Farm in Nottinghamshire and was kept at -20°C until used. The sample was separated into the outer crust, the inner core and blue veins. This sample was then processed together with samples which were collected in the preliminary study (pre-milling, post-milling and salting, pre-piercing (6 weeks old) and post-piercing (9 weeks old); Yunita, 2010) and stored at -80°C. All samples were thawed at room temperature (approx. 20°C) for 30 minutes before analysis.

2.2. CULTURE MEDIA

M17 agar, de Man Rogosa Sharpe (MRS) agar, Rogosa agar (ROS), Baird Parker agar (BP), KF Streptococcus agar (KFSA), Rose Bengal chloramphenicol agar (RBCA), nutrient agar (NA) and brain heart infusion (BHI) were used for microbial culturing. All media were obtained from Oxoid Ltd, Hampshire, UK except NA which was obtained from Acumedia, Neogen Europe Ltd., Scotland, UK. Also, bacteriological agar (Oxoid, 1.5% (w/v)) was needed to prepare BHI agar. All prepared media were stored in a cold room at 4°C until used. The media preparation details can be seen in Appendix 1.

2.3 MICROBIAL ISOLATION

The cheese samples (25 g) were weighed into a stomacher bag and diluted in quarter-strength Ringer's solution (225 ml) and homogenized in a stomacher at 230 RPM for 2 min (Stomacher 400 Circulator, Seward, West Sussex, UK). This sample was considered as the 10^{-1} dilution. Further 10-fold dilutions were prepared with the same diluent up to 10^{-8} . Raw milk collected from the Stichelton farm (Welbeck Farm) and their commercial liquid starter culture (a mixed of three species of *Lc. lactis*) samples were serially diluted in quarter-strength Ringer's solution.

Samples (0.1 ml) of each dilution were inoculated in triplicate on selective media by spread plating. All plates were incubated under appropriate conditions (Table 2.1). Anaerobic conditions were obtained by using AnaeroGen kits (Oxoid). Each sample was also analyzed for pH using a pH meter (Camlab, Cambridge, UK) with the specific pH electrode for cheese samples (VWR International, UK, Cat. number 662-0084 (spear tip)).

Table 2.1. Conditions for the various media during incubation.

Medium	Incubation Temperature (°C)	Incubation Time (hours)	Environment	Target Microorganisms
BHI	30	48	Aerobic	Total aerobic counts
NA	30	48	Aerobic	Total aerobic counts
BP	30	48	Aerobic	<i>Staphylococcus</i>
M17	30	48	Aerobic	<i>Lactococcus</i>
KFSA	30	48	Aerobic	<i>Enterococcus</i>
RBCA	25	120	Aerobic	Yeasts and moulds
MRS	30	48	Anaerobic	Lactic acid bacteria
ROS	30	48	Anaerobic	<i>Lactobacillus</i>

2.4 VIABLE COUNT

After incubation, the plates with the number of colonies in the range from 30 to 300 colonies were counted using a colony counter (SC6 Stuart Colony Counter, Staffordshire, UK). The formula for deriving the viable count is described below:

$$\text{Number of cells/g in original sample} = \frac{\text{Number of cells on plate}}{\text{sample volume}} \times \frac{1}{\text{dilution factor}}$$

2.5 HARVESTING OF BULK CELLS

After counting, the 'bulk cells' were collected from the plates by adding 1 ml of sterile Phosphate Buffered Saline (PBS; Oxoid code BR 0014G) onto the confluent growth plates (10^{-1} dilution). All the cells were scraped off by using a spreader. Then, the cell suspensions were collected using pipette, put into 1.5 ml microcentrifuge tubes and kept at -80°C until used.

2.6 CHARACTERIZATION OF STICHELTON ISOLATES

To analyze the cultured populations, a total of 86 isolates (47 isolates from cheese and 39 isolates from raw milk) with different colony types were randomly picked from the eight media used. Each colony was streaked three times onto BHI agar to get a pure culture. The isolates were stored using the Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostic) at -80°C . The isolates were also determined for their ability to grow at 10°C for 4 days and 45°C for 2 days on BHI medium.

2.7 BIOCHEMICAL CHARACTERISTICS

2.7.1. GRAM STAINING

One colony was dispersed in a loopful of maximum recovery diluent (MRD; Oxoid CM0733) on the surface of a glass slide using a sterile loop. The glass slide was left to dry for about 15 min at room temperature (~20°C) and was heat fixed by passing through a bunsen flame three times. The Gram staining was done by treating the slide with crystal violet (Prolab PL8000) for 1 min, Lugol's iodine (Prolab PL8010) for 30 seconds, general purpose methyalted spirit (Fisher Scientific M/4450/17) for 1 min and carbol fuschin (Prolab PL8004) for 30 seconds. Between each stain, the slide was rinsed with tap water to remove the excess stain. At the end, the slide was allowed to dry for about 15 min at room temperature.

Slides were examined under a microscope (PBA Microscopes T250, England) at X100 oil-immersion magnification. Gram negative cells have a pink or red colour while Gram positive cells are purple. *Escherichia coli* and *Staphylococcus epidermidis* from The University of Nottingham, Division of Food Sciences strain collection were prepared as the Gram negative and positive controls.

2.7.2. CATALASE TEST

One drop of 30% (v/v) hydrogen peroxide (Fisher Scientific H/1800/15) was placed on a clean petridish. One colony from overnight M17 or MRS agars was smeared into the H₂O₂ drop using a sterile loop. Bubbles or froth forming within 10 s are considered as catalase positive. Otherwise, the colony is catalase negative. *Enterococcus faecalis* and *Escherichia coli* from The University of

Nottingham, Division of Food Sciences strain collections were prepared as the catalase negative and positive controls.

2.7.3. OXIDASE TEST

An overnight colony from M17 or MRS agars was touched using an Oxidase Detection Strip (Microbact MB0266A). A deep blue or violet colour observed within 5 sec indicates the strain was oxidase positive. *Escherichia coli* and *Pseudomonas fluorescens* from The University of Nottingham, Division of Food Sciences strain collections were prepared as the oxidase negative and positive control.

2.7.4. COAGULASE TEST

The coagulase test was used to confirm that the isolates were coagulase negative. The Staphylase test kit (Staphytect Plus Test, Oxoid DR0850) was used according to the manufacturer's instructions. The test (Oxoid DR0851) and control (Oxoid DR0852) reagents were placed at room temperature and mixed by shaking for about 10 s. One drop of each reagent was put onto separate circles of the reaction card (Oxoid DR500). Colonies (3-5) were mixed with the reagents using sterile disposable loops. The card was rocked gently. A positive result can be seen from agglutination within 20 s. *S. aureus* and *S. epidermidis* from University of Nottingham, Division of Food Sciences strain collection were used as positive and negative controls respectively.

2.7.5. STREPTOCOCCAL GROUPING TEST (GROUP D)

The isolates were grown on Columbia blood agar base (Oxoid CM0331) supplemented with 5% defibrinated sheep blood (Oxoid SR0051B) for 24 h at

37°C. Groupings were determined using a Streptococcal Grouping Kit (Oxoid DR0585) based on Lancefield serogrouping. In Lancefield test, Group D strains are *Enterococcus*. Five colonies were mixed in the *Streptococcus* extraction enzyme (400 µl; Oxoid DR593) which were reconstituted to 1X with sterile deionised water (SDW) in a sterile 1.5 ml microcentrifuge tube using a sterile disposable loop. The mixture was incubated at 37°C for 10 min. After 5 min incubation, the mixture was shaken vigorously for 2-3 s. The mixture was brought to room temperature for about 15 min. A drop of Latex Grouping Reagent D (Oxoid DR0589) was put onto circles of the reaction card (Oxoid DR500) and one drop of the mixture was added into it. The mixture was mixed using a sterile wooden mixing stick and the card was rocked gently. A positive result can be seen from agglutination within 30 s.

2.8 DENATURING GRADIENT GEL ELECTROPHORESIS

2.8.1. DNA EXTRACTION

A DNeasy[®]Mericon Food Kit (Qiagen) was used to extract DNA from cheese and bulk cell suspensions. The following procedure was used in accordance with some modification of the manufacturer's instructions. Briefly, the cheese sample (200 mg), milk sample (0.2 ml) and bulk cell suspensions (0.2 ml) was placed into a sterile 1.5 ml microcentrifuge tube. Then, 1 ml of Food Lysis Buffer (cetyltrimethylammonium bromide, CTAB) and 2.5 µl of proteinase K solution were added. The mix was incubated for 30 min at 60°C in a thermomixer (Eppendorf, Germany) at 1,000 RPM followed by cooling for 1 min and centrifuging at 10,000 g for 5 min. The clear supernatant (700 µl) was placed into a new tube containing 500 µl of chloroform (Fisher Scientific, UK). Then, the mix was vortexed for 15 s and centrifuged at 14,000 g for 15 min. The upper solution

(250 µl) and 1 ml of Buffer PB (5M guanidine hydrochloride and 30% isopropanol) were mixed thoroughly in a new tube by vortexing then transferred into the QIAquick spin column prior to centrifuging at 17,000 g for 1 min. Buffer AW2 (a low salt buffer, 500 µl) was added onto the column and this was centrifuged at 17,000 g for 1 min. The flow-through was discarded and the column was centrifuged again at 17,000 g for 1 min. Finally, the column was transferred into a new tube and 100 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added. The mix was incubated at room temperature for 1 min before it was centrifuged at 17,000 g for 1 min to elute the DNA.

DNA from microbial colonies and selected reference strains (Table 2.2) was extracted by a boiling procedure in a heating thermocycler to lyse the cells (Cocolin *et al.*, 2001). A single colony was resuspended in 200 µl of sterile distilled water and 10 µl of Proteinase-K (20 mg/ml; Fermentas). The mixed suspension was incubated at 65°C for 1.5 h followed by 10 min at 99°C in a Thermomixer Comfort (Eppendorf). It was then centrifuged at 4°C and 8,000 g for 5 min. Five microliters from the DNA solution was used as template. The DNA concentration and purity were then measured by Nano drop spectrophotometer (Ultrospec 2000 Pharmacia Biotech).

Table 2.2. Reference strains used in this study.

Reference Strains	Origin
<i>Lactococcus lactis</i> NCIMB 9918	NCIMB*
<i>Lactococcus lactis</i> NCIMB 8763	NCIMB
<i>Lactobacillus plantarum</i> NCIMB 138914	NCIMB
<i>Enterococcus faecalis</i> NCTC 775	NCTC**
<i>Leuconostoc mesenteroides</i>	Food Microbiology Laboratory, The University of Nottingham, UK

*NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK.

**NCTC: The National Collection of Types Cultures, Central Public Health Laboratory, London, UK.

2.8.2. MEASUREMENT OF DNA CONCENTRATION

DNA concentration in a sample (2 µl) was measured at a wavelength of 260 nm by using a Nano Drop ND-1000 spectrophotometer. The value was obtained by using the associated computer software (The NanoDrop ND-1000 V3.1.0 software, USA). Nuclease-free water (Fisher Scientific, 2 µl) was also needed to set the blank before and after DNA sample measurements and a piece of lens tissue was used to clean the surface of the machine. A ratio of absorbance at 260 and 280 nm (260/280) of 1.8 is generally accepted as pure for DNA, while a ratio of absorbance at 260 and 230 nm (260/230) of 2.0 - 2.2 values are for pure nucleic acid. Lower ratios indicate the presence of protein, phenol or other contaminants which absorb at 280 (for 260/280) and at 230 (for 260/230) (NanoDrop Technologies, Inc., 2007).

2.8.3. PCR AMPLIFICATION OF BACTERIAL 16S rDNA

PCR amplification was performed with DreamTaqTM Green PCR Master Mix (2x) (Fermentas, Yorkshire, UK) in a programmable heating thermocycler (Biorad C1000TM Thermal Cycler). The DreamTaqTM Green PCR Master Mix (2x) (Fermentas) already consists of DreamTaqTM DNA polymerase, optimized DreamTaqTM Green buffer, MgCl₂ and dNTPs. Nuclease-free water (23.3 µl; Fermentas), 0.1 µl of each primer (MWG-Biotech) and 1.5 µl of sample DNA were added into the master mix (25 µl) to give a final volume of 50 µl. Primers used in this study can be seen in Table 2.3. For DGGE analysis, a GC-clamp was attached to the 5' end of each forward primer (Muyzer *et al.*, 1993).

PCR amplification of the V3 region was performed as follows: 5 min at 94°C to denature template DNA, 10 cycles decreasing by 1°C each round from 66°C, 20 cycles at 56°C for primer annealing, 3 min at 72°C for primer extension

and then the final extension was carried out at 72°C for 10 min (Muyzer *et al.*, 1993; Ercolini *et al.*, 2003). PCR amplification of the V4 and V5 regions was carried out as follows: 3 min at 94°C to denature template DNA, 35 cycles each consisting of 60 s at 94°C, 60 s at 50°C, and 90 s at 72°C for primer extension and then the final extension was carried out 72°C for 4 min (Schwieger and Tebbe, 1998). The cycling programme for amplifying V6 and V8 regions was as follows: 5 min at 94°C, 35 cycles which each cycle includes 60 s at 94°C, 60 s at 63°C, and 60 s at 72°C for primer extension and then the final extension was carried out at 72°C for 5 min (Nübel *et al.*, 1996). PCR amplification of the 16S rDNA was carried out as follows: 5 min at 94°C to denature template DNA, 30 cycles each consisting of 60 s at 94°C, 45 s at 54°C, and 120 s at 72°C for primer extension and then the final extension was carried out at 72°C for 7 min (Weisburg *et al.*, 1991).

Table 2.3. PCR primers used in this study.

Primer	Sequence (5'-3')	Annealing Position**	Target Gene	Reference
V3F*	CCT ACG GGA GGC AGC AG	341-357	V3 (200 bp)	Muyzer <i>et al.</i> (1993)
V3R	ATT ACC GCG GCT GCT GG	518-534		
Com1*	CAG CAG CCG CGG TAA TAC	519-536	V4-V5 (400 bp)	Schwieger and Tebbe (1998)
Com2-Ph	CCG TCA ATT CCT TTG AGT TT	907-926		
968F*	AAC GCG AAG AAC CTT AC	968-984	V6-V8 (347 bp)	Nübel <i>et al.</i> (1996)
1401R	GCG TGT GTA CAA GAC CC	1385-1401		
fD1	AGA GTT TGA TCC TGG CTC AG	8-17	16S rRNA (1,500 bp)	Weisburg <i>et al.</i> (1991)
rD1	AAG GAG GTG ATC CAG CC	1540-1524		

*GC clamp: 5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Muyzer *et al.*, 1993).

**The segment of Bacterial 16S rDNA (*E. coli* numbering) (Brosius *et al.*, 1981).

The PCR product (5 µl) was checked by electrophoresis on a 1% TAE agarose (Agarose 3:1, Melford Laboratories Ltd., Suffolk, UK) gel containing ethidium bromide (0.2 µg/ml) in 1X TAE running buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 75 volts for about 45 min. To determine the size of the amplified DNA, a 100 bp molecular weight marker (Promega, UK) was used. The gel was imaged under a UV transilluminator (GelDoc XR, BioRad) and gel pictures were recorded using the GelDoc system (The Quantity One 4.6.5 Basic software, USA).

2.8.4. DGGE ANALYSIS

Samples of 200 bp size PCR products (using V3 primer sets) were analysed in 8% (w/v) polyacrylamide gels in 1x TAE buffer while those of 400 bp sizes (using V4V5 and V6V8 primer sets) were run in 6.5% (w/v) polyacrylamide gels (Ercolini *et al.*, 2003). A 2% (v/v) solution of glycerol was added into 6.5% polyacrylamide gels (Sigler and Pasutti, 2006) to increase gel flexibility when the gel is removed from the plates for staining or placement and positioning on the transilluminator so that the risk of tearing the gel could be decreased. Parallel electrophoresis was performed at 20 V for 10 min followed by 16 h at 50 V by using a Bio-Rad Dcode apparatus (Universal Mutation Detection System, USA). The gel contained 20-80% urea formamide for which 100% denaturant solutions consisted of 40% (v/v) formamide and 7 M urea. All DGGE reagents were from Severn Biotech Ltd., Worcestershire, UK. The gel was stained with GelStar (Lonza Rockland, USA) and the images recorded as before in Section 2.8.3.

2.8.5. DNA REAMPLIFICATION, PURIFICATION AND SEQUENCING

Selected bands were excised from the gel using a sterile scalpel and kept at 4°C for about 24 hours in 20 µl of TE buffer (10 mM Tris Base, 1 mM EDTA; pH 7) to elute DNA into the buffer. The eluted DNA (1.5 µl) was reamplified with the same PCR programme (Section 2.8.3) but without the GC-clamp in the respective forward primer. The PCR products (15 µl) were purified with Zymoclean Gel DNA Recovery Kit (The Epigenetics Company; Cambridge, UK) according to the manufacturer's procedures. Following purification, 15 µl of purified DNA was sequenced (MWG Laboratory, Germany). The DNA sequences were compared with those in public data libraries by using BLAST (Basic Local Alignment Search Tool) search programme (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.9 PULSE-FIELD GEL ELECTROPHORESIS

2.9.1. PREPARATION OF *LACTOCOCCUS LACTIS* ISOLATES

L. lactis was isolated from two commercial starter cultures used alternately by the cheese producers (liquid and powdered) and fully ripened Stichelton cheese (12 weeks). The added starter cultures contained *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*. The liquid starter culture provided by Joe Schneider was used directly for isolation, while powdered starter culture was prepared in UHT milk which had been heated at 32°C for 24 h before use following the manufacturer's procedure; 0.1 g starter culture (OV100, Orchard Valley Dairy Supplies, Worchestershire, UK) in 10 ml heated milk. For Stichelton cheese, the crust was also tested separately from the core and blue veins. The cheese samples were then processed as in Section 2.3.

All samples of each dilution were spread plated (0.1 ml in duplicate) and grown aerobically on M17 agar (Oxoid CM 0785) and anaerobically (AnaeroGen kit; Oxoid AN0035A) on MRS agar (Oxoid CM 0361) at 30°C for 2 days. Twenty five isolates were picked from each sample on each media agar and were restreaked twice to get pure isolates for further characterization (Gram stain as in Section 2.7.1, catalase test as in Section 2.7.2, oxidase test as in Section 2.7.3 and Streptococcal grouping test (Lancefield test) as in Section 2.7.5). Isolates with the following characteristics: Gram positive cocci, catalase negative, oxidase negative and non-group D were presumptively identified as *Lc. lactis* isolates and were confirmed by sequencing (Section 2.8.3 and 2.8.5). *Lc. lactis* subsp. *lactis* NCIMB 6681, *Lc. lactis* subsp. *cremoris* NCIMB 8662 and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484 were used as controls throughout this study. These strains were grown in MRS broth (Oxoid CM0359) at 30°C and 100 RPM for 24 h.

2.9.2. PREPARATION OF GENOMIC DNA

Cells were harvested from 3 ml of an overnight culture grown in BHI broth under 100 RPM agitation (Gallenkamp), by centrifugation (Eppendorf) at 13,000 g for 60 s. In a microcentrifuge tube, the cell pellet was re-suspended and washed twice with 1 ml of TN buffer [1M NaCl (Fisher Scientific S/316/60) and 10 mM Tris-HCl (Sigma T6066)] at pH 6.7 by centrifugation (Hettich Zentrifugen Mikro200) at 13,000 g for 60 s. Re-suspended cells (300 µl) in TN buffer were warmed to 55°C (Thermomixer Eppendorf) and mixed with 300 µl of 1% (w/v) pulse-field certified agarose (Bio-Rad 161-3109) made up in TN buffer. The suspension mixture was poured into a CHEF plug mould (Bio-Rad) and allowed to solidify for 15 min at room temperature (20°C). The embedded cells were lysed

with 4 ml of lysis buffer [6 mM Tris-HCl, 0.1 M EDTA (Fisher Scientific D/0700/53) and 1% (w/v) N-lauryl-sarcosine sodium salt (Sigma L9150), pH 7.6] containing 10 mg/ml lysozyme (Sigma L6876) overnight at 37°C. The plugs were washed three times with 3 ml of TE buffer [10 mM Tris-HCl and 1 mM EDTA, pH 7.6] for 30 min at room temperature followed by incubation in 4 ml of proteinase-K buffer [0.5 M EDTA, 1% (w/v) N-lauryl-sarcosine sodium salt, and 1 mg/ml proteinase-K (Fermentas EO 0491), pH 8.5] for 24 h at 55°C (100 RPM; Gallenkamp). The plugs were treated twice with 4 ml of TE1 buffer [10 mM Tris-HCl and 1 mM EDTA, pH 8] containing 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma P7626) shaken at 100 RPM for 1 h at 55°C. The addition of PMSF was to obtain vivid, discrete and clear bands after running the gel (Obszańska *et al.*, 2015). The plugs were finally rinsed three times with TE2 buffer [10 mM Tris-HCl and 50 mM EDTA, pH 8] for 30 min at room temperature. The plugs were stored at 4°C in TE1 buffer until use.

2.9.3. DIGESTION WITH *Sma*I

Before digestion, the DNA plugs were rinsed in 500 µl sterile distilled water for 15 min at room temperature followed by rinsing in 200 µl restriction buffer-D [6 mM Tris-HCl, 6 mM MgCl₂, 150 mM NaCl, 1 mM dithiothreitol, pH 7.9; (Promega R004A)]. The digestion was carried out for 24 h at 37°C in a 100 µl of solution consisting of *Sma*I (0.4 U/µl; Promega R6121), bovine serum albumin (BSA; 0.2 µg/µl; Promega R396D) and buffer J (1X; Promega R009A). The digested plugs were removed from the reaction reagents and stored at 4°C in 0.5X TBE buffer [44.5 mM Tris-HCl, 44.5 mM boric acid (Sigma B7901), 1.25 mM EDTA, pH 8] until use.

2.9.4. PFGE ANALYSIS

The digested plugs were loaded into 1% (w/v) of pulse-field certified agarose (Bio-Rad 161-3109) prepared in 0.5X TBE buffer containing 100 μ M thiourea (Sigma 88810). Electrophoresis was run in 2 l of 0.5X TBE buffer containing 100 μ M thiourea, at 14°C for 16 h at 6 V/cm, using a CHEF DRII (counter-clamped homogenous electrophoresis cell, Bio-Rad). The pulsed time used was 4-45 s and pump pressure was 80 RPM. A 50-1000 kb DNA ladder (Sigma D-2416) was used as a molecular size marker. DNA was visualized by staining in 100 ml of sterile distilled water containing 10 μ l of ethidium bromide (10 mg/ml; Fisher Scientific) for 1 h at room temperature followed by de-staining in 100 ml of sterile distilled water for 30 min at room temperature. The images were imaged under a UV transilluminator (GelDoc XR, Bio-Rad) and recorded with Quantity One 4.6.5 Basic Gel Doc software (Bio-Rad).

2.9.5. CLUSTER AND STATISTICAL ANALYSIS

PFGE profiles were saved as 150 dpi tiff format and were analyzed using FPquest version 4.5 software (Bio-Rad). Calculation of the similarity of the band profiles and grouping of the PFGE patterns was based on the Dice correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. Calculation of the significance of the PFGE profiles was done by AMOVA GenAlEx 6.5: Genetic Analysis in Excel.

2.10 PLASMID PROFILING

Plasmid DNA of *L. lactis* isolates from Section 2.9.1 was extracted by using a QIAprep Spin Miniprep Kit (Qiagen Cat. No. 27104). The procedures followed the manufacturer's instructions for low-copy-number plasmids. The *Lc.*

lactis culture (10 ml) was incubated no longer than 16 h in MRS broth (Oxoid CM0359) at 30°C and 100 RPM and then centrifuged at 4,700 g for 10 min at 4°C. The bacterial pellet was then resuspended in 500 µl of Buffer P1 (50 mM Tris-HCl pH 8, 10 mM EDTA and 100 µg/ml RNase) and transferred to a 2 ml microcentrifuge tube. Buffer P2 (200 mM NaOH and 1% sodium dodecyl sulfate; 500 µl) was added and mixed by inverting the tube 4-6 times until the solution became clear. Buffer N3 (4.2 M guanidine hydrochloride, 0.9 M potassium acetate, pH 4.8; 700 µl) was then added and mixed with the same technique and continued by centrifuging at 17,900 g for 10 min. The supernatant was transferred to the QIAprep spin column by pipetting and centrifuged for 1 min at 4,700 g. The column was washed with 0.5 ml of Buffer PB (5 M guanidine hydrochloride and 30% isopropanol) by centrifuging for 1 min at 4,700 g and then with 0.75 ml of Buffer PE (10mM Tris-HCl, 80% ethanol, and HCl, pH 7.5). An additional 1 min centrifugation was needed to remove the residual wash buffer. Finally, the column was placed into a clean microcentrifuge tube and 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) was added into the centre of the column. The plasmid DNA was kept at -20°C until use.

The plasmid DNA (5 µl) was added to 1 µl of blue/orange loading dye 6X (Promega Cat. No. G1881) and run on a 0.7 % Agarose gel in 1X TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) for 3 hours at 85 Volts to separate the plasmids. Six µl of Quick-Load 1 kb Extend DNA ladder (5,000 – 48,500 bp) (New England Biolabs Cat. No. N3239S) was used as the plasmid size marker.

2.11 CHARACTERISATION OF *STAPHYLOCOCCUS EQUORUM*

2.11.1. PREPARATION OF *S. EQUORUM* ISOLATES

The *S. equorum* isolates were grown on mannitol salt agar (MSA; Oxoid CM0085) at 30°C for 2 days using the same method described in Section 2.3. Fifteen isolates from each sample were picked and restreaked twice and then subjected to a coagulase test (Section 2.7.4). The confirmed coagulase negative isolates were stored in Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostic) at -80°C. Seven *Staphylococcus* isolates previously isolated from Stichelton cheese (Yunita, 2010) were also tested using the coagulase test. *S. aureus* NCTC 1803, *S. epidermidis* NCTC 11047, *S. equorum* NCTC 12414 and *S. xylosus* NCIMB 13636 were used as reference strains in this work.

2.11.2. PCR AMPLIFICATION OF THE *sodA* GENE

DNA of the presumptive *S. equorum* isolates was prepared by a boiling method as in Section 2.8.1. PCR amplification of the *S. equorum sodA* gene was carried out as previously reported by (Blaiotta *et al.*, 2004). Primers used in this study can be observed in Table 2.4. PCR amplification of the *sodA* gene was performed as follows: 3 min at 95°C to denature template DNA and 30 amplification cycles (at 95°C for 5 s and at 61°C for 25 s; Blaiotta *et al.*, 2004).

Table 2.4. PCR primers used in *Staphylococcus equorum* study.

Primer	Sequence (5'-3')	Annealing Position*	Target Gene	References
SdAEqF	GTG GAG GAC ACT TAA ACC ATT C	173-194	<i>sodA</i> (193 bp)	Blaiotta <i>et al.</i> (2004)
SdAEqR	CAA TTT ACC ATC GTT TAC AAC TAG	363-340		

*The segment of Bacterial 16S rDNA (*E. coli* numbering) (Brosius *et al.*, 1981).

2.11.3. PFGE ANALYSIS

The same methods of PFGE and statistical analysis described in Section 2.9.4 and 2.9.5 were applied to *S. equorum* isolates. *Sma*I, *Sac*I and *Apa*I enzymes were used to digest the DNA plugs (Jaeger *et al.*, 2004; Leroy *et al.*, 2009; Coton *et al.*, 2010).

2.11.4. BIOCHEMICAL CHARACTERISTICS OF *S. EQUORUM*

The API Staph kit (BioMérieux) was used to examine the biochemical characteristics of *S. equorum* following the manufacturer's instructions. Sterile distilled water (SDW; 5 ml) was distributed into the combed wells of the tray to create a humid atmosphere. A homogenous bacterial suspension was prepared by harvesting cells grown on Columbia blood agar as in Section 2.5 and mixing with 2 ml normal saline (0.85% sodium chloride, pH 7.4±0.2). The turbidity of this bacterial suspension should equal McFarland standard 0.5 (BioMerieux). One drop of the suspension was filled into the microtubes gently to avoid bubbles. One drop of sterile mineral oil was filled into the ADH (L-arginine) and URE (urea) microtubes after filling them with the bacterial suspension to create anaerobic conditions. Then, the tray was placed in an incubation box and incubated at 37°C for 24 hours.

After incubation, one drop of VP1 and VP2 reagent (BioMérieux) were added into the VP (sodium pyruvate) microtube, NIT1 and NIT2 reagents (BioMerieux) were added into the NIT (potassium nitrate) microtube and ZYM A and ZYM B reagents (BioMerieux) were added into the PAL (β-naphthyl phosphate) microtube. A positive or negative reaction was indicated by comparing the colour in each microtube with the standard colour for a positive or negative reaction in the Analytical Profile Index.

For the lysostaphin resistance (LSTR) test, a Lysostaphin disc (MAST group 171048) was added into a homogenous bacterial suspension (18-24 h culture in 2 ml phosphate buffered saline) in a sterile bijou bottle. The suspension should equal McFarland standard 1 (BioMérieux). The second suspension without the disc served as a negative control. A positive result was indicated by complete clearing of the bacterial suspension with the disc while the suspension without a disc remained fully turbid. A negative result was indicated by both bacterial suspensions remaining turbid.

On the results sheet, the values corresponding to the positive reactions were added. The 7-digit profile numbers were entered into the API database V 4.1 in the apiwebTM (<https://apiweb.biomerieux.com>).

2.11.5. ACID AND SALT TOLERANCE

The response of *S. equorum* to acid and salt was examined following the procedures described by Succi *et al.* (2005) and Mugampoza (2013) but in BHI broth (Oxoid CM1135). In both analyses, one ml of an overnight BHI broth culture ($\sim 10^9$ CFU/ml) grown at 37°C and 100 RPM (Gallenkamp) was serially diluted to 10^6 CFU/ml so that 10^5 CFU/ml was used as the initial cell density (Mugampoza, 2013).

In the test of acid tolerance, one ml of the 10^6 CFU/ml suspension was transferred into 9 ml BHI broth acidified to pH 6, 5, 4 and 3 using lactic acid (Fisher Scientific L/0100/PB08). BHI broth without acidification was used as the control (pH 7). In the test of salt tolerance, one ml of the 10^6 CFU/ml suspension was transferred into 9 ml BHI broth containing 3.5%, 5%, 8% and 10% sodium chloride (Fisher Scientific S/3160/60). BHI broth without sodium chloride addition was used as the control (0%). The test cultures were incubated at 37°C

and 100 rpm (Gallenkamp). One ml of each culture broth was taken at 4, 8, 24, 48, 72 and 96 h for enumeration on BHI agar at 30°C for 48 h (Lab Companion IS-971R).

A viable count was conducted as described in Section 2.4 on BHI agar. All microbial counts were converted to log₁₀ CFU/ml and parametric statistics (means ± standard errors) were also calculated. Analysis of variance (ANOVA) was computed using IBM SPSS Statistics 22 with Tukey-HSD (honest significant difference) as the posthoc test.

2.12 INTERACTION BETWEEN *LACTOCOCCUS LACTIS*, *PENICILLIUM ROQUEFORTI* AND *STAPHYLOCOCCUS EQUORUM*

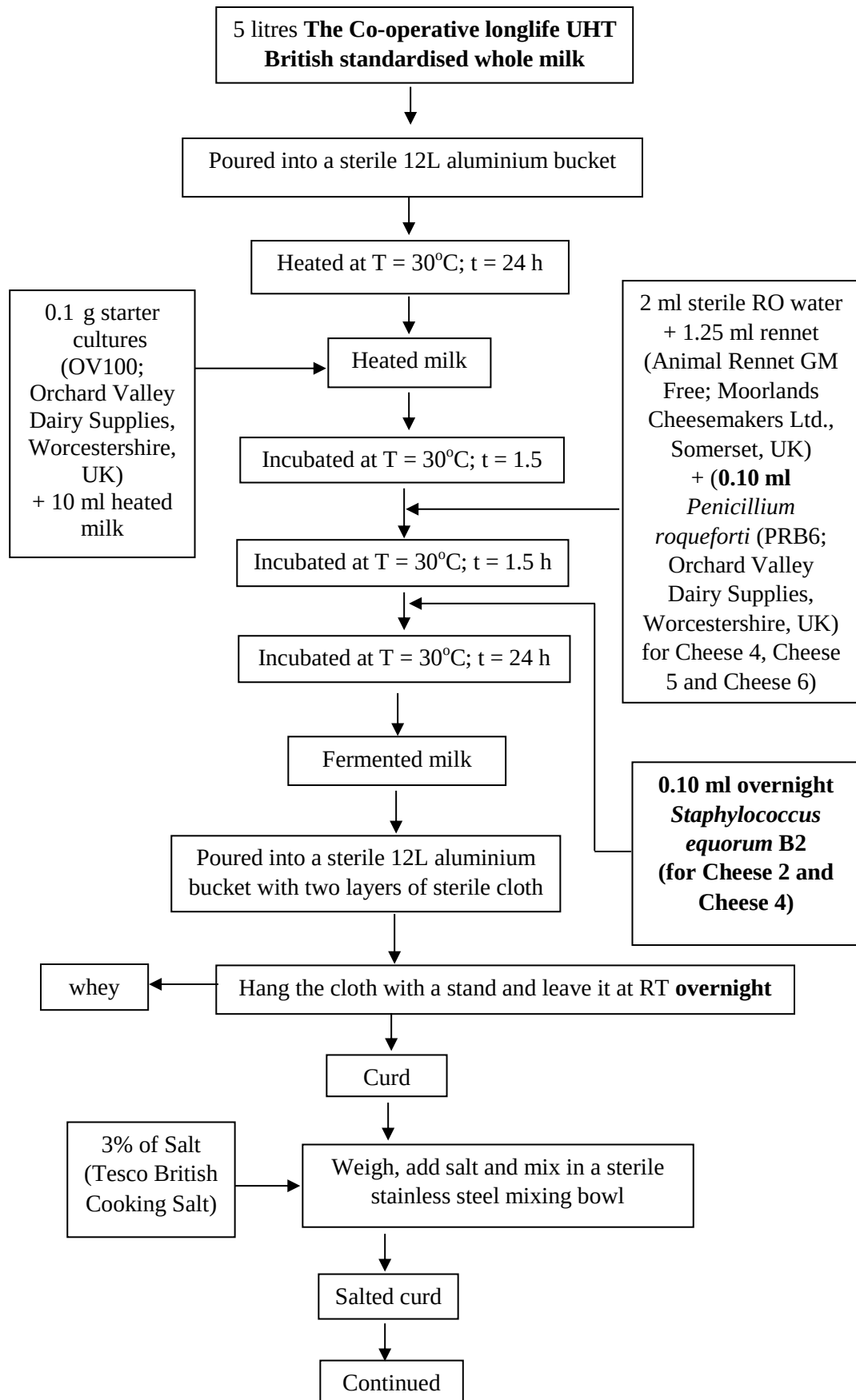
2.12.1. CHEESE MODEL MAKING

Cheese models were made based on Stilton cheese procedures with some modification for small-scale cheeses (Whiley, 2013). A few changes from this study were also added to improve the texture (Figure 2.1 and Figure 2.2). Cheese models were examined with three experimental factors (Table 2.5). The first factor was incorporation of *S. equorum* in two different ways: *S. equorum* was added 1.5 h after *L. lactis* addition to the milk or *S. equorum* was surface-spread after the rubbing-up process. These designated applications were chosen because of two expectations: *S. equorum* was introduced at early fermentation, potentially via raw milk in the case of Stichelton cheese, or its presence was due to contamination through handling prior to ripening. A control with no *S. equorum* was included. The second factor was parts of the cheeses examined which consisted of core and crust. The third factor was *P. roqueforti* addition, with cheeses made without addition of the mould culture and with addition.

Table 2.5. Combination of three different factors in cheese model making.

FACTORS		Application		
		Without <i>S. equorum</i> (X1)	<i>S. equorum</i> added 1.5 h after <i>P. roqueforti</i> addition (X2)	<i>S. equorum</i> spreaded after rubbing-up process (X3)
Core (Y1)	No <i>Penicillium</i> (Z1)	X1Y1Z1	X2Y1Z1	X3Y1Z1
	Yes <i>Penicillium</i> (Z2)	X1Y1Z2	X2Y1Z2	X3Y1Z2
Crust (Y2)	No <i>Penicillium</i> (Z1)	X1Y2Z1	X2Y2Z1	X3Y2Z1
	Yes <i>Penicillium</i> (Z2)	X1Y2Z2	X2Y2Z2	X3Y2Z2
		C1: cheese with <i>Lc. lactis</i> (X1Z1)		
		C2: cheese with <i>Lc. lactis</i> and <i>S. equorum</i> (X2Z1)		
		C3: cheese with <i>Lc. lactis</i> and spread surface <i>S. equorum</i> (X3Z1)		
		C4: cheese with <i>Lc. lactis</i> and <i>P. roqueforti</i> (X1Z2)		
		C5: cheese with <i>Lc. lactis</i> , <i>S. equorum</i> and <i>P. roqueforti</i> (X2Z2)		
		C6: cheese with <i>Lc. lactis</i> , spread surface <i>S. equorum</i> and <i>P. roqueforti</i> (X3Z2)		

UHT milk was used to control flora composition. Commercially used Stilton freeze dried starters *Lc. lactis*, liquid *P. roqueforti* and rennet were added to overnight incubated milk (30°C). The milk was then fermented at the same temperature overnight. The whey was then separated from the curd using two layers of sterile cloth and the cloth was hanging at room temperature overnight. The salt (3%) was added into the soft curd and the salted curd was loaded in to open ended cheese hoops. A post-salting curd sample was collected within 5 hours of salt addition. Model cheeses were ripened at 23°C for 4 days at 75% relative humidity (RH) which was reached using saturated NaCl solution (Greenspan, 1977) and turned daily. The cheeses were further ripened at 12°C for one month. They were pierced every week during the ripening period using a sterile skewer. Viable counts of lactococci, lactic acid bacteria (LAB), total aerobic counts, yeasts and moulds, staphylococci, and pH value were analysed throughout the process, whereas water activity, texture and flavour volatiles were analysed only at the final step (one month ripened cheese).



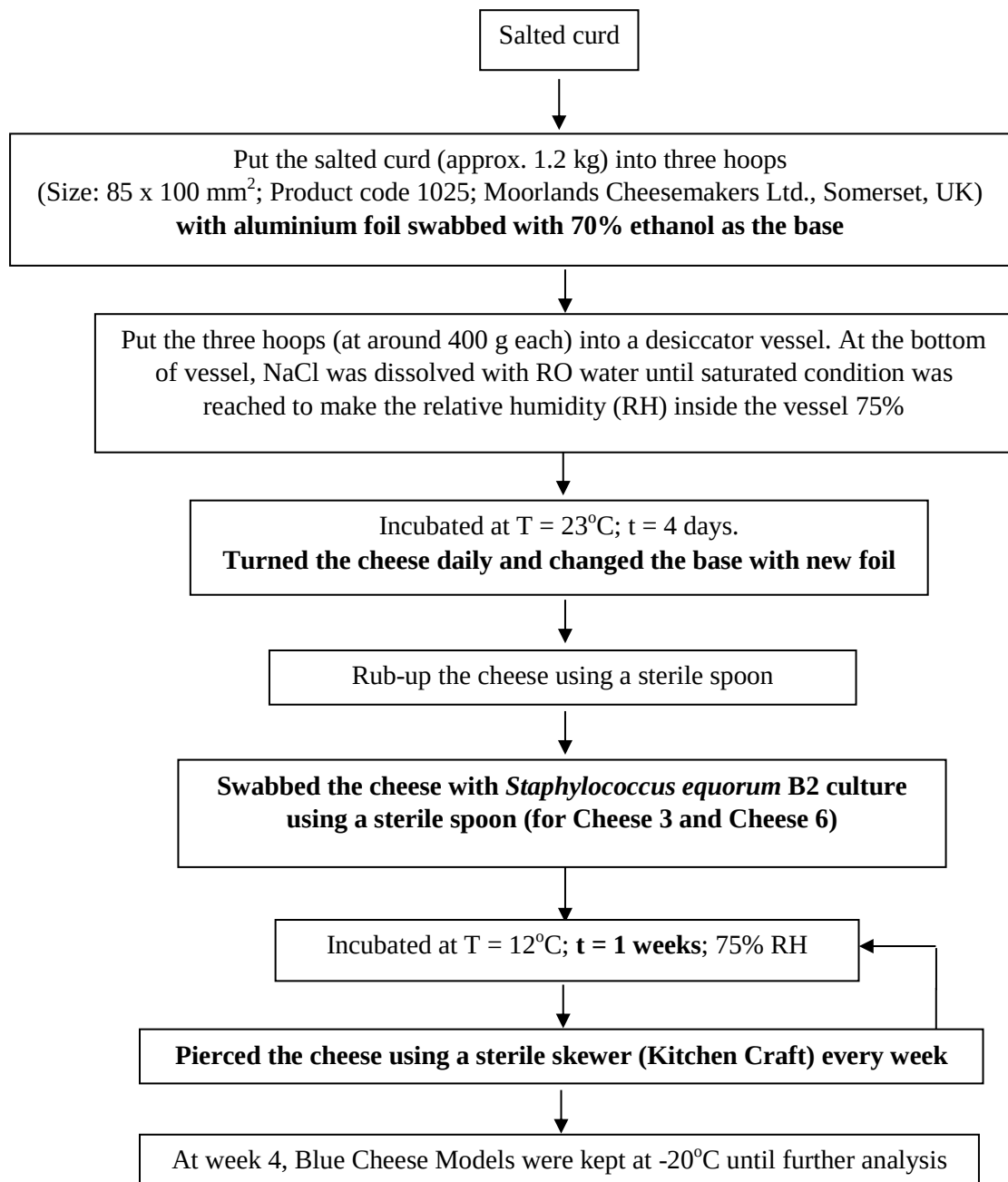


Figure 2.1. Production of cheese model. Changes from Whitley (2013) study are highlighted in bold. *Staphylococcus equorum* B2 was isolated from the crust of commercial Stilton cheese (Chapter 5). Cheese models were made with: *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6).

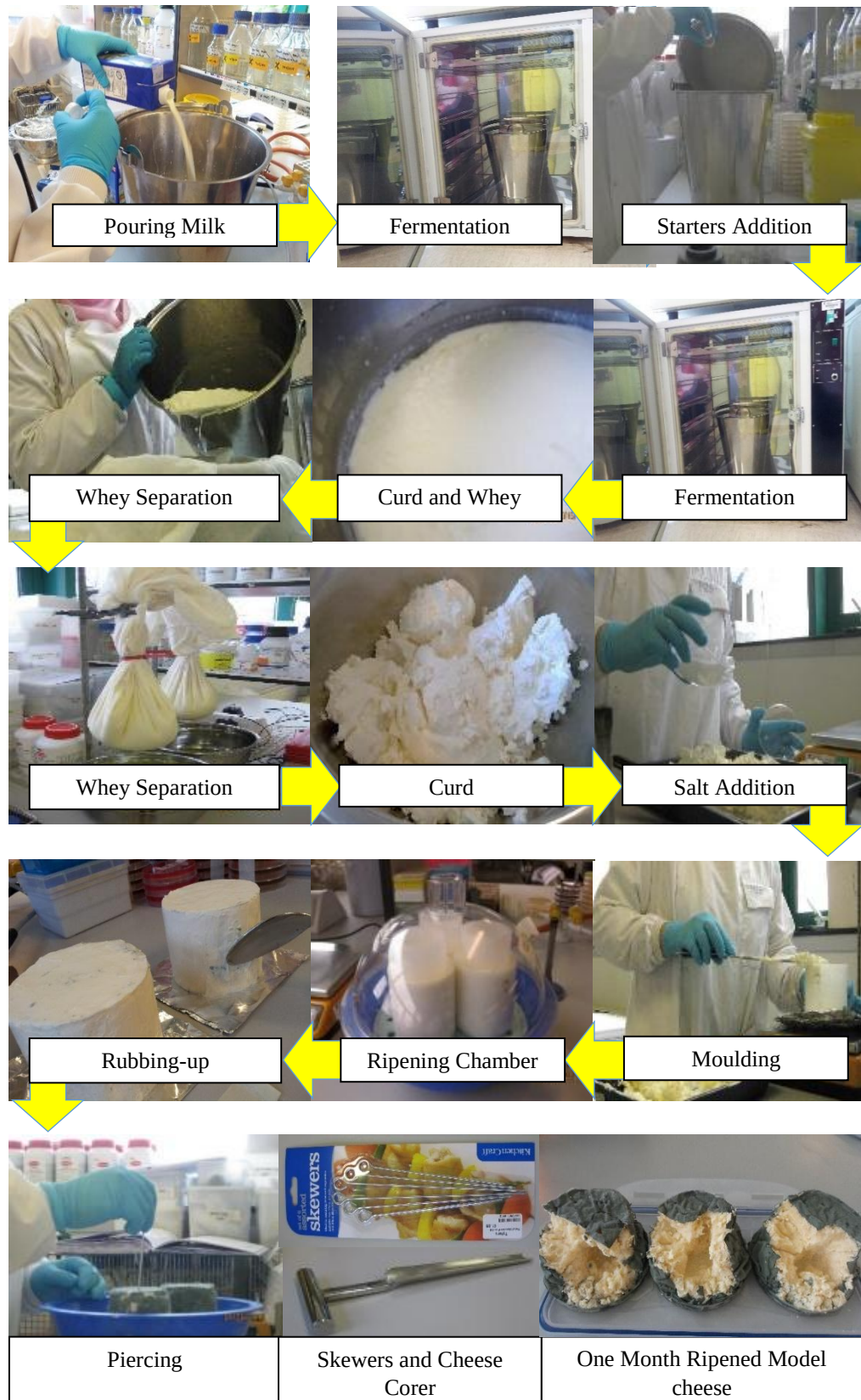


Figure 2.2. Production of cheese model on photos.

2.12.2. MICROBIOLOGICAL AND pH ANALYSIS OF CHEESE MODELS

Samples (1 ml or 1 g) were taken aseptically from the milk, the heated milk, during incubation (4, 8, 20, and 24 hours), the curd after whey separation, the salted curd, the soft cheese before rubbing-up and during ripening (1, 2, 3 and 4 weeks). During ripening, the cheese sample was collected from three model cheeses using a cheese corer then put the rest of the cheese back in to the cheese to seal the cheese for further ripening. The core and crust parts of the model cheese were analysed separately. For microbiological analysis, the sample preparation followed the same procedures previously described in Section 2.3. All samples were grown aerobically on M17 agar (Oxoid CM0785) and plate count agar (Oxoid CM0325) at 30°C for 2 days, anaerobically on MRS agar (Oxoid CM0361) at 30°C for 2 days and aerobically on Rose Bengal Chloramphenicol agar (Oxoid CM0549) at 25°C for 5 days. The pH value was analysed following procedures in Section 2.3. Analysis of variance (ANOVA) was computed using IBM SPSS Statistics 22 with Tukey-HSD (honest significant difference) as the post-hoc test.

2.12.3. WATER ACTIVITY

The water activity (a_w) of the cheese models was measured using an Aqua Lab Series 3TE (Decagon Devices Inc., Washington, USA). Distilled water (a_w 1.000 ± 0.003), 0.5 M KCl (a_w 0.984 ± 0.003) and 6.0 M NaCl (a_w 0.760 ± 0.003) were used as standard solutions. These solutions were measured before measuring the cheese samples. Stilton, Wensleydale and Danish Blue cheeses bought commercially were used for comparisons. A sample of cheese (1 g) was placed into a plastic cup. The top lip of the cup was checked to make sure it was free

from sample residue before it was put in the sample drawer to avoid contamination of the chamber's sensor. The sample drawer was closed carefully and the knob was turned to the "Read" position. The a_w measurement was read from the digital screen. This method was replicated three times technically from each cheese and three times biologically (three replicate cheeses). Analysis of variance (ANOVA) was computed using IBM SPSS Statistics 22 with Tukey-HSD (honest significant difference) as the post-hoc test.

2.12.4. TEXTURE ANALYSIS

The TA.HD Plus Texture Analyzer (Texture Technologies, New York, USA) was used to measure the hardness of the cheese models. It is the maximum force (peak force; g) during the first cycle of compression. Texture Exponent 32 software (Visual Components Inc., Surrey, UK) was used to set-up the machine and analyse the data. A 2-bite test method was set-up: the pre-test speed was 5 mm/s, the test speed was 1 mm/s and the post-test speed was 5 mm/s (Whiley, 2013). A plastic cylinder (diameter 3 cm, height 1 cm) was used as the probe (Figure 2.3). The distance between the probe and the cheese ($5 \times 5 \times 2 \text{ cm}^3$) was set at 10 mm, the compression distance was 5 mm and the force of the probe was 5 g.mm/s. Stilton, Wensleydale and Danish Blue cheeses were used as comparison cheeses.

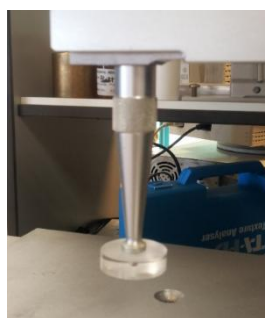


Figure 2.3. Plastic probe used in the texture analysis.

2.12.5. SPOT TEST AND SINGLE LAYER ASSAY

The spot test and single layer assay procedures followed Hansen and Jakobsen (1997) in analysing the interaction between LAB and *P. roqueforti* in Danablu cheese. *S. equorum* B2 was isolated from the crust of commercial Stilton cheese by Jihan Sherif (who was a project student under my supervision and did her project as part of her MSc in Applied Biomolecular Technology degree in 2012) and the culture broth was prepared from an overnight isolate grown on potato dextrose broth (PDB; Oxoid CM0962) at 37°C.

In the spot test (ST), one ml of *P. roqueforti* spore suspension (9 log CFU/ml; PRB6, Orchard Valley Dairy Supplies, Worcestershire, UK) was pour plated in 20 ml melted Potato Dextrose Agar (PDA; Oxoid CM0139). Once the plate was solidified, 40 µl of *S. equorum* B2 culture (9 log CFU/ml) was spotted onto the plate and was left until dried. The dried plate was then incubated at 25°C for 5 days in a box without a lid, covered by aluminium foil to create dark conditions. The plate with *P. roqueforti* and PDB was used as a control plate. In the single layer assay (SLA), one ml of *P. roqueforti* spore suspension (9 log CFU/ml) and one ml of *S. equorum* B2 culture (9 log CFU/ml) were mixed together with 20 ml melted PDA. Once the plate was solidified, the plate was incubated as described above. The control plates consisted of a plate with *P. roqueforti* and PDB and a plate with 2 ml of *P. roqueforti*.

The ST and SLA were repeated for cell-free supernatant (CFS) and the four treated CFSs. The CFS was prepared by centrifuging the overnight culture at 3,400 g and 4°C for 15 min followed by membrane filtration (0.2 µm; Ministart AG 37070, Sartorius). The treated CFSs were CFS neutralised by adding 1 N NaOH to pH 6.5-7 (CFS-N); the CFS treated with 500 Unit/ml Catalase (Sigma C1345) (CFS-C); and CFS treated with 1 mg/ml Proteinase-K (Fermentas EO

0491) (CFS-P) (Mugampoza, 2013). CFS-NC was CFS adjusted to pH 6.5-7 and treated with catalase. The treated CFSs were incubated at 37°C for 30 min and boiled for 2 min (Thermomixer, Eppendorf).

The CFS of *S. equorum* B2 was further tested in the presence of 0.05% and 0.5% hydrogen peroxide (H₂O₂). A plate without *S. equorum* B2 and H₂O₂ and plates without *S. equorum* B2 but with 0.05% and 0.5% H₂O₂ were used as control plates.

To confirm whether the sporulation activity of *P. roqueforti* could be recovered, a loop of mixed culture was picked-up from the clear zone of the spot test plate and was streaked onto PDA and BHI agar. The streaked plates were incubated at 25°C for 5 days.

2.12.6. MOULD STAINING

P. roqueforti grown on SLA plates was collected using Fungi Tape (Scientific Device Laboratory, USA; Catalogue No. 745). The tape was placed onto the microscope slide with a drop of Lactophenol Blue Solution (Fluka Analytical; 61335-100mL). The microscope slide was examined microscopically for spore formation at 40X magnification. The picture was viewed by TS View Digital Imaging Software (version 6.1.3.9) and was captured by eyepiece microscope digital camera (Premiere Model MA88-130).

2.13 FLAVOUR ANALYSIS (SPME GCMS)

The cheese samples (3 g) from Section 2.12.1 were taken using a cheese corer for aroma analysis. The core and the crust were analysed separately. The samples were transferred into 20 ml headspace vials (22.5 x 75.5 mm, Grace Alltech, UK) using a plastic sterile loop. The vials were immediately sealed with a

PTFE-Silicone lined magnetic cap (20 mm diameter, 5 mm centre, Grace Alltech) and gently hammered to create a flat surface on the cap. The sealed vials were stored at -20°C until analysis. Samples were defrosted overnight at 4°C and allowed to equilibrate at room temperature (20°C) for 30 min prior to analysis. Three technical replicates from each cheese and three replicate cheeses (biological replicates) which were made on the same occasion were prepared for all samples. Two additional samples were selected randomly to be used at the first run to check the machine was working properly.

A 1-cm Stableflex fibre coated with 50/30 µm divinylbenzene-carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA, USA) was used for the extraction of the volatile compounds. The fibre was conditioned for 60 min in the injection port at 230°C. The SPME needle was then introduced into the septum in the lid of the vial using a PAL autosampler (CTC Analytics, Switzerland) and the fibre exposed to the headspace for 20 min at room temperature. The fibre was then desorbed in the injector for 10 min, transferring the volatile compounds to the column. The temperature of the injection port was 230°C.

Chromatography was carried out with a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, UK) using a Zebron ZB-Wax GC column (30 m x 0.25 mm x 1 µm; Phenomenex, Cheshire, UK). Helium gas was employed as the carrier gas at a constant pressure of 18 psi. The GC oven temperature programme was as follows: initial temperature was 40°C maintained for 2 min and increased at a rate of 8°C/min to a final temperature of 220°C. The transfer line from GC to MS was held at 250°C. MS was performed with a DSQ mass spectrometer (Thermo Electron Corporation) operating in positive ionisation electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2

scans/s) scanning from m/z 20-250. Source temperature was 200°C and pressure 39 mtorr.

Data were processed with Xcalibur software v.14 (Thermo Fisher Scientific). Qual Browser was used to identify the ion and retention time of peaks. The compounds were identified by comparing their retention times (RT) and mass spectra with retention indices (RI) and mass spectra published in the databases of the National Institute of Standards and Technology (NIST) mass spectral library (2008). Peak areas were determined for each compound.

ANOVA was performed with Design Expert (Minneapolis, USA) in order to examine the significance of the differences between the quantities of the aroma compounds of the cheese models. The relationship between the different sections of the cheese models and their volatiles detected by SPME GC-MS analysis was evaluated by Principal Component Analysis (PCA) using Unscrambler v.9.0 (Camo Process AS., Norway). All data were standardised (1/standard deviation) prior to analysis. Duncan test was selected as the post-hoc test.

CHAPTER 3

MICROFLORA DYNAMICS OF STICHELTON CHEESE

3.1 INTRODUCTION

The preliminary study on microflora dynamics of Stichelton cheese was conducted in 2009. However, the final cheese could not be collected and the sequencing has been done only for the starter culture isolates. Following this study, the fully ripened cheese was collected in 2011. A combination of culture-dependent and culture-independent (PCR-DGGE) techniques were employed as suggested by previous studies to overcome the bias of the culture independent techniques (Muyzer, 1999; Cocolin *et al.*, 2001; Ercolini, 2004).

PCR is an *in vitro* method for replicating a target DNA sequence so that its amount is increased exponentially. The method consists of repetitive cycles of DNA denaturation through melting at an elevated temperature to convert double-stranded DNA to single-stranded DNA. This is followed by annealing of oligonucleotide primers to the target DNA, and extension of the DNA by nucleotide addition from the primers by the action of the enzyme DNA polymerase (Atlas and Bej, 1994).

The PCR product is then analyzed by DGGE, a method in which DNA amplimers of the same size or length but of different sequences will be separated. The DNA takes place through an increased concentration gradient of denaturant in the gel electrophoresis. When a strand reaches an appropriate concentration of denaturing reagents, the base pairs formed by nucleotides A (adenine) and T (thymine), and those formed by C (cytosine) and G (guanine) are chemically

melted apart. The greater bonding between G-C pairs than A-T pairs requires a greater level of denaturant to break the bonds. Where DNA amplimers have a different sequence, and thus different numbers of G-C and A-T bonds, the domains will melt at different denaturant concentrations. This has an effect on DNA migration and each amplimer stops at a different position in the gel (Muyzer *et al.*, 1993; Ercolini, 2004).

The 16S rDNA is the most common and targeted gene for the PCR-DGGE analysis of microbial communities in natural environments among the eubacterial species because of being genetically stable. It is also presenting all the species and functionally same. The genetic sequence variation was commensurate with the evolutionary distance. The conserved regions offer a target for common primer site so they get amplification regardless of the bacteria. The 16S rDNA has approximately 1500 base pairs from the variable regions V1 to V9 (Figure 3.1). These variable regions characterize the species variability and are used for microbial classification and identification (Muyzer *et al.*, 1993; Ercolini, 2004).

Briefly, in PCR-DGGE, the DNA is firstly extracted either from the whole community or single species. The targeted V-region of the DNA is then amplified by PCR. Separation of the amplified DNA is conducted in the DGGE gel. The DNA produced from one single band will be reamplified using same primer sets but without the GC-clamp and purified. The purified DNA is then sequenced. The sequence DNA of the species is finally compared with data in the Gen Bank. The diversity and dynamics of the microbial communities in various cheeses have been identified successfully by using a 16S rDNA PCR-DGGE method (Table 3.1).

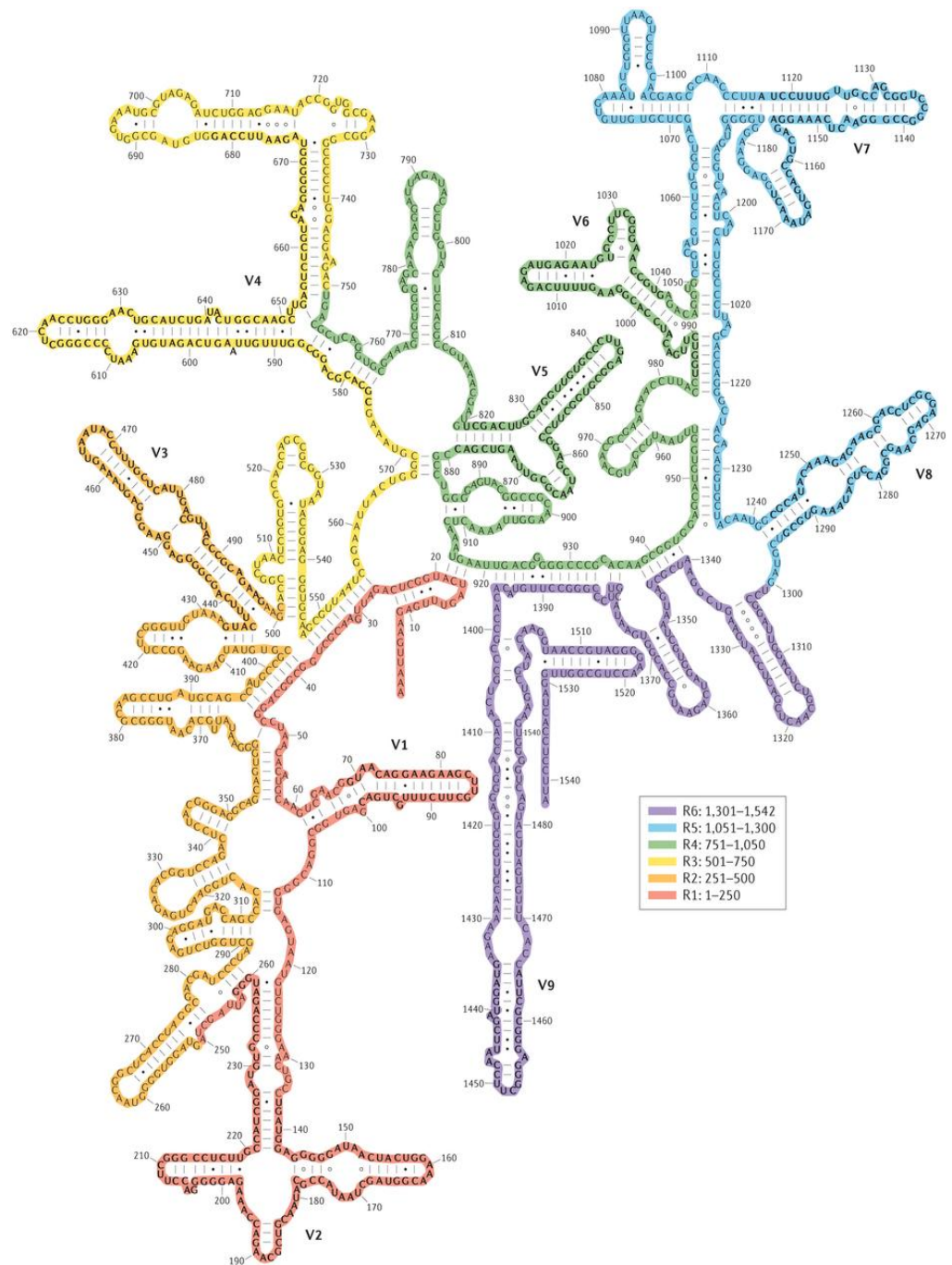


Figure 3.1. Variable region of the 16S ribosomal RNA (Yarza *et al.*, 2014)

Table 3.1. Targeted hypervariable regions for detection of microflora in various cheeses.

Cheese	Target*	DGGE Conditions			References
		Acrylamide	Urea-Formamide	Electrophoresis	
Stilton	V3	8%	30-50%	50 V, 10 min.	Ercolini <i>et al.</i> , (2003)
	V4V5	6.5%		170 V, 6 hours.	
Artisanal Sicilian	V6V8	8%	30-60%	85 V, 16 hours.	Randazzo <i>et al.</i> , (2002)
	16S				
	V1V3				
Cabrales	V3	8%	40-60%	75V, 17 hours	Flórez and Mayo, (2006)
	D1		30-50%	130 V, 4.5 hours	
Robiola di Roccaverano	V1	8%	40-60%	130 V, 4 hours	Bonetta <i>et al.</i> , (2008)
	D1D2		30-60%	120 V, 4 hours	

*V region is for 16S rRNA gene (bacteria) and D domain is for 26S rRNA gene (yeast).

Yu and Morrison (2004) compared DGGE amplicon lengths (V1, V3, V8, V1V3, V4V5, V3V5, and V6V8) to examine the population diversity of sheep gastrointestinal microbes. Their results showed that the DGGE profiles of the V3 or V1 regions produced the greatest number of resolved bands. However, multiple V-regions (V3 to V5 or V6 to V8 regions) should be targeted if a longer amplification product is required to provide a more detailed characterization of the species level with a higher degree of probability. Chakravorty *et al.* (2007) analysed 16S rRNA gene segments of V1V8 in 110 different bacterial species including common blood pathogens. Based on their result, the V3 region was found to be the most suitable for distinguishing all bacteria species to genus level except for the closely related Enterobacteriaceae. Ercolini *et al.* (2003) also compared the variable V3 region and V4V5 regions of Stilton cheese bacterial flora. Their results showed that *Lactobacillus curvatus* was identified by the variable V3 region amplification only, while *Leuconostoc mesenteroides* was detected by amplifying the V4V5 regions only. It seems that each bacterium has a different region of 16S rDNA from which it can be identified. Therefore, various

primers were used to support justification of primer choice in the subsequent PCR-DGGE analysis. The variable V6V8 region was evaluated to overcome possible misleading conclusions in this research.

3.2 OBJECTIVES

The objectives of this work were to identify the microbial diversity of the fully ripened Stichelton cheese by culture-dependent and culture-independent (PCR-DGGE) techniques and to characterise bacteria isolated in the raw milk as well as the matured Stichelton.

3.3 RESULTS

3.3.1. MICROBIAL COUNTS

In the preliminary study (Yunita, 2010), the microbial diversity of Stichelton cheese had been examined through the process until 9 weeks ripening. Therefore, a fully ripened Stichelton cheese (12 weeks old) sample was collected to analyse the microbial profiles and compare it with the unripened cheeses. The viable counts were enumerated by culturing as shown in Table 3.2.

Comparing the mature cheese (12 weeks) with 9 weeks cheese samples, the microbial counts have different trends. Particularly, the LAB, *Lactobacillus*, *Lactococcus*, total aerobic (NA medium) as well as yeasts and moulds counts decreased in all the parts of Stichelton cheese (inner core and outer crust). It is interesting to note that in the final product, the viable count of yeasts and moulds in the core (6.66 log CFU/g) was higher than that of in the crust (4.53 log CFU/g).

Table 3.2. Microbial counts^a in different steps of Stichelton cheese production.

Samples		pH	MRS (Lactic Acid Bacteria)	Rogosa (<i>Lactobacillus</i>)	M17 (<i>Lactococcus</i>)	BP (<i>Staphylococcus</i>)	KFSA (<i>Enterococcus</i>)	NA (Total Aerobic Counts)	BHI (Total Aerobic Counts)	RBCA (Yeasts and Moulds)
Starter Cultures ^c		4.47 (0.08)	0.00 (0.00)	0.00 (0.00)	8.46 (0.01)	0.00 (0.00)	0.00 (0.00)	6.81 (0.16)	9.10 (0.05)	0.00 (0.00)
Unpasteurised Milk ^c		6.65 (0.11)	2.76 (0.28) ^b	0.00 (0.00)	4.00 (0.07)	2.51 (0.30)	2.18 (0.54)	3.98 (0.13)	4.06 (0.00)	0.00 (0.00)
Pre-Milling ^c		4.80 (0.41)	5.26 (0.99)	0.00 (0.00)	9.00 (0.07)	4.51 (0.30)	3.84 (1.01)	8.48 (0.58)	9.06 (0.00)	0.00 (0.00)
Post Milling and Salting		4.80 (0.13)	7.61 (0.04)	7.58 (0.06)	8.73 (0.19)	5.88 (0.02)	7.51 (0.03)	8.37 (0.03)	8.72 (0.08)	4.88 (0.05)
Pre-Piercing ^c (6 weeks)	Core	3.60 (0.77)	8.13 (0.02)	7.79 (0.23)	8.16 (0.05)	2.96 (0.46)	7.98 (0.04)	8.18 (0.05)	8.14 (0.01)	5.49 (0.07)
	Crust	4.72 (0.27)	8.02 (0.04)	7.97 (0.02)	11.32 (0.05)	4.83 (0.04)	7.99 (0.02)	8.21 (0.08)	11.43 (0.03)	5.90 (0.03)
Post Piercing ^c (9 weeks)	Core + Veins	6.70 (0.58)	7.89 (0.06)	7.32 (0.04)	8.04 (0.04)	4.45 (0.26)	7.22 (0.05)	8.00 (0.07)	8.03 (0.16)	7.28 (0.01)
	Crust	6.32 (0.12)	10.70 (0.09)	7.08 (0.11)	11.00 (0.05)	10.60 (0.08)	10.68 (0.07)	11.04 (0.07)	11.28 (0.02)	9.99 (0.04)
Mature (12 weeks)	Core + Veins	6.36 (0.24)	7.41 (0.01)	6.18 (0.04)	7.83 (0.06)	5.35 (0.06)	7.44 (0.03)	7.83 (0.04)	8.17 (0.21)	6.66 (0.07)
	Crust	8.21 (0.31)	6.90 (0.06)	4.85 (0.05)	8.65 (0.05)	7.03 (0.03)	7.09 (0.03)	7.34 (0.04)	8.92 (0.02)	4.53 (0.09)

^alog CFU/ml for milk and starter cultures; log CFU/g for cheese samples.

^bMean (Standard Deviation, SD).

^cData was obtained from the preliminary research (Yunita, 2010).

However, the profiles of *Staphylococcus*, *Enterococcus* and total aerobic (BHI medium) counts in the core were different from the LAB, *Lactobacillus*, *Lactococcus*, total aerobic (NA medium), yeasts and moulds. These three counts in the core had increased trends during the last week of ripening while the crust had decreased trends. It is noteworthy that there were different trends of total aerobic counts between NA and BHI media with NA counts were lower than BHI counts. This supports the previous finding that NA is less rich than BHI. Therefore, the more fastidious microorganisms like lactic acid bacteria would grow much more on BHI than NA suggesting that the use of BHI agar is more useful in assessing microflora diversity from milk products.

3.3.2. PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERIZATION

From the matured Stichelton and raw milk culture plates, a total of 86 representative isolates were picked from eight medium. The isolates were streaked on to the corresponding media agar plates and incubated at 10°C to determine isolates which could grow under the cheese ripening conditions. The colonies were also incubated at 45°C to screen for thermotolerant isolates. From sequencing result, the eighty isolates were grouped into twelve different species (Table 3.3) while six yeast isolates were kept in -80°C for further study.

Table 3.3. Bacteria detected in raw milk and matured Stichelton using culture-based method.

Isolates	Total of Isolates (80)	Samples			Media (30°C)	Ability to grow at		Gram Staining	Cell Shape
		Raw Milk	Core and Veins	Crust		10°C	45°C		
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	33	+	+	+	M17	+	+	+	Cocci, small
<i>Lactobacillus plantarum</i>	4	+	+	+	MRS, KFSA, Rogosa	+	-	+	Rod, small
<i>Lactobacillus curvatus</i>	3	+	-	-	MRS	+	+	+	Rod, big
<i>Staphylococcus gallinarum</i>	3	+	-	-	MRS, M17	+	-	+	Cocci, big
<i>Staphylococcus devriesei</i>	2	+	-	-	M17	+	+	+	Cocci, big
<i>Microbacterium</i> sp.	1	+	-	-	M17	+	-	+	Rod, small
<i>Sphingobacterium</i> sp.	3	+	-	-	M17	+	-	-	Rod, very small
<i>Mycetocola</i> sp.	1	+	-	-	M17	+	-	+	Cocci, very small
<i>Brevundimonas</i> sp.	3	+	-	-	M17	+	-	-	Rod, very small
<i>Kocuria</i> sp.	13	+	+	-	M17, MRS, Rogosa	+	+	+	Cocci, big
<i>Enterococcus faecalis</i>	13	-	+	+	KFSA, M17	+	+	+	Cocci, small
<i>Proteus</i> sp.	1	-	-	+	BP, M17	-	-	-	Rod, very small

+ : Detected.

- : Not detected.

As given in Table 3.3, *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp. and *Brevundimonas* sp. were detected in the raw milk sample. Conversely, *Enterococcus faecalis* and *Proteus* sp. were found in the matured Stichelton. *Kocuria* sp. was detected in the milk and the core of the cheese samples, but not in the crust. *L. lactis* subsp. *lactis* and *Lactobacillus plantarum* were found in all samples. All isolates could grow at 10°C except *Proteus* sp. This bacterium did not grow at 45°C as well as *Sphingobacterium* sp., *Brevundimonas* sp., *Microbacterium* sp., *Mycetocola* sp., *Lactobacillus plantarum* and *Staphylococcus gallinarum*. It is interesting to note that M17 was not very selective for *Lactococcus* isolation. KFSA was also not for *Enterococcus* as *Lactobacillus plantarum* grew on this media. *Kocuria* sp. could grow on M17, MRS and Rogosa agar.

3.3.3. PCR-DGGE ANALYSIS

Three sets of primers were used to amplify the variable V3, V4-V5, V6-V8 regions of 16S rDNA of populations from mature Stichelton cheese. The DNA samples were extracted directly from the cheese as well as bulk cells which were collected on 8 different media after colony counting. The PCR products were analysed by DGGE using 8% acrylamide for V3 and 6.5 % acrylamide for V4-V5 and V6-V8 amplification with the percentage of denaturing gradient of 20-80%. The dominant bacterial species were screened by the intensity of DGGE bands and then cut out and sequenced. Figure 3.2 summarizes the DGGE profile for the V3 region of 16S rDNA of Stichelton cheese and bulk cells collected from MRS, Rogosa, and KFSA agar.

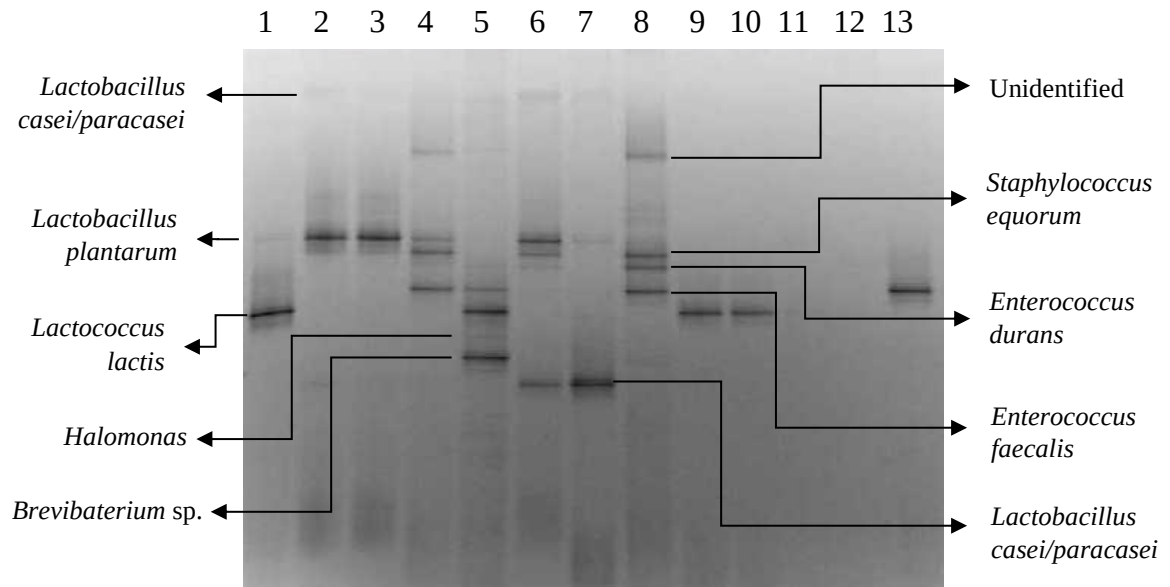


Figure 3.2. DGGE profiles of PCR amplicons of V3 region 16S rDNA of Stichelton cheese and bulk cells. Lanes: 1, Mature Stichelton core and veins (direct DNA extraction); 2, Cultured cells on MRS agar from mature Stichelton core and veins; 3, Cultured cells on Rogosa agar from mature Stichelton core and veins; 4, Cultured cells on KFSA agar from mature Stichelton core and veins; 5, Mature Stichelton crust (direct DNA extraction); 6, Cultured cells on MRS agar from mature Stichelton crust; 7, Cultured cells on Rogosa agar from mature Stichelton crust; 8, Cultured cells on KFSA agar from mature Stichelton crust; 9, *Lactococcus lactis* NCIMB 9918; 10, *Lactococcus lactis* NCIMB 8763; 11, *Lactobacillus plantarum* NCIMB 138914; 12, *Leuconostoc mesenteroides*; 13, *Enterococcus faecalis* NCTC 775.

Based on Figure 3.2, it is clear that the microbial species in the crust were more complex than those in the core. Two bands corresponding to *Lc. lactis* and *Lactobacillus plantarum* were found in the core (Lane 1, Figure 3.2) and there was one weak band found in the bulk cell samples from MRS and Rogosa agar (Lanes 2 and 3, Figure 3.2). *Enterococcus faecalis* and *Staphylococcus equorum* were found in

samples grown on KFSA agar. Also, there was an unidentified bacterium, weak bands of *Enterococcus durans* and *Lactobacillus casei/paracasei* (Lane 4, Figure 3.2), but the presence of these three bands was not shown clearly in the DNA sample extracted directly from cheese, only from colonies on KFSA agar. Presumptively, they were in very low numbers in the cheese itself. On the other hand, *Lc. lactis* was only detected in the DNA sample produced by direct extraction from cheese, not in bulk cells from MRS agar. No DNA was extracted from M17, BP, NA and BHI agar as the mixed solution of the bulk cells, Proteinase-K, food lysis buffer and chloroform formed a very sticky solution so the clear supernatant could not be separated with the pellet suggesting that lower dilution plates would be needed to obtain purified DNA from media.

The sample profile of the crust consisted of at least seven detectable bands (Lane 5, Figure 3.2) which sequencing showed were *Brevibacterium* sp., *Lc. lactis* and *Enterococcus faecalis* (three bright bands) as well as *Enterococcus durans*, *Halomonas* sp. and *Lactobacillus casei/paracasei* (three of the four weak bands). A prominent band of *Lactobacillus plantarum* was detected from MRS agar, but this was less evident from Rogosa agar (Lanes 6 and 7, Figure 3.2). Again, *Lc. lactis* was not detected from MRS agar. *Staphylococcus equorum* and *Enterococcus durans* were detected on MRS agar as well as KFSA agar. Moreover, *Enterococcus faecalis* was detected on KFSA agar.

The identification of the microbial community during production of Stichelton cheese was also extended to different 16S rDNA regions of amplification. Figure 3.3 shows a comparison of communities when V3, V4V5 and V6V8 are used as primer sets. It can be seen that the bacteria vary at each step and with each primer with an

increase in the diversity in the final Stichelton cheese. In general, *Lc. lactis* subsp. *lactis* and *Lactobacillus* sp. were the main representatives in all samples.

Using the V3 primer, *Lactococcus lactis* was seen in the milk sample and starter cultures (Lanes 1, 2 and 3, V3, Figure 3.3). *Lactobacillus plantarum* appeared at the post milling and salting stages (Lane 5, V3, Figure 3.3). These two bacteria were the main bacteria during production of Stichelton cheese and were still detected in the fully ripened cheese (12 weeks old). *Staphylococcus equorum* and *Enterococcus durans* were found in the crust of a pre-piercing sample but not in the core and veins (Lanes 6 and 7, V3, Figure 3.3). Then, after piercing, these species were found in all parts of the cheese, suggesting redistribution by the piercing process (Lanes 8 and 9, V3, Figure 3.3). *Lactobacillus casei/paracasei*, *Enterococcus faecalis* and *Brevibacterium* sp. were only found in the crust of matured Stichelton cheese suggesting late introduction or development of these populations.

The analysis of Stichelton cheese was repeated to amplify V4 and V5 regions. From Figure 3.3, milk samples contained of three weak bands (Lanes 1 and 2, V4V5, Figure 3.3); *Lc. lactis*, *Bacillus* sp. and an unidentified organism. The starter culture had 5 bands (Lane 3, V4V5, Figure 3.3). The three bands corresponding to *Lc. lactis* were prominent in all steps until pre-piercing. After piercing, these three bands were seen in the crust, but only one bright band was seen in the core which was also seen in the final product. The other two bands were weak bands. *S. equorum* was only detected in the post-piercing core and veins sample (Lane 8, V4V5, Figure 3.3). *Halomonas* sp. was firstly detected in the core of post-piercing but it was then found in the crust of the final cheese.

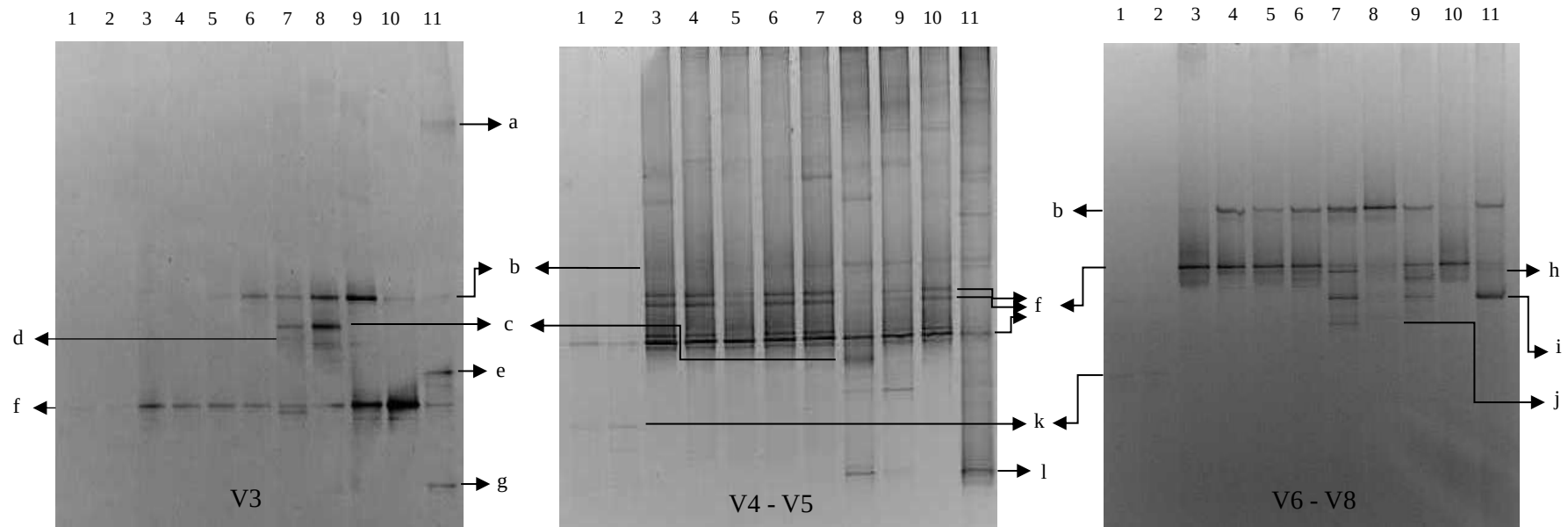


Figure 3.3. Comparison of bacterial DGGE profiles of PCR amplicons of 16S rDNA of Stichelton cheese production using three sets of different primers. Lane 1 and 2, Milk; 3, Starter cultures; 4, Pre-milling; 5, Post-milling and salting; 6, Pre-piercing core and veins; 7, Pre-piercing crust; 8, Post-piercing core and veins; 9, Post-piercing crust; 10, Matured Stichelton core and veins; 11, Matured Stichelton crust. Bands: a, *Lactobacillus casei/paracasei*; b, *Lactobacillus plantarum*; c, *Staphylococcus equorum*; d, *Enterococcus durans*; e, *Enterococcus faecalis*; f, *Lactococcus lactis*; g, *Brevibacterium* sp.; h, *Acinetobacter* sp.; i, *Alkalibacterium* sp.; j, *Corynebacterium casei*; k, *Bacillus* sp.; l, *Halomonas* sp.

The V6-V8 amplified regions, characterised weak bands in the milk sample identified as *Lc. lactis*, *Lactobacillus plantarum* and *Bacillus* sp. (Lanes 1 and 2, V6V8, Figure 3.3). *Lc. lactis* and *Lactobacillus plantarum* bands then were seen through the production process. In the crust of the pre-piercing sample (Lane 7, V6V8, Figure 3.3), three bands were found corresponding to *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei*, none of which were identified in DGGE using primers for the V3 and V4V5 regions. *Acinetobacter* sp. and *Alkalibacterium* sp. were still present in the crust of post-piercing and final product (Lanes 9 and 11, V6V8, Figure 3.3), but *Corynebacterium casei* was not. In the core of the pre-piercing sample (Lane 6, V6V8, Figure 3.3), only *Acinetobacter* sp. was detected and it was present after piercing and in the final product, while *Alkalibacterium* sp. and *Corynebacterium casei* were detected after piercing but then disappeared (Lanes 8 and 10, V6V8, Figure 3.3).

The results of DGGE profiles for the V3 region of 16S rDNA of starter cultures used in Stichelton cheese production, the bulk cells collected from nutrient agar and M17 agar and the isolates picked from nutrient agar, BHI agar, and M17 agar are summarized in Figure 3.4. It is clear that even for the starter culture used in the production of Stichelton cheese which is composed of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, these three strains could not be separated into three distinct DGGE bands. Isolates from nutrient agar and M17 agar also produced one band which was at the same position on the gel as the *Lc. lactis* band from direct DNA extract of the starter culture. For BHI agar, two other bands were detected in one isolate (Figure 3.4, Lane 8) and one faint band in another three isolates (Figure 3.4, Lanes 6, 7 and 9). Unfortunately, they were unidentified.

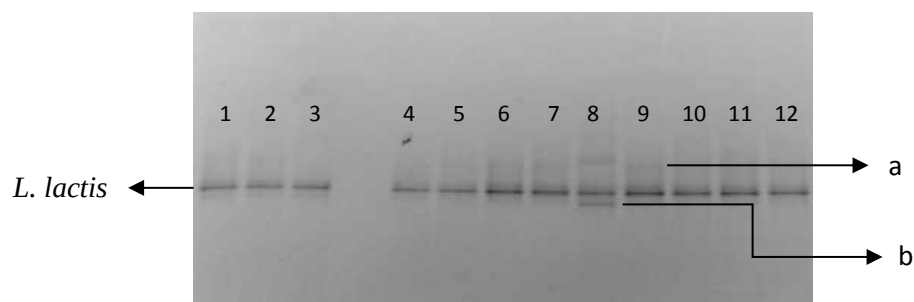


Figure 3.4. DGGE profiles of PCR amplicons of V3 region 16S rDNA of starter culture and bulk cells. Lanes: 1, starter culture (direct DNA extraction); 2, starter culture (nutrient agar, 10^{-1}); 3, starter culture (M17 agar, 10^{-1}); 4-5, starter culture isolates from nutrient agar; 6-9, starter culture isolates from BHI agar; 10-12, starter culture isolates from M17 agar. Bands: a and b, unidentified.

3.4 DISCUSSION

In the present study, the bacterial species present during the production of Stichelton cheese has been examined by using traditional culturing methods and PCR-DGGE analysis of the variable V3, V4V5, and V6V8 regions of the 16S rDNA genes. This method has been successfully used to identify the microbial communities in Artisanal Sicilian cheese (Randazzo *et al.*, 2002), Stilton cheese (Ercolini *et al.*, 2003), Mozzarella cheese (Ercolini *et al.*, 2004), Cabrales cheese (Florez and Mayo, 2006), Domiati cheese (El-Baradei *et al.*, 2007) and Lighvan cheese (Kafili *et al.*, 2009).

According to Table 3.2, the highest viable count of microorganisms was in the post piercing step (9 weeks old), then the viable bacterial counts decreased. Compared to Stilton cheese (Ercolini *et al.*, 2003), the viable counts of bacterial groups of Stichelton cheese on M17 (lactococci), MRS (lactic acid bacteria), Rogosa (lactobacilli) and NA agar (total aerobic microflora) were lower. Also, *Proteus* sp. was only found in the pre-milling step of Stichelton cheese. It then

disappeared in the post-piercing step. This may be because of nutrient competition and pH effect as a consequence of the number of microbes growing at this stage.

Moreover, in terms of pH analysis, in Stichelton, like Stilton and other mould ripened cheese, the pH of the outer crust was higher than that of the inner regions indicating greater mould growth in the crust than in the core. In the beginning of the process, LAB produce acid so that the pH drops, but as the moulds grow, these break down proteins producing ammonia and use up lactic acid, so the pH rises again. Compared to preliminary findings, the pH of the crust of three commercial Stilton cheeses were in the range 6.5 and 7.1 (Yunita, 2010), while in this study, the pH of the outer crust was 8.21 which was at a high level. This may represent that there are different mould species growing or they grow to a different extent.

From DGGE analysis, the initial bacterial communities in the raw milk were quite different from that of the fully ripened cheese. *Lc. lactis* was the main bacteria identified in milk as well as being added as a starter culture and it survived until the end of the process. According to the V4V5 DGGE profile, there were at least three bands identified as *Lc. lactis* in the starter and only one band of *Lc. lactis* was identified in the milk; the three *Lc. lactis* bands were found in the further process. However, only one band of *Lc. lactis* which was identified both in milk and starter culture survived in to the crust of the final cheese.

In milk samples, *Bacillus* sp. was identified using V4V5 and V6V8 primers. The presence of *Lactobacillus curvatus*, *Staphylococcus gallinarum*, *Staphylococcus devriesei*, *Microbacterium* sp., *Sphingobacterium* sp., *Mycetocola* sp. and *Brevundimonas* sp. were detected by the culturing method. However, those microorganisms did not survive in the process. They were not found in Stilton cheese, but Mounier *et al.* (2005) detected *Bacillus* sp. in four Irish smear-

ripened cheeses (Gubbeen, Durrus, Ardrahan and Milleens cheeses). It is likely to be eliminated by low pH (< 4.5) but may depend on the species.

A close relative of *Lactobacillus plantarum* was also found during processing. It was detected from the milk using culturing methods and V6V8 amplification and from the pre-milling step using V3 and V4V5 amplification. It can be concluded that *Lactobacillus plantarum* which was found in the pre-milling sample came from the milk. In the matured Stichelton, *Lactobacillus plantarum* was clearly shown in the core and crust although this was not always evident using all primers or in all samples. This inconsistency of results needs further investigation because *Lactobacillus plantarum* has an important role not only in flavour development (Amarita *et al.*, 2001), but has also been shown to produce inhibition of *Listeria monocytogenes* in a smear-surface soft cheese (Ennahar *et al.*, 1998) as well as *Staphylococcus aureus* and *Salmonella* Typhimurium in Montasio cheese (Stecchini *et al.*, 1991). Moreover, *Lactobacillus curvatus* was also identified in Stichelton cheese which was one of the members of the microbial communities in Stilton (Ercolini *et al.*, 2003). In addition, *Lactobacillus casei/paracasei* was detected in Stichelton, but was not found in Stilton cheese.

It was not surprising that *S. equorum*, *Enterococcus faecalis* and *Enterococcus durans* were found because these bacteria had been shown to be present from preliminary research of Stichelton based on DGGE profiles of cultured cells on BP and KFSA agar (Yunita, 2010). However, the presence of these bacteria in cheese is controversial. According to Gelsomino *et al.* (2001), *Enterococcus faecalis* in cheese is usually assumed to be from faecal contamination and can cause urinary tract infections in immune compromised patients (Coque *et al.*, 1996). On the other hand, these species contribute to

flavour development (Moreno *et al.*, 2006); *Enterococcus* sp. has an impact on glycolysis and proteolysis, whereas *Staphylococcus* sp. have an impact on lipolysis (Molimard and Spinnler, 1996; Smit *et al.*, 2005). It is noticeable that *Enterococcus faecalis* was detected in the V3 profile of mature Stichelton cheese as well as culturing method. No evidence of these organisms was found during processing which suggests these populations may develop towards the end of the ripening period when the pH rises.

Interestingly, the following species: *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were not identified in Stilton cheese (Ercolini *et al.*, 2003). These bacteria are salt-tolerant, less acid-tolerant and usually found in the smear of surface-ripened cheese (Ratnayake and Fox, 1999; Maoz *et al.*, 2003; Mounier *et al.*, 2005; Mounier *et al.*, 2007). It is expected that the presence of these bacteria is caused by salt addition but they grew optimally during ripening when a suitable environment had been reached. However, the influence of *Halomonas* sp., an indicator of hygiene, on final cheese characteristics needs to be analysed further. The presence of *Proteus* sp. in the crust of matured Stichelton and *Kocuria* sp. in the core of the cheese using the culturing method also needs to be analysed.

It is also noteworthy that those surface-ripened cheese bacteria were detected using different primers. *Brevibacterium* sp. was found using the V3 primer, *Halomonas* sp. was detected using the V4V5 primers and *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were amplified using the V6V8 primers. These results suggest that analyzing 16S regions of mixed bacterial population needs various primers, as different bacteria has different region selectivity (Ercolini *et al.*, 2003; Flórez and Mayo, 2006b).

According to Figure 3.4, *Lc. lactis* isolated directly from liquid starter cultures, single colonies and the bulk cells were all detected at the same position in DGGE. However, a faint unidentified band found in all isolates from BHI agar (Figure 3.4, Lane 6-9, Band a) shows that a pure culture can have more than one rDNA sequence as most strains have multiple ribosomal operons and not all the sequences are the same. It is well known that some bacteria produce more than one band because of heterogeneous rDNA copies (von Wintzingerode *et al.*, 1997). Flórez and Mayo (2006b) found two bands in both *Lactobacillus casei* and *Enterococcus faecium*.

3.5 CONCLUSION

The bacterial composition of Stichelton was more complex than that of Stilton cheese. These bacteria came from the raw milk and the salt and different regions of 16S rDNA of the bacteria were amplified producing different DGGE profiles. The study also showed how the piercing process can introduce surface bacteria into the core of the cheese during the ripening process. Differences were seen in the cultured and directly analysed samples. *Lactobacillus casei/paracasei*, *S. equorum*, *Bacillus* sp., *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were only found by molecular methods. However, culturing was still valuable as it detected a large number of raw milk microbiota. It was notable that the *Lc. lactis* survived into the core and crust parts of the final cheese using both methods and it was confirmed as subspecies *lactis*. However, it would need to be established if these are *Lc. lactis* which was found in the milk or in the starter culture. This would establish if raw milk flora are important in contributing to the final flora and its sensory properties.

CHAPTER 4

GENOTYPIC AND PHENOTYPIC DIVERSITY OF *LACTOCOCCUS LACTIS* IN THE PRODUCTION OF STICHELTON CHEESE

4.1 INTRODUCTION

A combination of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* is used as the starter culture in the production of Stichelton. However, unlike Stilton which is made from pasteurised milk, Stichelton uses unpasteurised milk. This may allow the natural microflora in the raw milk to contribute to the fermentation of the milk and also to take part in the ripening process and survive until the final stage of cheese ripening.

From the study on identification of microorganisms in Stichelton cheese production (Chapter 3), *Lc. lactis* was detected in the raw milk, was present as the dominant bacterium in all cheese parts during processing and was detected in the crust and core of fully ripened cheese. The question was therefore whether the *Lc. lactis* isolates surviving in a mature Stichelton comes from the raw milk or the added starter culture. Therefore, in the current work, the survival of *Lc. lactis* isolates through production into a mature Stichelton was examined by sub-species differentiation of the isolates using pulsed-field gel electrophoresis (PFGE). This method was chosen based on the study of Fernández *et al.* (2011) who used this technique to compare phenotypic, genotypic and technological characteristics of *Lc. lactis* isolated from starter-free raw milk cheeses. Their results showed that all 10 strains of a subspecies *lactis* genotype and 8 out of 10 strains of a subspecies

cremoris genotype, isolated from Spanish cheeses (Cabralés, Penamellera, Genestoso and Casín), could be differentiated by this method.

Lc. lactis are also known to carry plasmids of various sizes. Promoting efficient growth in milk (metabolism of lactose, milk proteins and citrate), resistance to environmental stresses (bacteriophages, heavy metals and temperature/osmotic stress) and selective colonization advantages (bacteriocins and exopolysaccharides production) are important plasmid-encoded traits. Unfortunately, plasmid DNA can be lost or acquired because of its mobility (Sewaki *et al.*, 2001; Siezen *et al.*, 2005; Mills *et al.*, 2006; Fallico *et al.*, 2011). So, a second question was whether the surviving *Lc. lactis* isolates still contained important plasmids or not.

4.2 OBJECTIVES

The objectives of this work were to detect the origin of the matured Stichelton *Lc. lactis* isolate and to determine the plasmid carriage and its stability in the *Lc. lactis* isolate.

4.3 RESULTS

4.3.1. PHENOTYPIC CHARACTERIZATION OF STARTER CULTURES AND CHEESE ISOLATES

A total of two hundreds isolates were grouped into seven groups on the basis of different biochemical and morphology characteristics which were Gram positive-cocci-catalase negative-non group D of Lancefield grouping test (106 isolates; presumptive *Lactococcus*), Gram positive-cocci-catalase negative-group D (31 isolates; presumptive *Enterococcus*), Gram positive-cocci-catalase positive (40 isolates; presumptive *Staphylococcus*), Gram positive-rod-catalase negative

(22 isolates; presumptive *Lactobacillus*) and Gram negative-rod-catalase negative (1 isolate) (Table 4.1). The number of *Lactococcus* isolates in the isolated population of Stichelton cheese both inner core and outer crust parts were lower than that of in both starter culture. Also, while powdered starter culture contained a presumptive *Lactococcus* only, the liquid starter culture contained a presumptive *Lactococcus*, a presumptive *Enterococcus* and a presumptive *Lactobacillus*. From Figure 4.1, it is not surprisingly that presumptive *Lactococcus* was a dominant flora in starter culture, whereas the cheese had a more mixed flora. Furthermore, it is interesting to note that M17 agars were not very selective for *Lactococcus* as *Staphylococcus* and *Enterococcus* was found on this agar.

Table 4.1. Characteristics and numbers of bacterial isolates isolated from liquid starter cultures, powdered starter cultures, Stichelton core and veins, and Stichelton crust.

SAMPLES	Non group D				Group D			Total
	Gram positive			Gram negative	Gram positive			
	Catalase positive	Catalase negative		Catalase negative	Catalase positive	Catalase negative		
	cocci	cocci	rods	rods	cocci	cocci	rods	
Liquid Starter Cultures	0	47	1	0	0	2	0	50
Powdered Starter Cultures	0	50	0	0	0	0	0	50
Stichelton Core & Veins	18	2	7	0	4	19	0	50
Stichelton Crust	9	7	9	1	9	10	5	50
Total	27	106	17	1	13	31	5	200

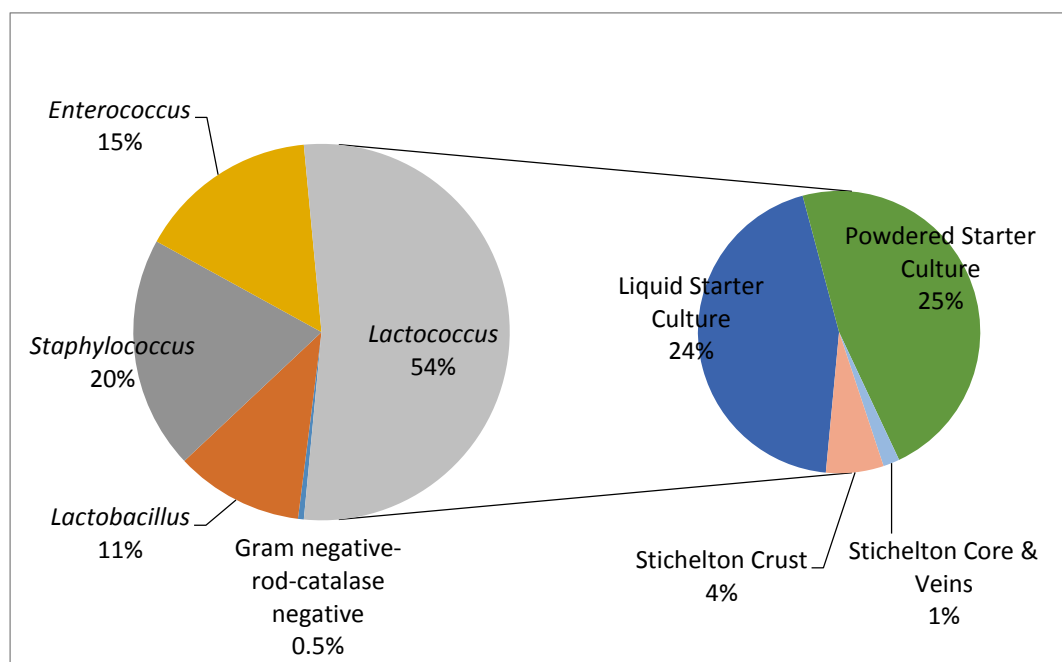


Figure 4.1. The percentage of bacterial isolates isolated from liquid starter cultures, powdered starter cultures, Stichelton core and veins, and Stichelton crust.

4.3.2. PFGE PROFILES OF *LACTOCOCCUS LACTIS* STRAINS

A dendrogram of the *Sma*I restriction patterns of twenty-six *Lc. lactis* strains which were confirmed by sequencing and three *Lc. lactis* controls is shown in Figure 4.2. The PFGE profiles of *Lc. lactis* strains produced good profiles and were significantly differentiated into three clusters at 40% similarity (Appendix 2). All strains from powdered starter culture were clustered together with eight strains from liquid starter culture, one strain from Stichelton crust and *Lc. lactis* subsp. *cremoris* NCIMB 8662. Two strains found in the liquid starter culture were similar with *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484, but not similar with any strain from Stichelton core and veins or crust. Two strains found in the core and veins of mature Stichelton had profiles different from those obtained from the liquid and powdered starter culture strains. One of these strains had a similar profile with *Lc. lactis* subsp. *lactis* NCIMB 6681.

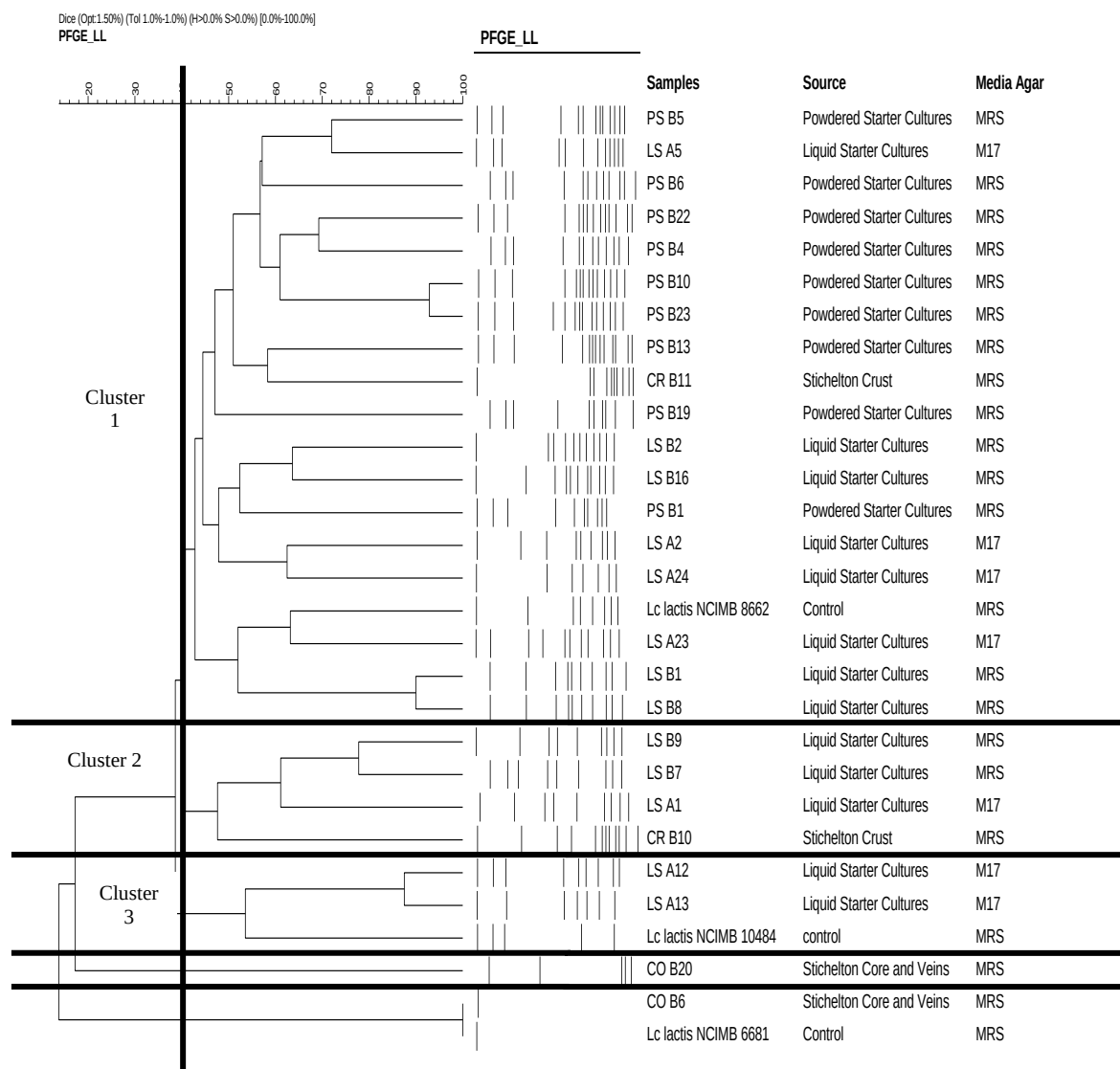


Figure 4.2. Dendrogram and *Sma*I restriction patterns of *Lactococcus lactis*.

Clustering was performed by the UPGMA method. The coefficient of similarity (40%) is indicated on the figure by the bold vertical line.

4.3.3. PLASMID PROFILE OF *LACTOCOCCUS LACTIS* ISOLATES

Six strains from powdered starter, seven strains from liquid starter and four strains from Stichelton cheese out of the twenty six *Lc. lactis* strains shown in the PFGE profile were subjected to plasmid analysis. Based on Figure 4.3,

powdered starter and liquid starter strains contained plasmid DNA, but Stichelton strains did not have any plasmids.

From powdered starter, strains PS B1, PS B20 and PS B23 had the same three plasmids with molecular weights at 2 kb, 48.5 kb and more than 48.5 kb, strain PS B5 and PS B22 were lacking plasmid DNA and strain PS B13 had plasmids ranging from 3 to 5 kb in size. From liquid starter, three out of the seven isolates showed unique patterns which were different from the powdered starter isolates plasmid profiles. Strain LS A12 carried 10 plasmids ranging between 2.8 kb and 50 kb while strain LS A13 showed 8 bands of similar sizes to the plasmids of LS A12. Strain LS A24 had a weak single band corresponding to 4 kb.

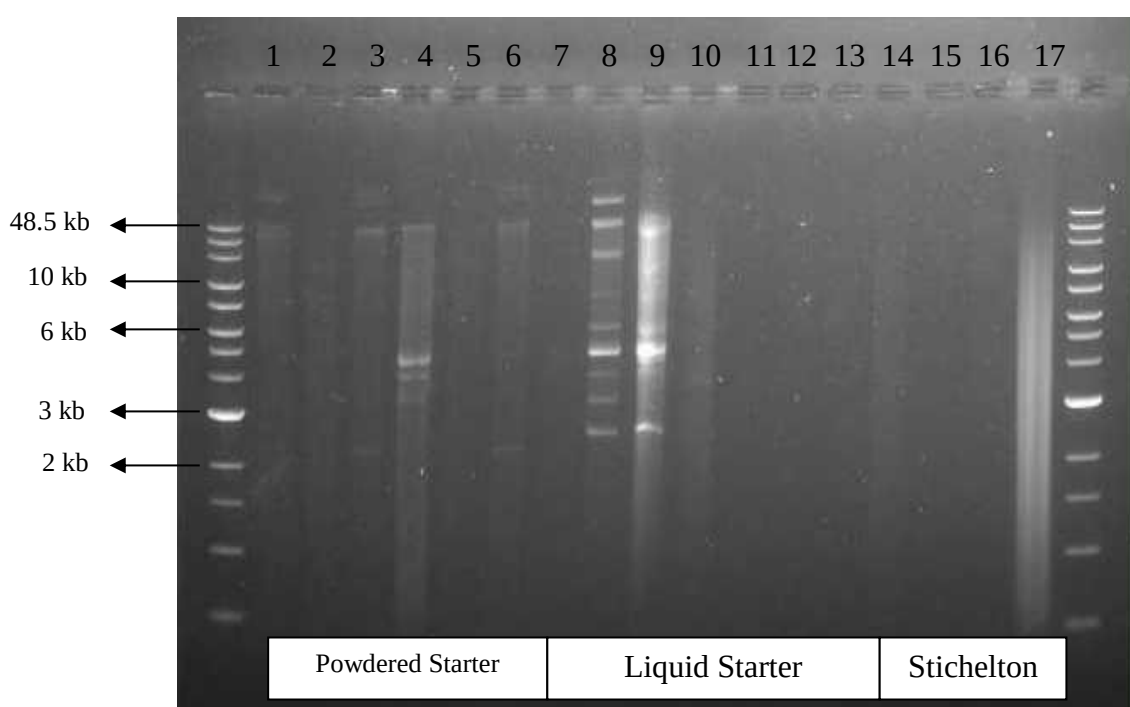


Figure 4.3. Plasmid profiles of *Lactococcus lactis* in Stichelton cheese and the added starter cultures. Lanes: 1, PS B1; 2, PS B5; 3, PS B10; 4, PS B13; 5, PS B22; 6, PS B23; 7, LS A2; 8, LS A12; 9, LS A13; 10, LS A24; 11, LS B2; 12, LS B9; 13, LS B16; 14, CO B6; 15, CO B20; 16, CR B10; 17, CR B11.

4.3 DISCUSSION

The bacterial flora was dominated by *Lactococcus* in the starter culture as expected. The presence of only *Lactococcus* in the powdered starter culture showed that this starter culture was pure so it is recommended to be used in Stichelton cheese production. In the liquid starter culture however, 5% of the isolates were not morphologically *Lactococcus* but included Gram positive-catalase negative-cocci, Gram positive-catalase negative-rods and Gram negative-catalase negative-cocci groups (Table 4.1). According to Bergey's Manual of Determinative Bacteriology (2000) and other published studies, these groups could belong to *Enterococcus* spp. and *Bacillus* spp./*Lactobacillus* spp./*Corynebacterium* spp. (Brennan *et al.*, 2002; Mounier *et al.*, 2005). The third group, Gram negative-catalase negative-cocci, showed high similarity to a soil bacterium by 16S rDNA sequencing.

In mature Stichelton cheese, the *Enterococcus* spp., and presumptive *Staphylococcus* spp. and *Bacillus* spp./*Lactobacillus* spp./*Corynebacterium* spp. became dominant whereas the *Lactococcus* population dropped. This result supports findings in traditional water buffalo mozzarella cheese and Artisanal Sicilian cheese where the number of *Lc. lactis* decreased in line with increasing ripening time (Randazzo *et al.*, 2002; Ercolini *et al.*, 2004). Mounier *et al.* (2005) also detected only two lactic acid bacteria isolates out of 50 isolates in Gubbeen cheese, an Irish farmhouse smear-ripened cheese. From unpublished results by Hansen, lactococci counts in the rind of Danablu cheese decreased rapidly while *Lactobacillus* spp. became a dominant bacterium (Cantor *et al.*, 2004).

The genetic diversity and relatedness of 26 selected strains from liquid starter culture, powdered starter culture and matured Stichelton cheese were examined further using PFGE analysis. This method has been successfully used to

see diversity at the strain level of 39 presumed lactococcal strains in Spanish starter-free farmhouse cheeses (Delgado and Mayo, 2004), 40 *Lc. lactis* strains from Batzos, a Greek PDO raw goat milk cheese in different seasons (Psoni *et al.*, 2007) and 20 *L. lactis* strains from five raw milk cheeses without added starter culture (Fernández *et al.*, 2011).

In this study, the patterns of *Lc. lactis* from Stichelton were compared to *Lc. lactis* of two commercial starter cultures used in its production. Based on Figure 4.2, reference strains which consisted of *Lc. lactis* subsp. *lactis* NCIMB 6681, *Lc. lactis* subsp. *cremoris* NCIMB 8662, and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* NCIMB 10484 were grouped in different clusters whereas all selected strains from powdered starter culture formed one cluster. It seems that only one subspecies of *Lc. lactis* which was similar with *Lc. lactis* subsp. *cremoris* NCIMB 8662 was detected in this type of starter culture. As it was also clustered with one strain from the crust of Stichelton cheese, we can conclude that *Lc. lactis* subsp. *cremoris* could survive until the end of processing. This is an expected result as the subspecies *cremoris* is the preferred cheese starter culture due to typical aroma compound production (Salama *et al.*, 1993; Nomura *et al.*, 1999).

Lc. lactis subsp. *lactis* biovar. *diacetylactis* NCIMB 10484 clustered together with LS B9 and LS B7 from liquid starter culture and no isolates from any part of Stichelton cheese were present in cluster 3. However, there is no information which type of starter cultures was used in this particular batch of cheese making. Therefore, it cannot be concluded whether the biovar from liquid starter could not survive into fully ripened Stichelton cheese or diacetyl was developed at the beginning of Stichelton production only as different batch may use different type of starter cultures.

One strain found in the core and veins, CO B20, was significantly different from those in the commercial starter cultures ($P < 0.01$) at 40% similarity. The same result applied to CO B6 strain where this strain had a similar PFGE pattern to *Lc. lactis* subsp. *lactis* NCIMB 6681. Thus, both CO B20 and CO B6 strains probably survived from the raw milk. Many studies reported that raw milk is the source of *Lc. lactis* subsp. *lactis*. According to Nomura *et al.* (1999), *Lc. lactis* subsp. *lactis* are able to produce γ -aminobutyric acid (GABA) which could maintain acidic conditions.

Furthermore, looking at Cluster 2 in Figure 4.2, there were two liquid starter strains from MRS grown anaerobically grouped with one liquid starter strain from M17 grown aerobically and one Stichelton crust strain. No reference strains belong to this cluster showing that these three strains had different PFGE pattern from the reference strains.

The *Lc. lactis* strains were subjected to plasmid profiling to examine whether these strains had plasmids or not. Strains displaying completely different plasmid profiles were considered as different genotypes (Psoni *et al.*, 2007). Compared to powdered starter, *Lc. lactis* from liquid starter had more plasmid bands. This may indicate the liquid starter strains had more important factors which could have an impact on sensory properties than the powdered starter. It is interesting to note that all *Lc. lactis* isolated from Stichelton were a plasmid-free. It suggests the *Lc. lactis* strains which survived until the final product was a natural strain as suggested by the PFGE study or it was a mutant starter. So, the significance of this study is that the raw milk used in the production of Stichelton cheese has *Lc. lactis* strains which have the potential to be a starter organism which have characteristics which would allow them to survive until the final step of maturing.

4.4 CONCLUSION

Identification of the *Lc. lactis* strains found in raw milk and in the final cheese was important as they could be a potential starter culture for Stichelton cheese production. Therefore, the phenotypic diversity of *Lc. lactis* strains isolated from a raw milk blue-vein Stichelton cheese was compared to two types of starter cultures used by the cheese makers. PFGE analysis was effective in distinguishing three different subspecies of *Lc. lactis*. *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* was detected in liquid starter culture only and the subspecies *cremoris* in both starters. The survival of *Lc. lactis* subsp. *lactis* in the core of Stichelton cheese was presumed to be from the raw milk. Indeed, no plasmids were observed in Stichelton strains, which distinguish the strains from the starter culture.

CHAPTER 5

CHARACTERISTICS OF *STAPHYLOCOCCUS* *EQUORUM* ISOLATED FROM CHEESES

5.1 INTRODUCTION

In Stilton and Stichelton cheeses production, *S. equorum* is not an added starter culture. From Chapter 3, *S. equorum* was detected in the outer crust of the Stichelton cheese pre-vein piercing and in all parts of the cheese post-vein piercing by PCR-DGGE analysis, but this bacterium was not detected in the fully ripened Stichelton. There is no published study about the role of *S. equorum* in the production of Stichelton cheese. Therefore, *S. equorum* was first isolated from Stichelton cheese by culturing method using mannitol salt agar (Oxoid CM0085). For comparative purpose, *S. equorum* was also isolated from Stilton (core and crust), Danish Blue and Reblochon (core and crust). The isolation from these three cheeses was done in association with Jihan Sherif, an MSc Applied Biomolecular Technology student in 2012.

Presumptive *S. equorum* isolates were easily distinguished from other staphylococci by their ability to produce acid from mannitol after 2 days incubation at 30°C so that the colour of the mannitol salt agar was changed from pink into yellow. This ability is usually associated with presumptive coagulase positive staphylococci which it can be distinguished from on the basis of the coagulase test. The biochemical characteristics of the isolates were examined using API Staph (BioMérieux). The PCR assay targeting the manganese-dependent superoxide dismutase (*sodA*) gene was then used to identify *S. equorum* because of its rapid and reliable identification (Blaiotta *et al.*, 2004). The

diversity of the isolates was then evaluated by PFGE. Five isolates chosen from different cheeses and different PFGE clusters were then examined for tolerance to acid and salt.

5.2 OBJECTIVES

The objectives of this work were to look for the presence of *S. equorum* in fully ripened Stichelton cheese compared to Stilton, Danish Blue and Reblochon, and to characterize the five selected *S. equorum* isolates so that one of these could be evaluated as a potential adjunct culture in cheese making.

5.3 RESULTS

5.3.1. IDENTIFICATION OF *STAPHYLOCOCCUS EQUORUM*

A total of 82 *Staphylococcus* was isolated from Stilton core, Stilton crust, Danish Blue, Reblochon core, Reblochon crust (15 isolates from each sample) and Stichelton (2 isolates from pre-piercing crust, 3 isolates from post-piercing core and 2 post-piercing crust; Yunita, 2010). From coagulase tests, 61 isolates (11 Stilton core, 9 Stilton crust, 14 Danish Blue, 13 Reblochon core, 10 Reblochon crust, 1 pre-piercing Stichelton crust, 1 post-piercing Stichelton core and 2 post-piercing Stichelton crust) were confirmed as coagulase negative *Staphylococcus*. Unfortunately, no isolates were identified as *S. equorum* by API tests. Isolates were identified as *S. xylosus*, *Micrococcus* sp., *Kocuria varians* and *S. sciuri*.

Therefore, the coagulase negative *Staphylococcus* were analysed by PCR amplification of the *sodA* gene to identify *S. equorum* and were confirmed by 16S rDNA sequencing. The results showed that a total of 26 *Staphylococcus* isolates from Stilton, Danish Blue and Reblochon were confirmed as *Staphylococcus*

equorum subsp. *equorum*, whereas four Stichelton isolates were not. Figure 5.1 shows all the confirmed *S. equorum* isolates.

The colony morphology of *S. equorum* on mannitol salt agar can be observed in Figure 5.2b and 5.2c and the cell morphology of *S. equorum* can be observed in Figure 5.2d. The colony morphology was yellow, opaque, puntiform, convex, entire edge, butyrous, less than 1 mm in diameter after 2 d on MSA plate at 30°C.

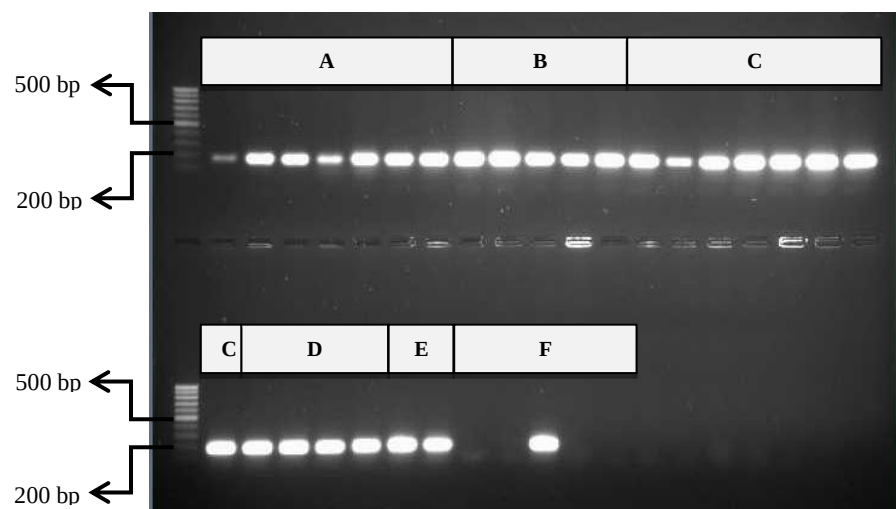


Figure 5.1. The *sodA* gene PCR of *Staphylococcus equorum* isolated from: A, Stilton core; B, Stilton crust; C, Danish blue; D, Reblochon core; E, Reblochon crust; and F, Controls.

Section A: A2, A5, A8, A21, A26, A27, A28

Section B: B2, B3, B7, B9, B12

Section C: C6, C8, C9, C11, C13, C14, C15, C18

Section D: D9, D11, D13, D19

Section E: E3, E19

Section F: *Staphylococcus aureus* NCTC 1803, *Staphylococcus epidermidis* NCTC 11047, *Staphylococcus equorum* NCTC 12414 and *Staphylococcus xylosus* NCIMB 13636, negative control

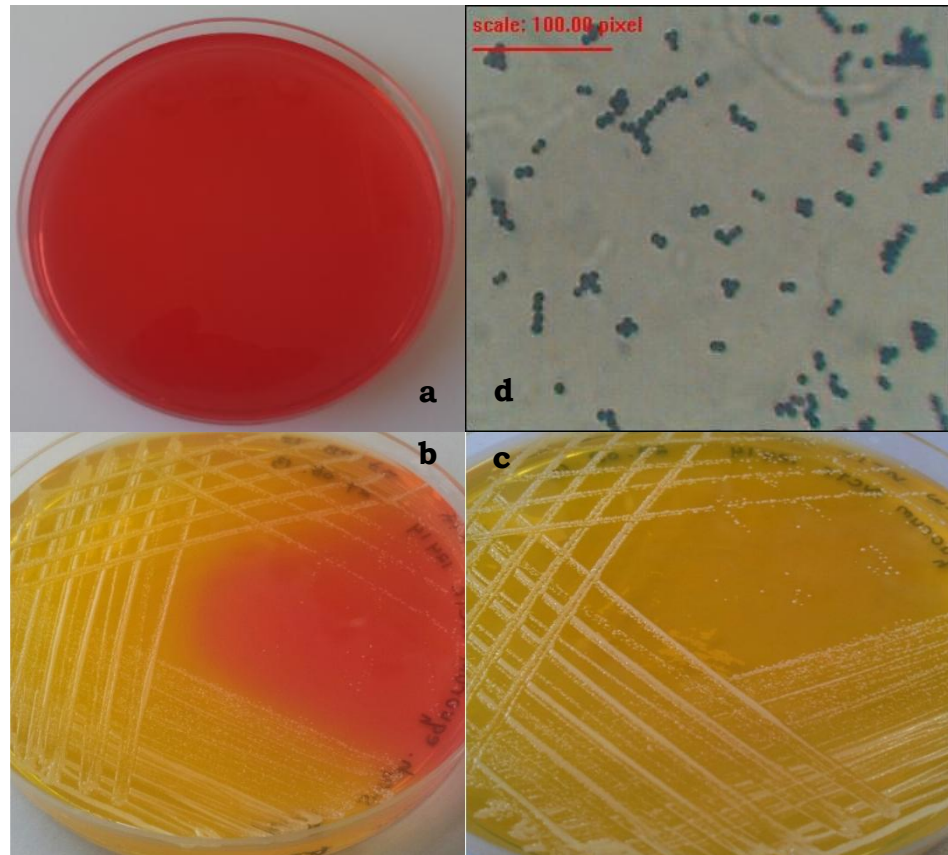


Figure 5.2. *Staphylococcus equorum*: a, on mannitol salt agar (MSA) before incubation; b, on MSA after incubation at 30°C for 2 days; c, on MSA after incubation at 30°C for 2 days and kept at 4°C for 2 days; d, microscopic appearance of Gram stain at 100X magnification.

5.3.2. PFGE PROFILE OF *STAPHYLOCOCCUS EQUORUM* ISOLATES

The dendrograms of *S. equorum* subsp. *equorum* isolated from Stilton, Danish Blue and Reblochon cheese using *Sma*I, *Sac*I and *Apa*I are shown in Figures 5.3, 5.4 and 5.5 respectively. The control strains *S. equorum* NCTC 12414 and *S. xylosus* NCIMB 13636 gave distinct but closely related profiles and were clustered together in all analyses.

In *Sma*I profile (Figure 5.3), clusters were significantly different from each other at 50% similarity (Appendix 3). All isolates in the main cluster, cluster 1, were grouped with *S. equorum* NCTC 12414 and *S. xylosus* NCIMB 13636. This indicates the isolates were similar although they were from different cheeses. However, there were some subclusters which related to cheese of isolation. Isolates B2 and B3 from Stilton cheese crust and isolate E3 from Reblochon crust clustered separately from the main cluster indicating that these isolates were different from other strains.

In the *Sac*I profile (Figure 5.4), characterization of the seventeen *S. equorum* isolates by PFGE analyses revealed a significant difference at 50% (Appendix 3). This profile had two main clusters in which all Reblochon isolates grouped together in one cluster. The Danish Blue and Stilton isolates formed another main cluster. The C6 and C9 isolates from Danish Blue cheese were distinguish from the main clusters.

In the *Apa*I profile (Figure 5.5), the analysis of PFGE profile showed that the majority of isolates were clustered with 55% relatedness among the profiles (Appendix 3). The cluster groupings were similar to the *Sma*I groupings.

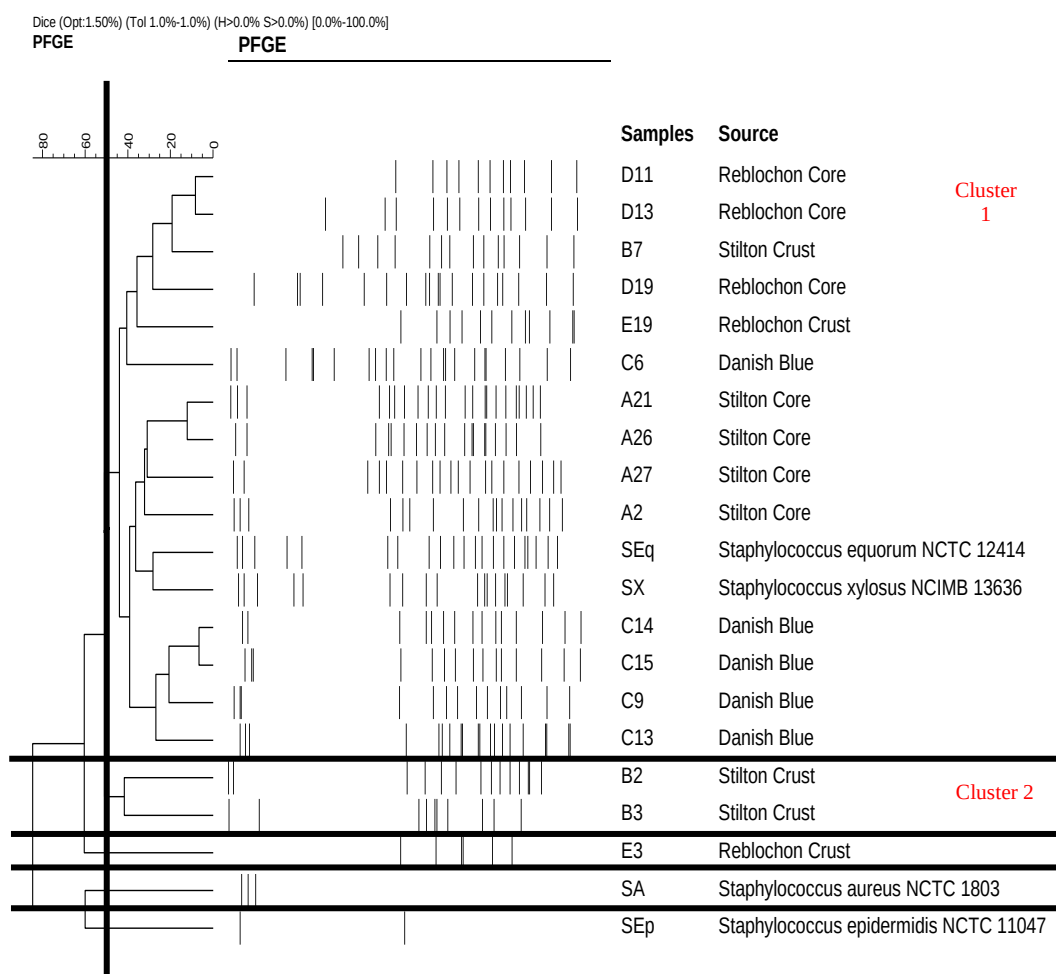


Figure 5.3. Dendrogram and *Sma*I restriction patterns of *Staphylococcus equorum*. Clustering was performed by the UPGMA method. The level of similarity for cluster distinction (50%) is indicated on the figure by the bold vertical line.

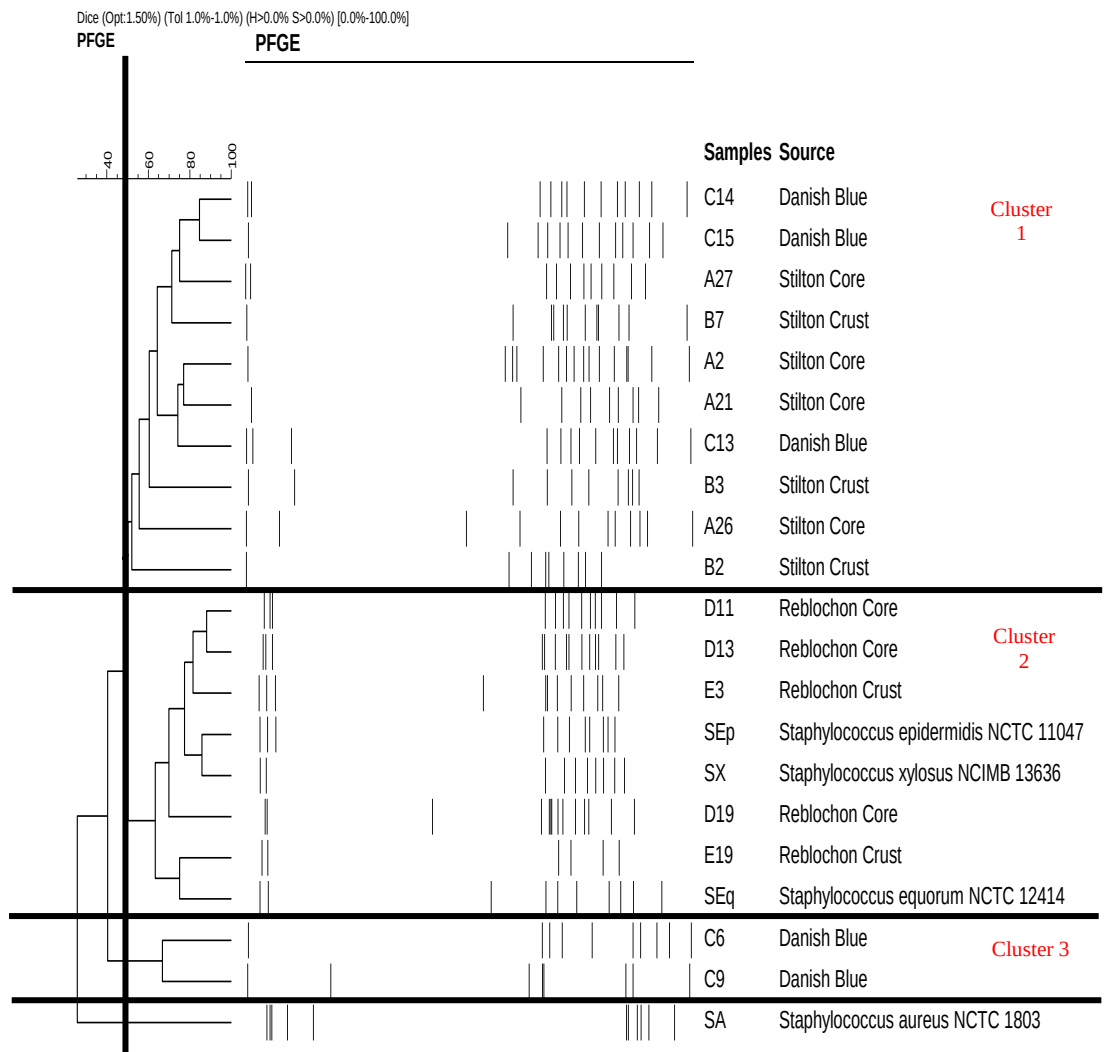


Figure 5.4. Dendrogram and *SacI* restriction patterns of *Staphylococcus equorum*. Clustering was performed by the UPGMA method. The level of similarity for cluster distinction (50%) is indicated on the figure by the bold vertical line.

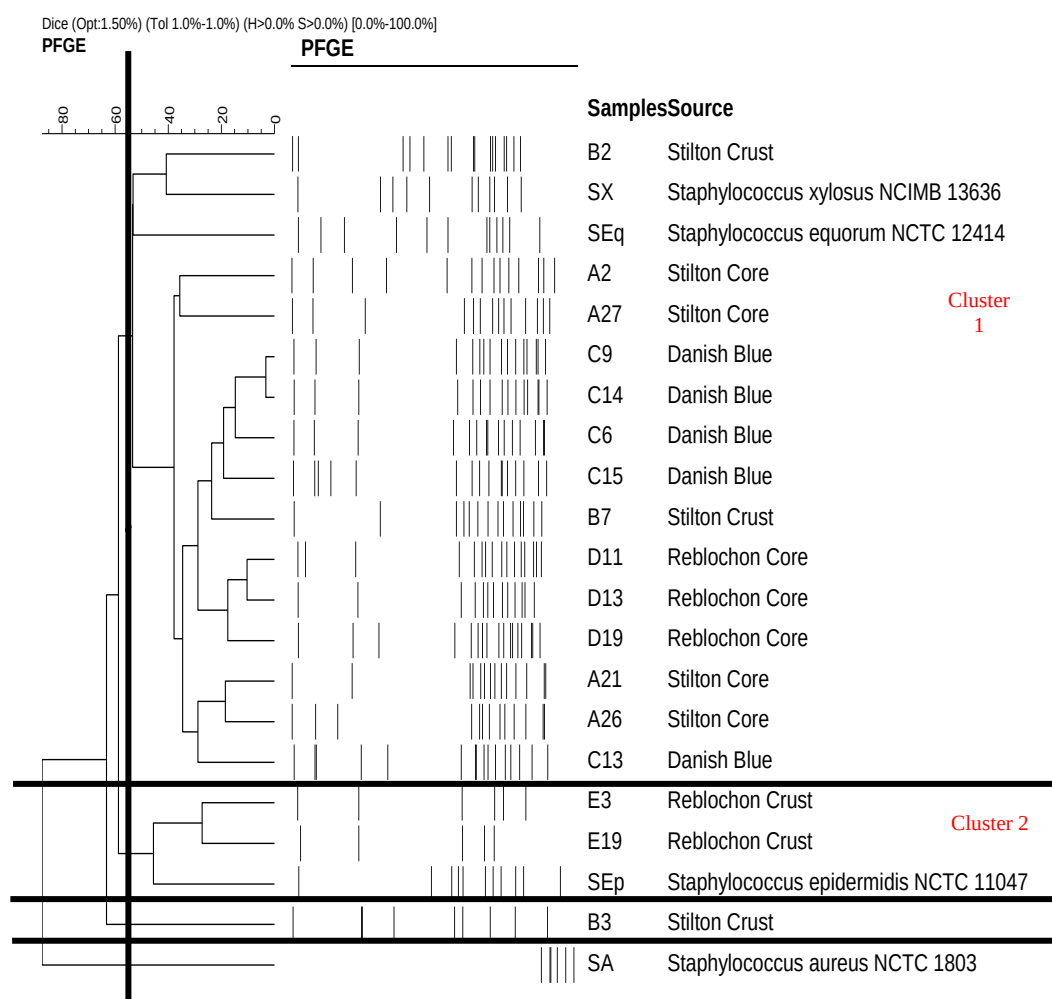


Figure 5.5. Dendrogram and *ApaI* restriction patterns of *Staphylococcus equorum*. Clustering was performed by the UPGMA method. The level of similarity for cluster distinction (55%) is indicated on the figure by the bold vertical line.

5.3.3. BIOCHEMICAL CHARACTERISTICS OF *STAPHYLOCOCCUS EQUORUM* SUBSP. *EQUORUM*

From Table 5.1, it is clear from these results that *S. equorum* subsp. *equorum* isolates had different characteristics in fermentation. While isolates A2, B2, D19 and E3 formed acid from D-glucose, D-fructose, D-mannose, D-maltose, D-lactose and D-trehalose, isolate C13 which was isolated from Danish Blue, did not ferment these sugars. All isolates showed activity with D-mannitol, β -naphthyl phosphate, sodium pyruvate, D-xylose and N-acetyl-glucosamine but no activity with L-arginine was identified. Activity with potassium nitrate was shown only on Stilton isolates (A2 and B2).

5.3.4. THE RESPONSE OF *STAPHYLOCOCCUS EQUORUM* TO ACID

Acid tolerance of five *S. equorum* isolates (A2, Stilton core; B2, Stilton crust; C13, Danish Blue; D19, Reblochon core; and E3, Reblochon crust) was examined at 37°C in BHI broth acidified with lactic acid to pH 3, 4, 5, 6 and 7 (control). One ml of each culture broth was collected at 0 h, 4 h, 8 h, 24 h and 48 h and was serially diluted in BHI broth for plating. The viable count of the *S. equorum* isolates was then analysed using the statistics as mentioned in Section 2.11.5. Figure 5.6 shows the response of these five isolates to acid. The significance of acid tolerance (as evaluated by ANOVA) of the *S. equorum* isolates can be seen in Appendix 4. The pH of different parts of the cheeses (Table 5.2) was also analysed to identify the pH of the isolates in their original sources. The hypothesis was *S. equorum* isolates might give different responses to the respective pH in an acidified broth.

Table 5.1. Biochemical characteristics of *Staphylococcus equorum* subsp. *equorum* isolated from Stilton core (A), Stilton crust (B), Danish blue (C), Reblochon core (D) and Reblochon crust (E).

Biochemical Assay	A2	B2	C13	D19	E3
D-Glucose	+	+	-	+	+
D-Fructose	+	+	-	+	+
D-Mannose	+	+	-	+	+
D-Maltose	+	+	-	+	+
D-Lactose	+	+	-	+	+
D-Trehalose	+	+	-	+	+
D-Mannitol	+	+	+	+	+
Xylitol	-	-	-	-	+
D-Melibiose	-	-	-	-	+
Potassium nitrate	+	+	-	-	-
β -Naphthyl phosphate	+	+	+	+	+
Sodium pyruvate	+	+	+	+	+
D-Raffinose	+	-	-	-	-
D-Xylose	+	+	+	+	+
D-Saccharose (sucrose)	+	-	+	+	+
Methyl- α D-glucopyranoside	+	-	-	+	+
N-acetyl-glucosamine	+	+	+	+	+
L-Arginine	-	-	-	-	-
Urea	+	+	-	-	-

Table 5.2. pH of different parts of Stilton, Danish Blue and Reblochon.

SAMPLES		Replicates			Average	SD
		R1	R2	R3		
Stilton	Crust	6.36	6.24	6.66	6.42	0.22
	Veins	8.02	7.95	7.35	7.77	0.37
	Core	4.80	5.98	4.32	5.03	0.85
Danish Blue	Veins	7.52	8.98	7.72	8.07	0.79
	Core	5.74	5.40	5.61	5.58	0.17
Reblochon	Crust	7.43	7.03	7.20	7.22	0.20
	Core	5.37	5.28	5.11	5.25	0.13

Based on Figure 5.6, the five *S. equorum* isolates could grow for 96 h at pH 7. However, isolates from different cheeses showed different trends over 72 h incubation. While *S. equorum* isolated from Reblochon-crust, Stilton-core, Stilton-crust and Danish Blue grew after a short lag, the isolate from Reblochon core showed an extended lag over 48 h (Figure 5.6d; 9 log increased from 3 log₁₀ CFU/ml). At pH 6 and 5, the numbers decreased and finally were undetectable at 24 h or 48 h depending on the isolate. Distinct differences were found between *S. equorum* isolate E3 from Reblochon crust (cheese pH > 7) which remained steady at pH 6 for 72 h (Figure 5.6e; 7 log₁₀ CFU/ml) before loss of viability and *S. equorum* isolate C13 from Danish Blue (cheese pH 6) which was not detected at pH 6 after 8 h (Figure 5.6c). The two Stilton isolates (A2 and B2) showed similar acid tolerance despite being isolated from different pH environments (Stilton cheese core pH 5.03±0.85; Stilton cheese crust pH 6.42±0.22). None of the isolates grew at pH 4 and 3 confirming that *S. equorum* is not very tolerant to acid.

5.3.5. THE RESPONSE OF *STAPHYLOCOCCUS EQUORUM* TO SALT

Salt tolerance of the same five *S. equorum* isolates was examined at 37°C in BHI broth containing 10, 8, 5, 3.5 and 0% (control) (w/v) sodium chloride. The viable count of the *S. equorum* isolates was also analysed statistically as mentioned in Section 2.11.5. Figure 5.7 shows the response of these five isolates to salt and the significance in salt tolerance (as measured by ANOVA) of the *S. equorum* isolates can be seen in Appendix 5.

From Figure 5.7, the five *S. equorum* isolates could grow up to 10% salt, but different genotypes showed different growth characteristics again showing

strain variability. Decreasing the percentage of salt increased the final level of growth of *S. equorum*. At 10% salt, the Stilton core isolate A2 reached 8 log₁₀ CFU/ml in 24 h and grew better than the Stilton crust B2 isolate which grew to less than 6 log₁₀ CFU/ml, however the core isolate started to decrease after 72 h (Figure 5.7a and 5.7b). The Danish Blue isolate C13 was quite sensitive showing no increase in counts ($P>0.05$; Figure 5.7c) at 10% salt. In contrast to the Stilton isolates, the Reblochon crust isolate E3 grew better at 10% and grew to almost 8 log₁₀ CFU/ml in 48 h, while the core isolate only grew to 7 log₁₀ CFU/ml after 96 h and so has a slower growth (Figure 5.7d and 5.7e). However, the Reblochon crust isolate started to die off after 48 h. This may probably be because this isolate has gone through the whole growth cycle faster and has used up a key component in the medium preventing continued growth. At 8 and 5% salt, the trends of growth were very similar with the lower percentage of salt giving higher counts. At 3.5% salt, all *S. equorum* entered stationary phase after 72 h and grew to 10 log₁₀ CFU/ml except the Reblochon crust isolate E3 which stopped growing after 48 h and grew to 9 log₁₀ CFU/ml. Interestingly, in salt tolerance, the growth trend of Stilton and Reblochon isolates from the crust was more varied than the core.

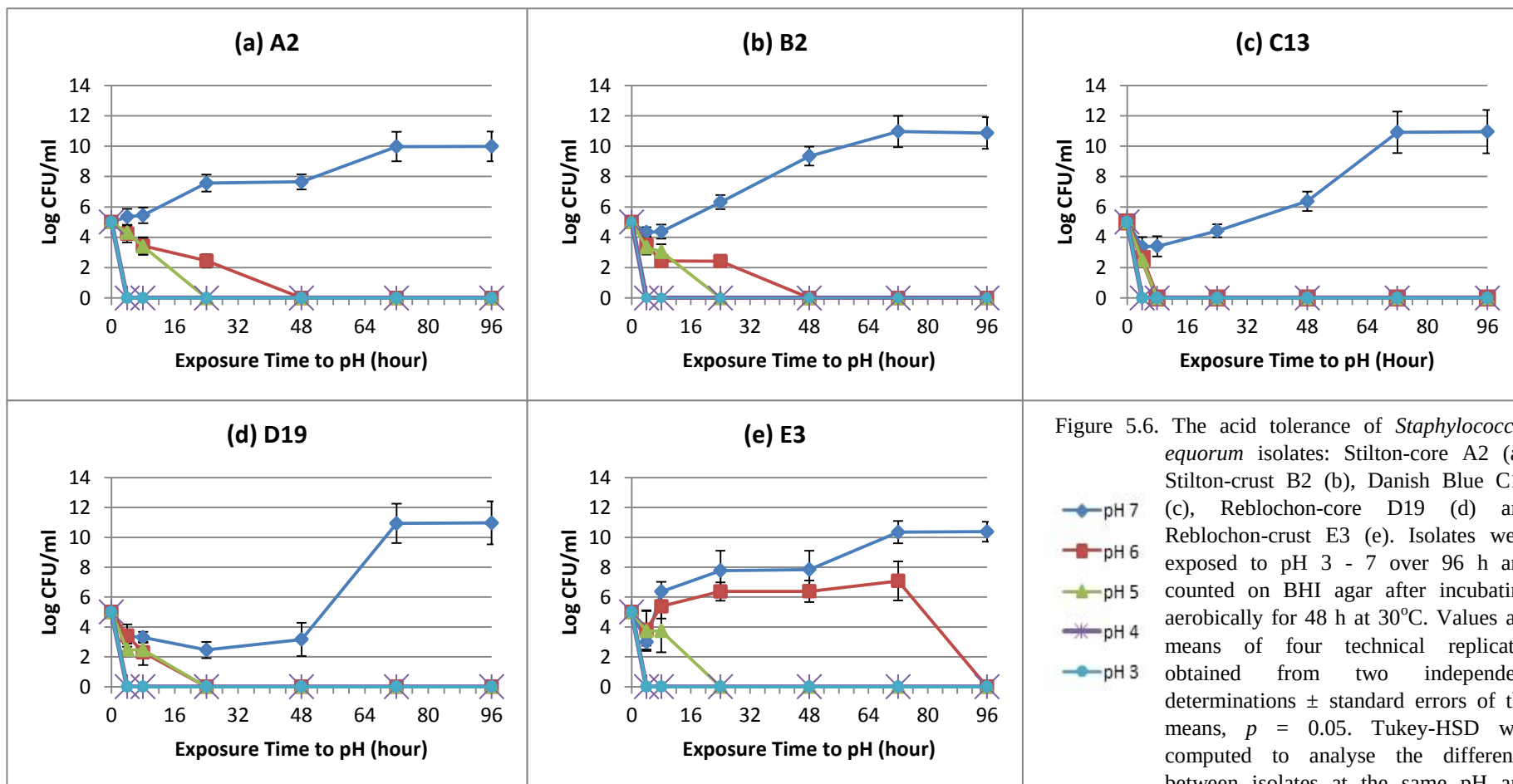


Figure 5.6. The acid tolerance of *Staphylococcus equorum* isolates: Stilton-core A2 (a), Stilton-crust B2 (b), Danish Blue C13 (c), Reblochon-core D19 (d) and Reblochon-crust E3 (e). Isolates were exposed to pH 3 - 7 over 96 h and counted on BHI agar after incubating aerobically for 48 h at 30°C. Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors of the means, $p = 0.05$. Tukey-HSD was computed to analyse the difference between isolates at the same pH and same exposure time (Appendix 4).

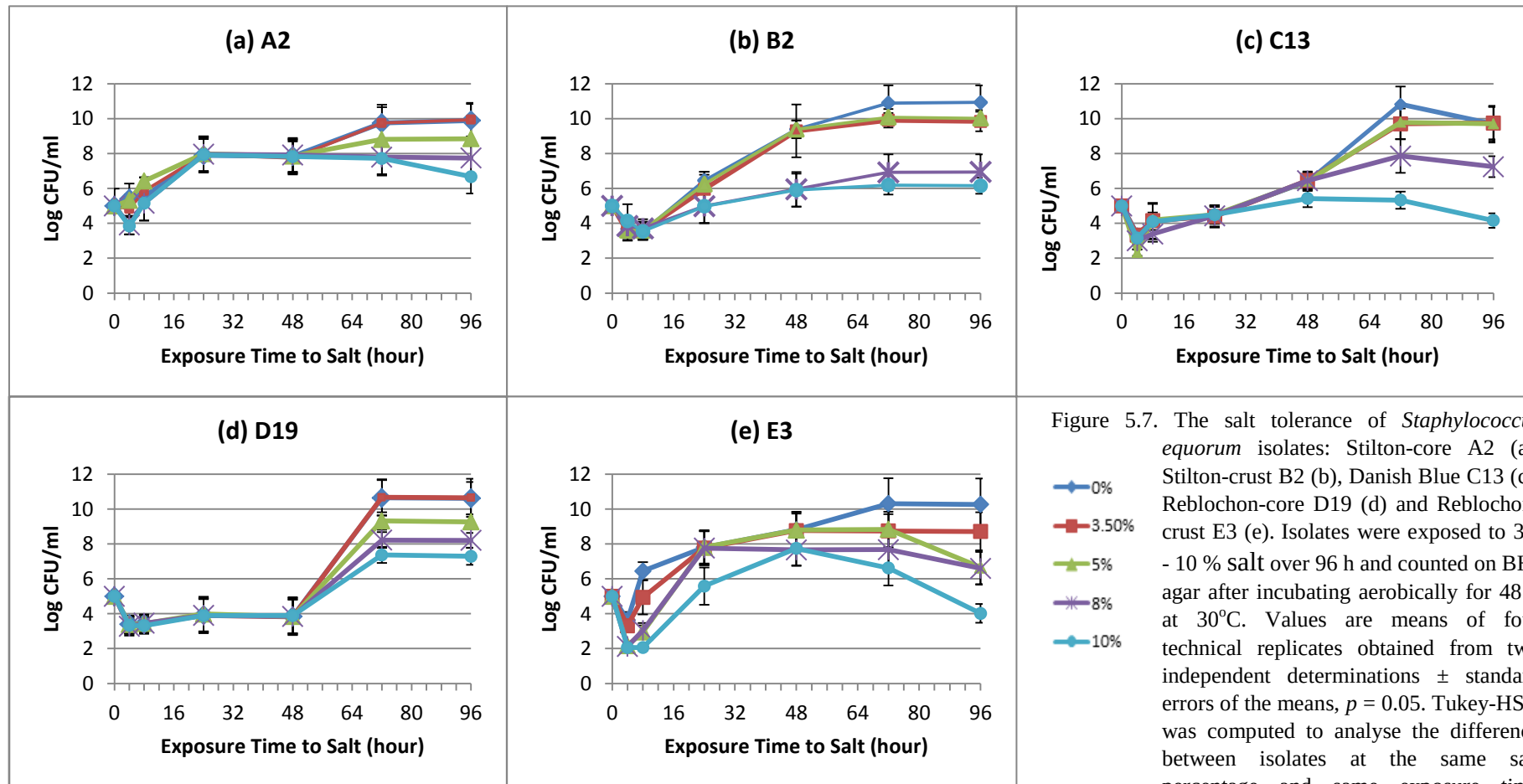


Figure 5.7. The salt tolerance of *Staphylococcus equorum* isolates: Stilton-core A2 (a), Stilton-crust B2 (b), Danish Blue C13 (c), Reblochon-core D19 (d) and Reblochon-crust E3 (e). Isolates were exposed to 3.5 - 10 % salt over 96 h and counted on BHI agar after incubating aerobically for 48 h at 30°C. Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors of the means, $p = 0.05$. Tukey-HSD was computed to analyse the difference between isolates at the same salt percentage and same exposure time (Appendix 5).

5.4 DISCUSSION

A total of twenty six *S. equorum* isolated from Stilton (crust and core), Danish Blue and Reblochon (crust and core) were confirmed by PCR amplification of the *sodA* gene (Sherif, 2012). This method was suggested by Poyart *et al.* (2001) and was successfully used to identify *S. equorum* in Southern Italian sausages and water buffalo milk (Blaiotta *et al.*, 2004).

The use of *sodA* gene as a target gene to identify *S. equorum* is more recommended than API tests because of the rapidity (18 mins PCR) and accuracy. It can be used to overcome the inaccurate designation given by the API Staph test which usually placed *S. equorum* isolates as *S. xylosus* and/or *S. sciuri*. Some studies have previously reported that the database was not able to discriminate *S. equorum* (Ieven *et al.*, 1995; Meugnier *et al.*, 1996; Irlinger *et al.*, 1997). Hoppe-Seyler *et al.*, (2004) and Irlinger *et al.*, (2008) also did not suggest the API tests for *S. equorum* identification because of less reliable.

However, the API kit is still useful to characterize the biochemical characteristics of the staphylococci isolates. The ability of *S. equorum* subsp. *equorum* isolates to ferment D-mannitol supports the find that these isolates produced acid from this sugar causing the phenol red in the mannitol salt agar turn to yellow. In addition, the fact that this bacterium is coagulase negative means this distinguishes it from other coagulase negative bacteria and thus means this selective and diagnostic agar could be used in the first step selection of *S. equorum* species.

In this study, *S. equorum* isolates showed a low genetic diversity with only 6 out of 17 isolates having distinct PFGE profiles. A high diversity of the PFGE profiles of *S. equorum* (52 out of 118 isolates) isolated from naturally fermented sausages and their manufacturing environment was reported by Leroy *et al.*

(2009). According to Coton *et al.* (2010), *S. equorum* is one of the food-related staphylococci species and has been proposed as a starter culture for smear-ripened cheese by Bockelmann (2002). Both subspecies *equorum* and *linens* could be used as a component of starter cultures in smear-ripened semi-hard cheeses (Irlinger *et al.*, 2012). Therefore, distinct isolates could be used as a potential starter culture.

Unfortunately, in the PFGE analysis, the use of *smaI*, *sacI* and *apaI* enzymes failed to discriminate the control isolates *S. equorum* and *S. xylosus* as they were clustered together. The percentage of similarity of these species was 50% for *smaI* and *sacI* and 55% for *apaI*. It seems that different restriction enzymes may be used to solve the problem. *EcoRI* and *HindIII* could be used as another option (Meugnier *et al.*, 1996; Nováková *et al.*, 2006). In 1996, Meugnier *et al* mentioned that *S. equorum* can be distinguished from *S. xylosus* by colony size and morphology on P agar. While *S. equorum* forms small colonies (<6 mm) without pigmentation and grows preferably at 30°C, *S. xylosus* forms larger colonies (>6 mm) with yellow pigment and grows preferably at 37°C.

Five isolates of *S. equorum* which were genotypically distinct (A2, B2, C13, D19 and E3) were chosen from different cheeses and different PFGE clusters. These isolates were examined to see their response to acid and salt which were associated with the cheese microenvironment conditions. In this experiment, the response of the *S. equorum* isolates to ionised acid (H^+ ion concentration) was analysed by adding the lactic acid (non-ionised acid). Both molecules were present in various proportions which depend on the selected pH range (3 - 7) so that the microbial stress effect is due to presence of both molecules.

The non-dissociated form of lactic acid is a small molecule and tends to diffuse through the cell wall into the cytoplasm causing intracellular pH (pHi) decline. A larger intracellular pH gradient (ΔpHi) is the effect of acid-sensitive

cells failing to buffer H^+ ions. This inactivates the microbial enzymes' activity by collapsing the electrochemical proton gradient and stops their growth by having a bactericidal effect (Pan *et al.*, 2009). At higher concentrations, the organic anion (lactate) will be accumulated leading to failure of material transport across the membrane. This causes cell starvation and death (Pieterse *et al.*, 2005). Because of that, all *S. equorum* isolates examined in this study could not grow at pH 3 and 4 so they were not very tolerant to low pH.

This is totally different from some species of the *Lactobacillus* genus which can grow at a low pH. *Lactobacillus rhamnosus* isolated from Parmigiano Reggiano cheese exhibited a good survival after 2 and 4 h of incubation at pH 3 (Succi *et al.*, 2005) and *L. plantarum* isolated from Stilton cheese showed a good growth at pH 4.5 - 6 (Mugampoza, 2013). A similar study was conducted by Pan *et al.* (2009) on *L. acidophilus* NIT isolated from infant faeces. The results showed that *L. acidophilus* NIT could survive at pH 3 and 4 for 3 h incubation while at pH 2, it could not survive after 2 h. Most strains (66%) of *L. casei* and *L. paracasei* isolated from cheese, sourdoughs, wine and the human body were also able to grow after 2 h incubation at pH 2.5 (Reale *et al.*, 2015).

In salt tolerance, the present work showed that even though the *S. equorum* isolates were isolated from Stilton (2.2 – 2.7 % salt; Cantor *et al.*, 2004), Danish Blue (3 – 3.9 % salt; Cantor *et al.*, 2004) and Reblochon cheese (1.3 – 1.5 % salt; Farnham, 2015), they were all tolerant to high levels of sodium salt (up to 10 %), with the higher salt percentage giving the lower viable counts of *S. equorum*. Unlike lactobacilli which grow at low salt levels (<5%), all bacteria present on the cheese surface like coryneforms and staphylococci are salt tolerant up to 15% NaCl (Ferchichi *et al.*, 1985; Collin and Law, 1989). According to Jeong *et al.* (2014), all *S. equorum* (39 isolates) from Jeotgal, a Korean high-salt fermented

seafood, had sustained growth on tryptic soy agar containing 15% NaCl, 37 isolates could grow in 20% NaCl condition and 13 isolates could grow in 25% NaCl condition for 72 h.

In Stichelton cheese, *S. equorum* was firstly detected in the crust of pre-piercing cheese by PCR-DGGE method only (Chapter 3). It was not detected in the matured Stichelton, even though the pHs of the cheeses at this step (pH 6.36 ± 0.24 , core and veins; pH 8.21 ± 0.31 , crust; Table 3.2) were at the optimal pH for *S. equorum* growth. The 2.5 – 3 % salt which was added into the curd after the milling process and the increasing salt percentage because of whey syneresis were also a suitable condition for *S. equorum* growth. This indicates that there is another factor preventing this bacterium being found in the final step. According to Brennan *et al.* (2002), *Staphylococcus* spp. are replaced by coryneform bacteria after about 15 days ripening supporting the findings in the PCR-DGGE study.

5.4 CONCLUSION

S. equorum does not tolerate low pH levels suggesting it must have been introduced at the initial fermentation as the pH at this stage is around 5 and once the mould grew on the surface and the pH rose. Hence including this organism in a mixed culture with the initial starter bacteria is unlikely to prove successful. *S. equorum* does tolerate high salt levels, but the numbers of *S. equorum* were much higher at lower salt concentrations. Surface inoculation prior to piercing (for blue-veined cheeses) is one potential method of introduction of this bacterium. No potential isolates of *S. equorum* were detected by *sodA* gene-PCR from the crust pre-piercing, the core and the crust post-piercing Stichelton cheese, meaning that this bacterium was in a very low number. From PFGE, three *S. equorum* isolates (B2 and B3, Stilton Crust; E3, Reblochon crust) were differentiated from other

isolates using the *SmaI* restriction enzyme. Two isolates (C6 and C9, Danish Blue) were differentiated using *SacI* enzyme and three isolates (B3, Stilton crust; E3 and E19, Reblochon crust) were differentiated using *ApaI* enzyme making these isolates distinct. However, further study is needed to make this bacterium as a part of a potential starter culture. In addition, the source of *S. equorum* needs to be further investigated as this is not an added starter culture. Salt, shelving, knife for rubbing-up and cheesemakers' hand are some potential sources.

CHAPTER 6

INTERACTION BETWEEN STARTERS

LACTOCOCCUS LACTIS, *PENICILLIUM*

ROQUEFORTI AND NON-STARTER

STAPHYLOCOCCUS EQUORUM

6.1 INTRODUCTION

Cheese is a complex ecosystem of microorganisms. In blue-veined Stilton cheese, the addition of the mould *P. roqueforti* and the presence of non-starter bacteria, mould and yeast together with the cheese structure (core, veins and crust) make the cheese ecosystem more complex. The use of raw milk in blue-veined Stichelton cheese adds to this complexity from the beginning of the cheese-making process. Interaction between these microorganisms is not only a result of nutrient competition, but also metabolite production and in blue cheeses these interactions could have an effect on the growth of *P. roqueforti* (Beresford and Williams, 2004) and therefore the final cheese characteristics.

In the previous chapter, *S. equorum* was shown to be isolated from blue-veined and smear-ripened cheeses. However, the contribution this non-starter bacterium to cheese characteristics and potential interactions with the starters *Lc. lactis* and *P. roqueforti* have not previously been studied. Therefore, in this chapter, the interaction between a mixed commercial starter of *Lc. lactis*, *P. roqueforti* and non-starter *S. equorum* B2 isolated from the crust of commercial Stilton cheese (Chapter 5) was investigated in a blue-veined cheese model and on media agar. The procedures of blue-veined cheese making were adopted from

those used for Stilton cheese (personal communication with Joe Schneider and Randolph Hodgson) and were modified for a lab-scale model in a study by Whiley (2013).

6.2 OBJECTIVES

The objectives of this work were to determine if *S. equorum* survives early fermentation or whether its presence is due to contamination during ripening as suggested by the PCR DGGE study (Chapter 3); to investigate the role of *S. equorum* in influencing microbial and physical characteristics of mould-ripened cheese; and also to examine the antifungal activity of *S. equorum* which could inhibit the sporulation of *P. roqueforti*.

6.3 RESULTS

Interaction between starters *Lc. lactis*, *P. roqueforti* and non-starter *S. equorum* B2 was examined in a cheese model which was made following the method given in Section 2.12.1. Six batches of model cheeses (Table 2.5) which were based on three experimental factors were examined for the physical characteristics (appearance, texture and water activity), pH, microbial counts and flavour volatiles. The difference between factors in water activity, pH and microbial counts were statistically analysed with Tukey-HSD test. The flavour volatiles are presented in Chapter 7. The antifungal activity of *S. equorum* was then examined on Potato Dextrose Agar (PDA; Oxoid CM0139) as described in Section 2.12.5.

6.3.1. PHYSICAL CHARACTERISTICS OF CHEESE MODELS

The appearance of the six model cheeses every week for 4 weeks of ripening can be observed in Figures 6.1 – 6.6. It is clear to see that in the model cheeses made without the addition of starter *P. roqueforti* (C1, C2 and C3), a surface layer of *Penicillium* growth still occurred. This is likely to be due to environmental contamination with this organism which is hard to exclude during the production process. With the addition of *P. roqueforti* (C4), a thick surface layer of mould developed in one week. An effect of the addition of *S. equorum* (C5 and C6) is the equivalent growth of *Penicillium* appears to develop only after two weeks. This is true for both the contaminant mould and the added starter. A greater suppressive effect was seen by spreading *S. equorum* on the surface of the models after rubbing-up. This caused the colour of the crust to be paler than for models C4 and C5 and could be an influence on sporulation which is what gives the mould its characteristic colour. Lack of vein development was also seen in all cheeses even piercing was done every week during ripening. The core appearance of the model cheeses can be observed in Figure 6.7.



Figure 6.1. Cheese made with *Lactococcus lactis*.

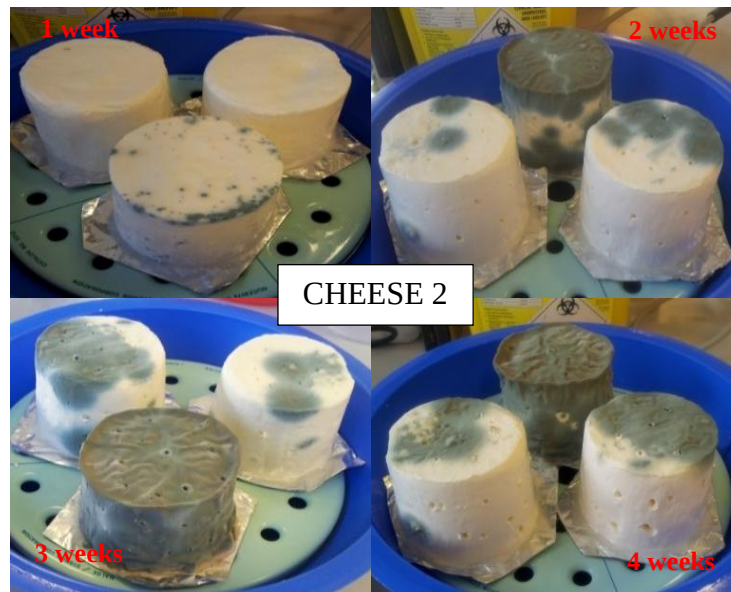


Figure 6.2. Cheese made with *Lactococcus lactis* and incorporated *Staphylococcus equorum*.



Figure 6.3. Cheese made with *Lactococcus lactis* and surface-spread *Staphylococcus equorum*.



Figure 6.4. Cheese made with *Lactococcus lactis* and *Penicillium roqueforti*.



Figure 6.5. Cheese made with *Lactococcus lactis*, incorporated *Staphylococcus equorum* and *Penicillium roqueforti*.



Figure 6.6. Cheese made with *Lactococcus lactis*, surface-spread *Staphylococcus equorum* and *Penicillium roqueforti*.

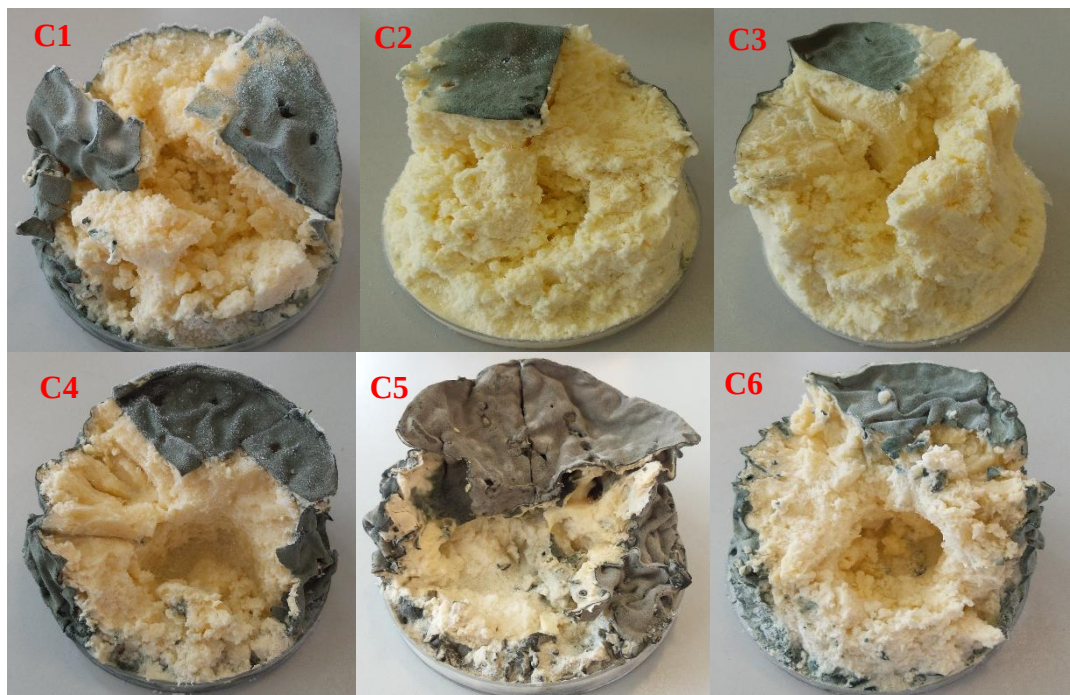


Figure 6.7. The core appearance of model cheeses after one month ripening. Cheese made with: *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6).

The hardness of the model cheeses (Figure 6.8) was analysed using a texture analyzer (Section 2.12.4). From all model cheeses, the highest peak force of the first compression cycle was seen for the cheese with *Lc. lactis*, *P. roqueforti* and surface-spread *S. equorum* (C6). In comparison with commercially produced cheeses, this model is harder than Danish Blue, but still a lot softer than Stilton. Compared to the model cheeses made by Whiley (2013) where the hardness of the cheese made with *Lc. lactis* and *P. roqueforti* was at around 15 g, the hardness of the cheese made with *Lc. lactis* and *P. roqueforti* and the addition of surface-spread *S. equorum* at the beginning of ripening (C6) slightly increased to 17 g. However, the addition of *S. equorum* during fermentation (1.5 h after the addition of starter *Lc. lactis*) either with or without the starter *P. roqueforti* (C5 and C2 respectively) produced the softest cheese (approx. 8 g; Figure 6.8).

Water activity (a_w) of the models was tested to determine whether the softness of C2 and C5 was because these retained more water. Figure 6.9 shows in cheeses made without *P. roqueforti* (C1, C2, C3), either incorporated *S. equorum* (C2) or surface-spread *S. equorum* (C3) did not affect the water activity ($P > 0.05$). In cheeses made with *P. roqueforti* (C4, C5, C6), the different application of how *S. equorum* was introduced into the models (C5 and C6) also had no effect on the a_w at all. This suggests that C2 and C5 were softer than other cheeses examined because of other factors such as more proteolysis.

The average of the water activity of all model cheeses was then compared with the control cheeses. The results (Figure 6.10) showed that the a_w of model cheeses was higher than that of commercially produced Stilton cheese and more closely resembled the moisture content of Wensleydale. Also, the a_w of the core of the model cheeses was significantly higher than the a_w of the crust of the model cheeses ($P < 0.05$).

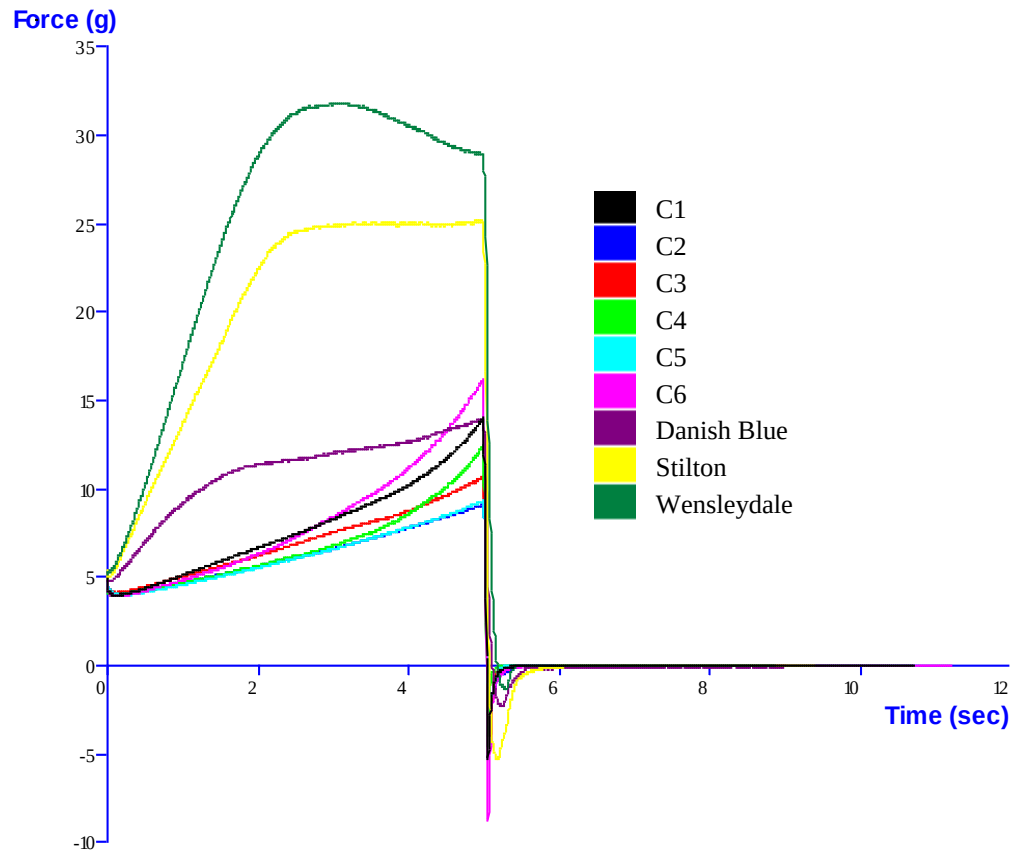


Figure 6.8. Texture of cheese models compared to the commercial cheeses. Cheese made with: *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6).

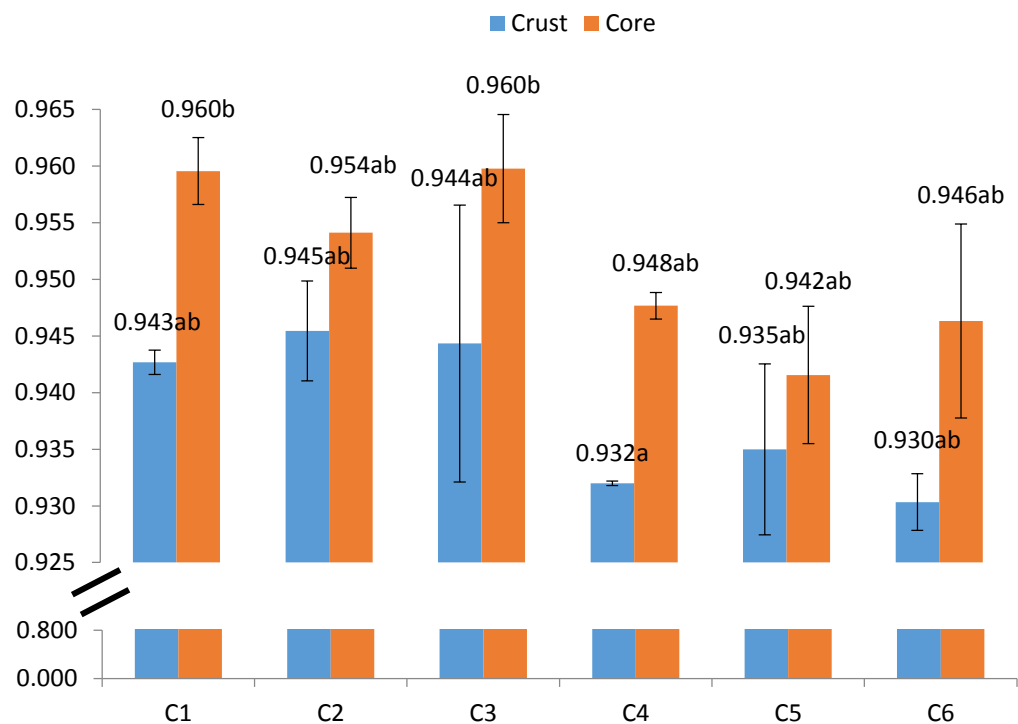


Figure 6.9. Water activity of crust and core of six model cheeses after one month ripening. Values are means of nine technical replicates obtained from three independent experiments and error bars are $\pm$ standard errors of the means, $P = 0.05$. Bars with the same letter are not different ($P > 0.05$). Tukey-HSD was computed to analyse the difference between water activity of the crust and core of the model cheeses. The significant ANOVA of the water activity can be seen in Appendix 6a. Cheese made with: *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6).

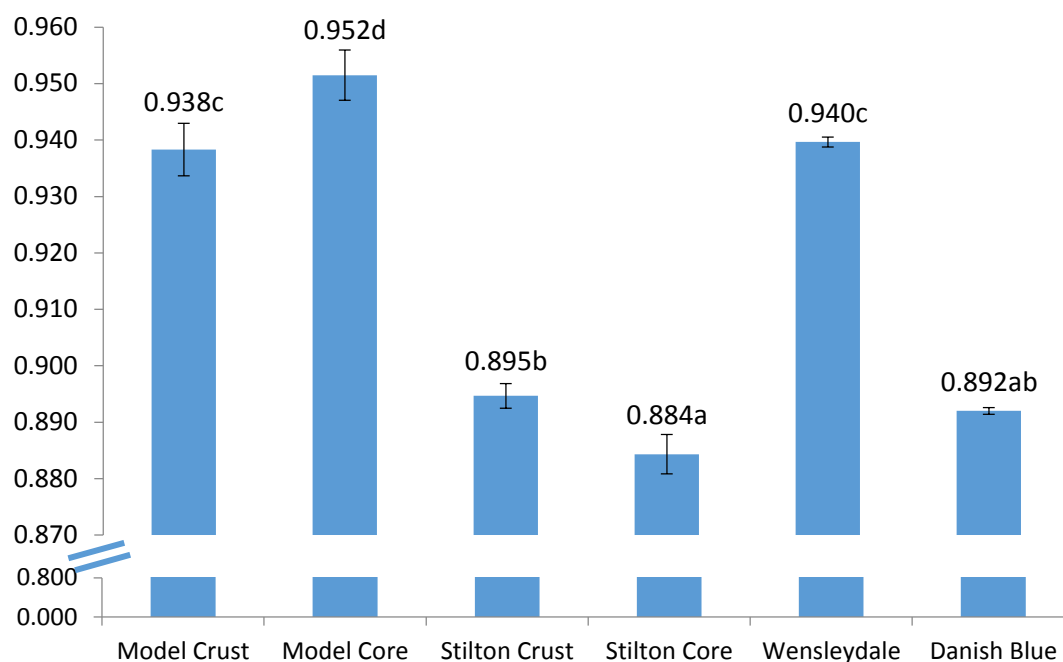


Figure 6.10. Water activity of average model cheese in comparison to commercially produced cheeses. Values are means of nine technical replicates obtained from three independent experiments and error bars are $\pm$ standard errors of the means, $P = 0.05$. Bars with the same letter are not different ($P > 0.05$). Tukey-HSD was computed to analyse the difference between water activity of the average model cheese and control cheeses. The significant ANOVA of the water activity can be seen in Appendix 6b.

6.3.2. pH

The pH of the six model cheeses throughout the production can be observed in Tables 6.1. Tukey-HSD was computed to analyse the difference between pH of the the model cheeses in each cheese making step. The significant ANOVA of the microbial counts can be seen in Appendix 7.

From Table 6.1, the different methods of application of *S. equorum* into the models and the use of the starter *P. roqueforti* did not produce significant changes in the pH of samples obtained from milk, after fermentation, pre-salting, post-salting and pre-rubbing up ($P>0.05$). The pH profiles of all cheese models decreased at the beginning of production reaching a pH of around 4 in 4 hours which was expected as the pH of Stilton curd during fermentation was about 4.5 (Whitley, 2002).

During ripening, the core pH did not increase significantly but the pH increased in the crust of some of the cheeses, as is seen in commercial Stilton (Brindley, 1954), which is due to mould growth. Noticeably, there was no significant difference in pH between the crust and core of the model cheeses with the contaminant *Penicillium* (C1, C2, C3). It was different for model cheeses with the added *P. roqueforti* (C4, C5, C6) which showed the pH of the crust was significantly higher than those of the core ($P<0.05$). This demonstrates that the starter mould has specific properties needed to give typical Stilton characteristics.

Incorporation of *S. equorum* into the models by both methods produced similar pH profiles until 4 weeks of ripening. At week 4, in cheeses made without *P. roqueforti* (C1, C2, C3), the pH of the crust for the cheeses without *S. equorum* (C1; 5.15) were significantly higher than these of the cheeses with *S. equorum* (C2 and C3; 3.99; $P<0.05$), indicating less mould growth in these two models from the visual results. However, in cheeses made with *P. roqueforti* (C4, C5, C6), the presence of *S. equorum* in C5 and C6 had no effect on the pH which was not significantly different with the C4.

Table 6.1. The pH value at each sampling point of cheeses production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors (SE) of the means, $P = 0.05$.

SAMPLES			C1		C2		C3		C4		C5		C6	
			pH	SE	pH	SE	pH	SE	pH	SE	pH	SE	pH	SE
Milk			6.6a	0.15	6.60a	0.08	6.69a	0.04	6.67a	0.03	6.64a	0.13	6.70a	0.06
Incubated milk (hours)	0		5.68bc	0.25	4.49a	0.14	5.03ab	0.10	4.87a	0.09	4.52a	0.08	5.88c	0.13
	4		5.16b	0.04	4.27a	0.05	4.34a	0.10	4.31a	0.13	4.34a	0.03	4.62ab	0.32
	8		4.68b	0.11	4.26ab	0.07	4.19a	0.04	4.21a	0.11	4.22ab	0.04	4.38ab	0.16
	24		4.21a	0.10	4.10a	0.07	4.05a	0.15	4.08a	0.21	4.08a	0.08	4.19a	0.09
Pre-salting curd			3.96a	0.06	3.54a	0.12	3.65a	0.11	3.67a	0.09	3.63a	0.11	3.78a	0.05
Post-salting curd			3.62a	0.04	3.57a	0.22	3.64a	0.13	3.70a	0.09	3.64a	0.10	3.74a	0.11
Pre-rubbing up			4.45b	0.31	4.50b	0.09	4.41ab	0.09	4.55b	0.13	3.53a	0.14	3.80ab	0.27
Ripened cheese (weeks)	1	Crust	3.72a	0.17	3.73a	0.16	3.63a	0.13	7.08b	0.33	7.08b	0.02	7.17b	0.13
		Core	3.65a	0.07	3.73a	0.02	3.71a	0.10	3.74a	0.21	3.74a	0.38	3.68a	0.30
	2	Crust	3.87a	0.19	3.81a	0.04	3.91a	0.07	7.05b	0.04	7.09b	0.11	7.25b	0.07
		Core	3.8a	0.16	3.68a	0.07	3.86a	0.04	3.85a	0.10	3.65a	0.10	3.75a	0.25
	3	Crust	4.65b	0.07	4.23ab	0.09	3.95a	0.11	7.59c	0.14	7.16c	0.08	7.18c	0.08
		Core	3.89a	0.06	3.75a	0.13	3.67a	0.06	3.95a	0.39	3.97a	0.11	3.76a	0.06
	4	Crust	5.15b	0.38	3.88a	0.12	3.99a	0.15	7.64c	0.10	7.28c	0.11	7.34c	0.04
		Core	3.93a	0.04	3.76a	0.26	3.77a	0.05	4.21a	0.10	4.17a	0.07	4.10a	0.16

Means in the same row labelled with different letters (a, b, c) are significantly different ($P < 0.05$) as shown by ANOVA.

6.3.3. MICROBIAL COUNTS

The viable counts of aerobic and anaerobic microorganisms on five media (PCA, MRS, M17, RBCA and MSA) throughout the production of the six model cheeses can be observed in Tables 6.2 – 6.6. Tukey-HSD was computed to analyse the difference between microbial counts of the the model cheeses in each medium and in each cheese making step. The significant ANOVA of the microbial counts can be seen in Appendix 8. No microorganisms were detected in the UHT milk each time the model cheeses were made indicating a good starting point for a model. During the fermentation, the number of lactococci, LAB and total aerobic counts increased gradually from about 11 log₁₀ CFU/ml before fermentation to 15 log₁₀ CFU/ml after 24 h fermentation. There was no significant impact on *Lactococcus*, LAB and total aerobic counts by adding *S. equorum* 1.5 h after *Lc. lactis* addition in cheeses C2 and C5 (Tables 6.2, 6.3, and 6.4 respectively; P>0.05). From Table 6.5, yeasts and moulds were not detected in the cheeses without starter *P. roqueforti* (C1, C2 and C3) indicating the models were not contaminated by *Penicillium* at this stage. In the cheeses with starter *P. roqueforti* (C4, C5 and C6), the moulds had similar growth characteristics at 0, 4, 8 and 24 h fermentation (P>0.05).

In the next step of cheese making, there is no significant effect between pre-salting and post-salting curd on any microflora counts in all models (P>0.05). However, before rubbing-up, in the model cheese with starter *P. roqueforti* and non-starter *S. equorum* (C5 and C6), there were lower counts of *Lactococcus*, LAB and total aerobic microflora in comparison to when *S. equorum* was not added (C4; Table 6.2, 6.3, and 6.4 respectively; P<0.05) even though the presence of this bacterium was not detected on MSA (Table 6.6). In the equivalent models without added *Penicillium* (C1, C2, and C3), there was no significant difference

between these three counts (Tables 6.2, 6.3 and 6.5 respectively; $P>0.05$). In yeasts and moulds count (Table 6.5), the cheeses without starter *P. roqueforti* (C1, C2 and C3) were free from contaminant *P. roqueforti*, while the mould counts in the cheeses with starter *P. roqueforti* were slightly increased at the pre-rubbing up step.

In the first week of ripening, model cheeses which were made without the addition of *P. roqueforti* (C1, C2, and C3) had no impact on lactococci, lactic acid bacteria and total aerobic counts either in the crust or in the core. However, these three counts of the model with starter *P. roqueforti* and incorporated *S. equorum* (C5) were significantly lower than those of the control counts (C4) (Table 6.2, 6.3, and 6.4; $P<0.05$) and at the same time, the staphylococci was detected ($4 \log_{10}$ CFU/ml; Table 6.7). Interestingly, while the lactococci counts in the C4 cheese decreased gradually until 4 weeks ripening, the lactococci counts in C5 models remained steady.

A contaminant *Penicillium* started to grow in the cheeses without the added starter *P. roqueforti* (C1, C2 and C3) from the first week of ripening in the crust and second week of ripening in the core (Table 6.6). However, introducing *S. equorum* on the surface of model C3 after rubbing-up decreased the counts of contaminant *Penicillium* in the first week of ripening significantly ($P<0.05$). In the model cheeses with the added *P. roqueforti* (C4, C5 and C6), the decreased trend of mould counts in the crust when *S. equorum* is added was true for both ways of introduction.

S. equorum was never detected during fermentation, pre-salting, post-salting and pre-rubbing up steps in all models (Table 6.6) although in C2 and C5 models, it was added 1.5 h after *P. roqueforti* addition. *S. equorum* was also never detected in the core of the cheeses into which it was incorporated but was detected

intermittently in the crust after 1 week ripening of C5 ($4 \log_{10}$ CFU/g) and again after 4 weeks ripening ($5 \log_{10}$ CFU/g). The surface-smeared *S. equorum* was also detected in the crust of C6 but at week 4 only ($5 \log_{10}$ CFU/g) although it was smeared onto the surface of the cheeses before ripening.

It is interesting to note that *S. equorum* was not found in all the model cheeses to which it was added in the absence of the added starter *P. roqueforti*. This could be because the mould raises the pH (especially in the crust; Table 6.1) allowing the *S. equorum* to grow whereas in absence of the mould the pH of cheeses' core may be too low (at around 3-4). From Chapter 5, *S. equorum* used in this study was isolated from the crust of Stilton cheese for which the pH was 6.42 ± 0.22 (Table 5.2) and it could not grow at pH 3 and 4 (Figure 5.6).

Table 6.2. Total cell counts (TCC) of lactococci on M17 agar (Log CFU/ml) at each sampling point of cheese production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors (SE) of the means, $P=0.05$.

SAMPLES			C1		C2		C3		C4		C5		C6	
			TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE
Milk			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Incubated milk (hours)	0		10a	0.47	11a	1.01	11a	0.98	11a	0.94	11a	0.55	12a	0.96
	4		11a	0.51	12a	1.06	12a	1.03	12a	0.97	12a	0.58	13a	0.56
	8		12a	0.49	13a	0.97	13a	0.51	13a	0.90	13a	0.45	14a	0.45
	24		13a	0.47	14a	1.02	15a	0.53	15a	1.01	15a	0.43	15a	0.47
Pre-salting curd			14a	0.46	13a	0.99	13a	0.46	14a	0.53	14a	0.48	14a	1.03
Post-salting curd			13a	0.98	13a	0.51	13a	0.51	14a	1.04	15a	1.00	15a	0.99
Pre-rubbing up			11a	0.98	13a	0.95	13a	0.48	12a	0.98	9a	0.54	9a	0.47
Ripened cheese (weeks)	1	Crust	7ab	0.95	6a	0.49	9ab	0.47	11b	1.52	5a	0.48	7ab	0.51
		Core	6abc	0.51	5a	0.44	9c	0.52	8bc	0.44	5a	0.93	6ab	0.00
	2	Crust	6a	0.47	5a	0.48	5a	0.53	10b	0.49	5a	0.51	7ab	0.46
		Core	5a	0.47	5a	0.45	5a	0.53	7a	1.49	4a	0.94	6a	0.96
	3	Crust	5ab	0.54	5ab	0.53	4b	0.46	9b	0.48	5b	1.04	5b	0.95
		Core	4a	0.46	5a	1.01	4a	0.44	6a	0.48	4a	0.54	5a	0.43
	4	Crust	4a	0.45	3a	0.01	4a	0.52	7a	1.01	5a	0.91	6a	1.01
		Core	4a	0.49	4a	0.48	3a	0.52	6a	1.05	3a	0.42	5a	1.00

Means in the same row labelled with different letters (a, b, c) are significantly different ($P < 0.05$) as shown by ANOVA.

Table 6.3. Total cell counts (TCC) of lactic acid bacteria on MRS agar (Log CFU/ml) at each sampling point of cheese production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors (SE) of the means, $P=0.05$.

SAMPLES			C1		C2		C3		C4		C5		C6	
			TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE
Milk			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Incubated milk (hours)	0		10a	0.45	11a	0.46	11a	0.48	11a	0.44	11a	1.04	12a	0.52
	4		11a	0.52	12a	0.50	12a	0.53	12a	0.47	12a	0.99	13a	1.01
	8		12a	1.00	13a	0.53	13a	1.02	13a	0.51	13a	0.99	14a	0.48
	24		13a	0.51	14a	0.99	15a	1.47	15a	0.49	15a	0.53	15a	1.52
Pre-salting curd			14a	0.51	13a	0.51	13a	1.51	14a	1.02	14a	0.52	14a	0.99
Post-salting curd			13a	0.50	13a	1.43	13a	1.54	14a	0.51	15a	0.54	15a	0.48
Pre-Rubbing up			11a	1.51	13a	0.51	13a	1.53	12a	1.08	9a	1.03	9a	1.47
Ripened cheese (weeks)	1	Crust	7a	1.10	6a	0.45	8ab	0.57	11b	0.47	5a	0.54	7a	0.99
		Core	6ab	0.53	5a	0.48	9b	0.52	8ab	0.56	5a	0.46	6ab	0.95
	2	Crust	6ab	0.51	5a	0.46	5a	0.51	9b	0.47	4a	0.47	7ab	1.02
		Core	5ab	0.48	5ab	0.92	5ab	0.51	7b	0.56	3a	0.02	6ab	0.45
	3	Crust	4a	0.51	5ab	0.52	4a	0.46	8b	0.95	4a	0.49	5ab	0.06
		Core	4a	0.53	5a	0.54	4a	0.49	6a	0.94	4a	0.47	5a	0.52
	4	Crust	4ab	0.55	4ab	0.51	4ab	0.51	7b	0.51	3a	0.96	6ab	0.44
		Core	4a	0.49	4a	0.51	4a	0.51	6a	1.04	3a	0.18	5a	0.53

Means in the same row labelled with different letters (a, b) are significantly different ($P < 0.05$) as shown by ANOVA.

Table 6.4. Total cell counts (TCC) of aerobic bacteria on PCA agar (Log CFU/ml) at each sampling point of cheese production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors (SE) of the means, $P=0.05$.

SAMPLES			C1		C2		C3		C4		C5		C6	
			TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE
Milk			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Incubated milk (hours)	0		10a	1.06	11a	0.49	11a	0.46	11a	0.96	11a	0.57	12a	0.52
	4		11a	1.48	12a	0.47	12a	0.51	12a	0.44	12a	0.48	13a	0.52
	8		12a	0.48	13a	1.00	13a	0.53	13a	0.42	13a	0.45	14a	0.98
	24		13a	1.52	14a	0.50	15a	0.49	15a	1.95	15a	0.55	15a	0.49
Pre-salting curd			14a	0.97	13a	0.84	13a	0.98	14a	1.03	14a	1.48	14a	1.01
Post-salting curd			13a	0.99	13a	0.50	13a	0.54	14a	0.49	15a	0.55	15a	0.97
Pre-rubbing up			11a	1.49	13a	1.97	13a	0.44	12a	0.48	9a	0.47	9a	0.47
Ripened cheese (weeks)	1	Crust	8bc	0.88	6ab	0.47	10cd	0.49	11d	0.41	4a	0.53	7ab	0.01
		Core	6a	0.48	5a	0.49	9a	0.52	8a	1.54	5a	0.52	6a	0.53
	2	Crust	6a	0.49	5a	1.60	5a	0.44	10a	1.04	5a	0.49	7a	0.58
		Core	5a	0.65	5a	0.50	5a	0.52	7a	0.91	4a	0.48	6a	1.03
	3	Crust	6a	1.07	5a	1.51	4a	0.51	9a	0.96	5a	0.47	5a	1.01
		Core	4a	1.53	5a	0.46	4a	0.49	6a	0.46	3a	0.99	5a	0.43
	4	Crust	5a	1.00	5a	0.49	4a	0.48	7a	1.02	3a	1.06	6a	1.07
		Core	4a	0.01	3a	0.50	4a	0.47	6a	1.09	3a	0.47	5a	1.45

Means in the same row labelled with different letters (a, b, c) are significantly different ($P < 0.05$) as shown by ANOVA.

Table 6.5. Total cell counts (TCC) of yeasts and moulds on RBCA agar (Log CFU/ml) at each sampling point of cheese production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors (SE) of the means, $P=0.05$.

SAMPLES			C1		C2		C3		C4		C5		C6	
			TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE
Milk			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Incubated milk (hours)	0		0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	4		0a	0.00	0a	0.00	0a	0.00	3b	0.37	3b	0.51	3b	0.51
	8		0a	0.00	0a	0.00	0a	0.00	3b	0.48	4b	0.51	3b	0.01
	24		0a	0.00	0a	0.00	0a	0.00	3b	0.48	4b	0.46	3b	0.48
Pre-salting curd			0a	0.00	0a	0.00	0a	0.00	4b	0.44	4b	0.02	4b	0.98
Post-salting curd			0a	0.00	0a	0.00	0a	0.00	4b	0.49	4b	0.53	4b	1.01
Pre-rubbing up			0a	0.00	0a	0.00	0a	0.00	6b	0.93	6b	0.46	7b	0.55
Ripened cheese (weeks)	1	Crust	8bc	0.57	10c	0.54	3a	0.98	8bc	0.51	6abc	0.52	6ab	0.98
		Core	0a	0.00	0a	0.00	0a	0.00	5b	0.52	5b	0.53	5b	0.99
	2	Crust	7a	0.98	4a	0.44	4a	1.51	7a	0.97	8a	1.02	9a	0.52
		Core	4ab	0.99	4ab	0.51	0a	0.00	6b	1.07	6b	0.99	5ab	1.03
	3	Crust	8ab	0.51	7a	0.51	7a	0.52	11b	0.47	10ab	0.96	9ab	0.96
		Core	5a	1.04	5a	0.54	4a	0.53	3a	1.01	6a	0.98	5a	1.02
	4	Crust	8a	1.03	9ab	0.52	9ab	0.47	12b	0.56	10ab	0.54	10ab	0.44
		Core	6a	0.53	7a	0.47	4a	0.53	7a	0.98	6a	0.97	6a	1.48

Means in the same row labelled with different letters (a, b, c) are significantly different ($P < 0.05$) as shown by ANOVA.

Table 6.6. Total staphylococci counts on MSA agar (Log CFU/ml) at each sampling point of cheese production with *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6). Values are means of four technical replicates obtained from two independent determinations $\pm$ standard errors (SE) of the means, P=0.05.

SAMPLES			C1		C2		C3		C4		C5		C6	
			TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE	TCC	SE
Milk			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Incubated milk (hours)	0		0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	4		0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	8		0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	24		0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Pre-salting curd			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Post-salting curd			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Pre-rubbing up			0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
Ripened cheese (weeks)	1	Crust	0a	0.00	0a	0.00	0a	0.00	0a	0.00	4b	0.46	0a	0.00
		Core	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	2	Crust	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
		Core	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	3	Crust	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
		Core	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00
	4	Crust	0a	0.00	0a	0.00	0a	0.00	0a	0.00	5b	1.08	5b	1.01
		Core	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00	0a	0.00

Means in the same row labelled with different letters (a, b) are significantly different (P < 0.05) as shown by ANOVA.

6.3.4. ANTIFUNGAL ACTIVITY OF *STAPHYLOCOCCUS EQUORUM*

S. equorum B2 which was isolated from the crust of commercial Stilton cheese was evaluated for antifungal activity against commercial starter *P. roqueforti* on Potato Dextrose Agar (PDA) by single layer assay (SLA) and spot test (ST) (Hansen and Jakobsen, 1997; Section 2.12.5) shown in Figure 6.11. Control plates from both analyses gave a uniform green colour associated with fungal growth and sporulation. In SLA, the mould grew on the surface of agar but lack of sporulation so that the plates became yellow. In ST, non-greening zones were produced in the lawn of *P. roqueforti* by each spot of *S. equorum* and there was mould growth within the zone but no sporulation. This also indicates an inhibition of sporulation by *S. equorum*. Figure 6.11 shows the *S. equorum* isolate impacted on the *P. roqueforti* growth in both SLA and ST analyses.

To confirm if sporulating *Penicillium* were recoverable from the yellow spots on the ST plate, a loopful of growth was streaked onto PDA and BHI agar (Section 2.12.5). Both *P. roqueforti* and *S. equorum* were recovered on the plates (Figure 6.12). The sporulation activity of *P. roqueforti* was seen on PDA (Figure 6.12a), but on BHI agar, *P. roqueforti* showed growth only (Figure 6.12b), suggesting that *P. roqueforti* could not grow on rich nutrient agar like BHI so it needs a specific medium to sporulate.

To examine the effect of *S. equorum* on mould sporulation on the SLA plates, *P. roqueforti* was collected using a fungi tape subjected to mould staining (Section 2.12.6). Under a microscope, the complete spore characteristics of *P. roqueforti* from the control plate (P.roq + P.roq) were evident (Figure 6.13a) while from a plate containing these two microorganisms, *P. roqueforti* produced

less spores (Figure 6.13b) suggesting *S. equorum* could inhibit *P. roqueforti* sporulation.

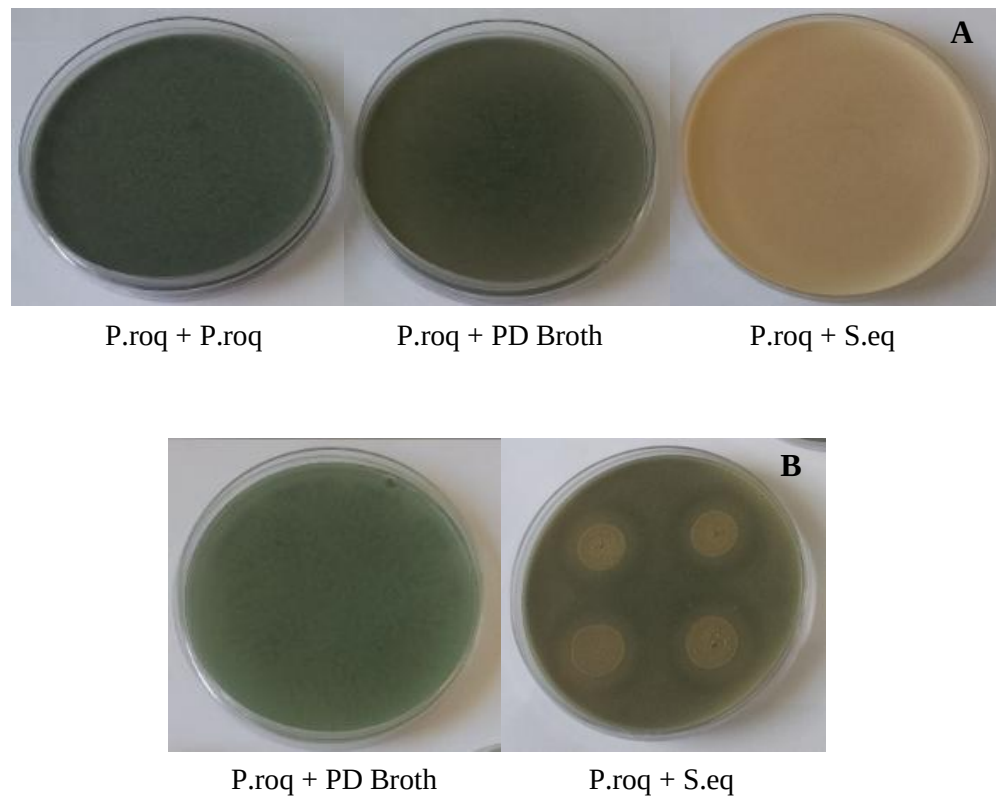


Figure 6.11. Single layer assay (A) and spot test (B) between *Penicillium roqueforti* (P.roq) and *Staphylococcus equorum* (S.eq) on potato dextrose (PD) agar.

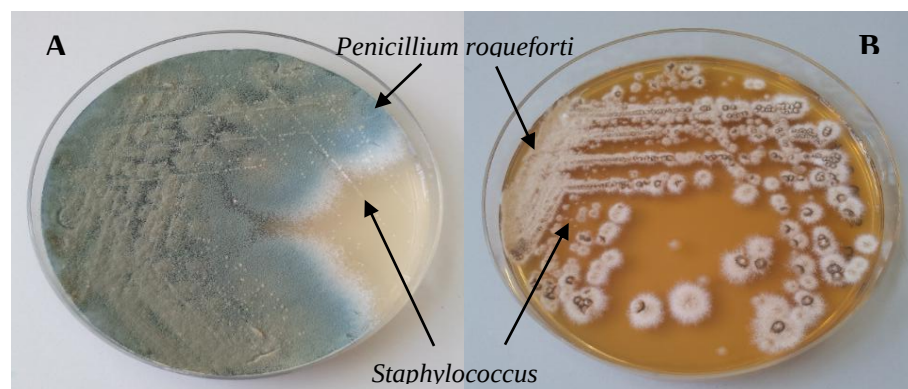


Figure 6.12. Confirmation of viability of *Penicillium roqueforti* and *Staphylococcus equorum* isolated from spot test plate on PD agar (A) and BHI agar (B).

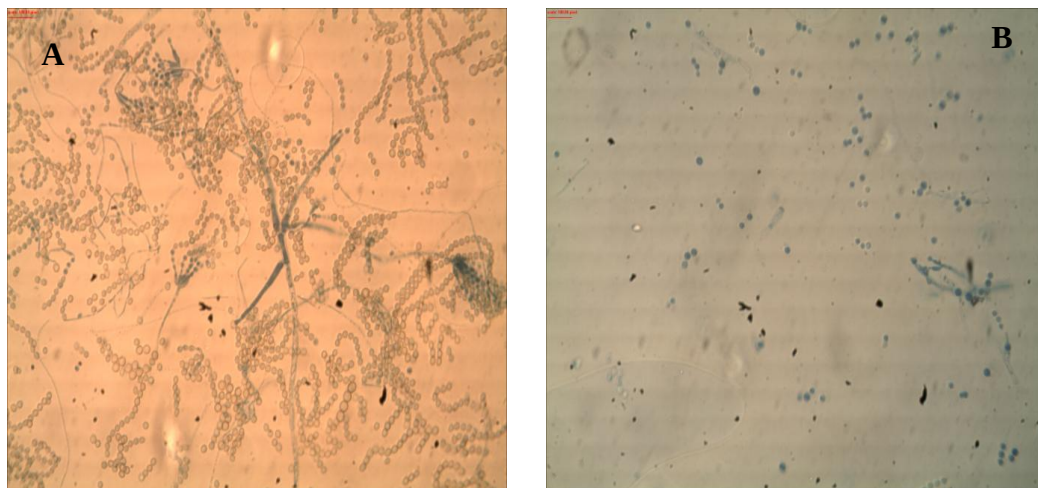


Figure 6.13. *Penicillium roqueforti* (A) and *Penicillium roqueforti* with *Staphylococcus equorum* (B) grown on PD agar at 25°C for 5 days and observed at 40X magnification.

6.3.5. ANTIFUNGAL ACTIVITY OF CELL-FREE SUPERNATANT OF *STAPHYLOCOCCUS EQUORUM*

From the previous results it was evident that *S. equorum* could affect the sporulation activity of *Penicillium*. This could be through direct nutrient competition effects or through the production of an antimicrobial inhibiting *Penicillium* growth and/or sporulation. To examine this, cell-free supernatants of an *S. equorum* overnight culture were produced (Section 2.12.5) and used in ST and SLA tests. The SLA and ST of *S. equorum* cell-free supernatant (CFS; pH 5.36) gave the same results as the culture broth (Figures 6.14 and 6.15) suggesting that the inhibitory effect was because of antifungal agents produced by *S. equorum*, not the cell competition.

Therefore, the CFS was then neutralised (CFS-N), treatment with catalase to remove hydrogen peroxide (CFS-C) or treatment with proteinase to remove bacteriocins (CFS-P) (Section 2.12.5) to detect which metabolites were causing the inhibitory effect. A combined treatment of neutralised and catalase treated

(CFS-NC) was also included. The results showed that the treated CFS gave various responses on *P. roqueforti* sporulation.

From Figure 6.14 and 6.15, the yellow colour on CFS-N SLA plate and the halo zone on CFS-N ST plate showed the treated supernatant still had activity, indicating that the antifungal activity was not due to acid production. By treating with the proteolytic enzyme proteinase-K, it was similarly shown that the antifungal activity was not due to bacteriocin either. From CFS treated with catalase, green plate was seen and it was similar with the control plate. Therefore, hydrogen peroxide (H_2O_2) is potentially affecting sporulation in the untreated CFS. This is linked to the presence of *S. equorum* and is unclear as this species produces catalase and would be expected to neutralise H_2O_2 .

The untreated CFS was then tested with added H_2O_2 in the overlay to check whether *P. roqueforti* sporulation was affected by H_2O_2 . However, the addition of H_2O_2 into an untreated CFS plate did not affect the sporulation *P. roqueforti*. H_2O_2 is interacting with the plates (Figure 6.16a) and being neutralised hence no activity (Figure 6.16b). In addition, the higher the H_2O_2 concentration added, the greener the resulting SLA plates.

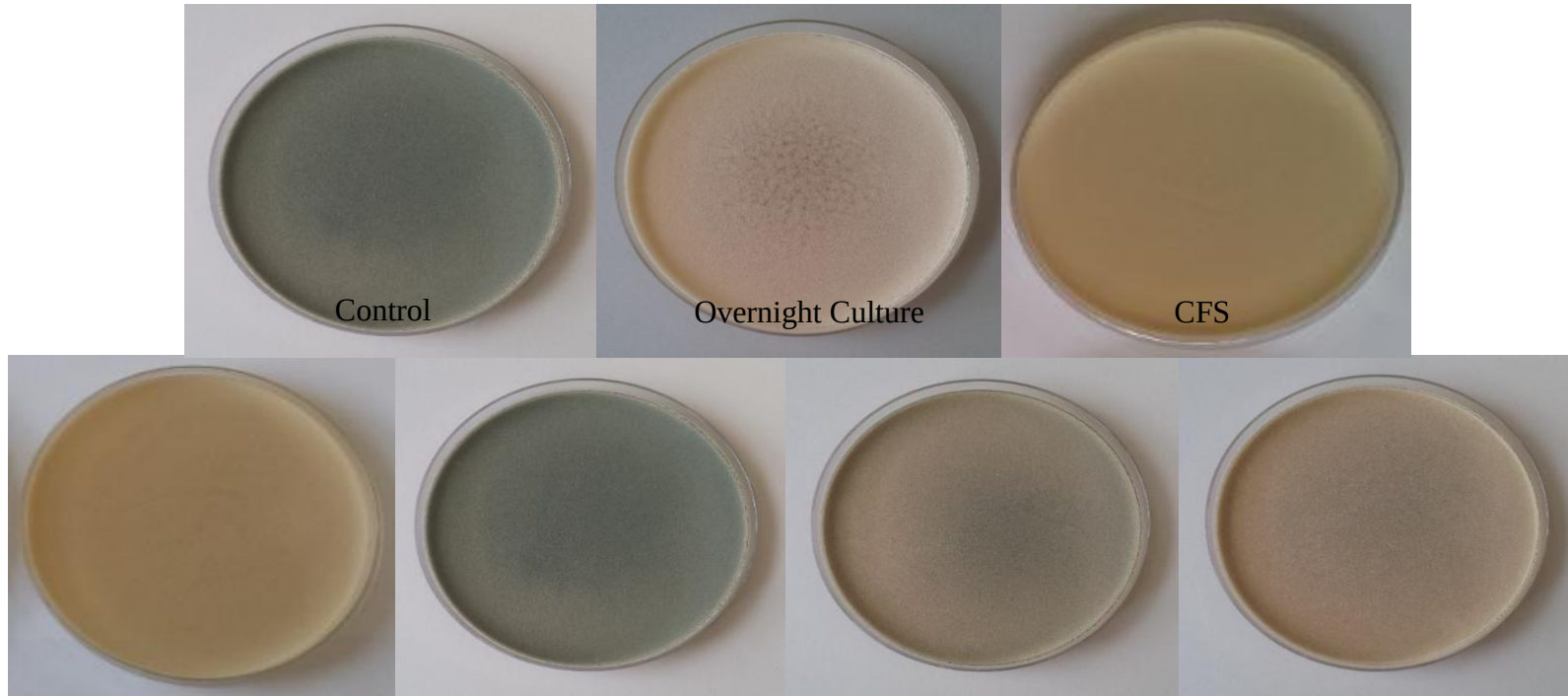


Figure 6.14. Single layer assay on PD agar between *Penicillium roqueforti* and cell-free supernatant (CFS) of *Staphylococcus equorum* adjusted to pH 7 (CFS-N), treated with catalase (CFS-C), adjusted to pH 7 and treated with catalase (CFS-NC) and treated with proteinase (CFS-P). Control is *P. roqueforti* only. Overnight culture is *S. equorum* with the cell.

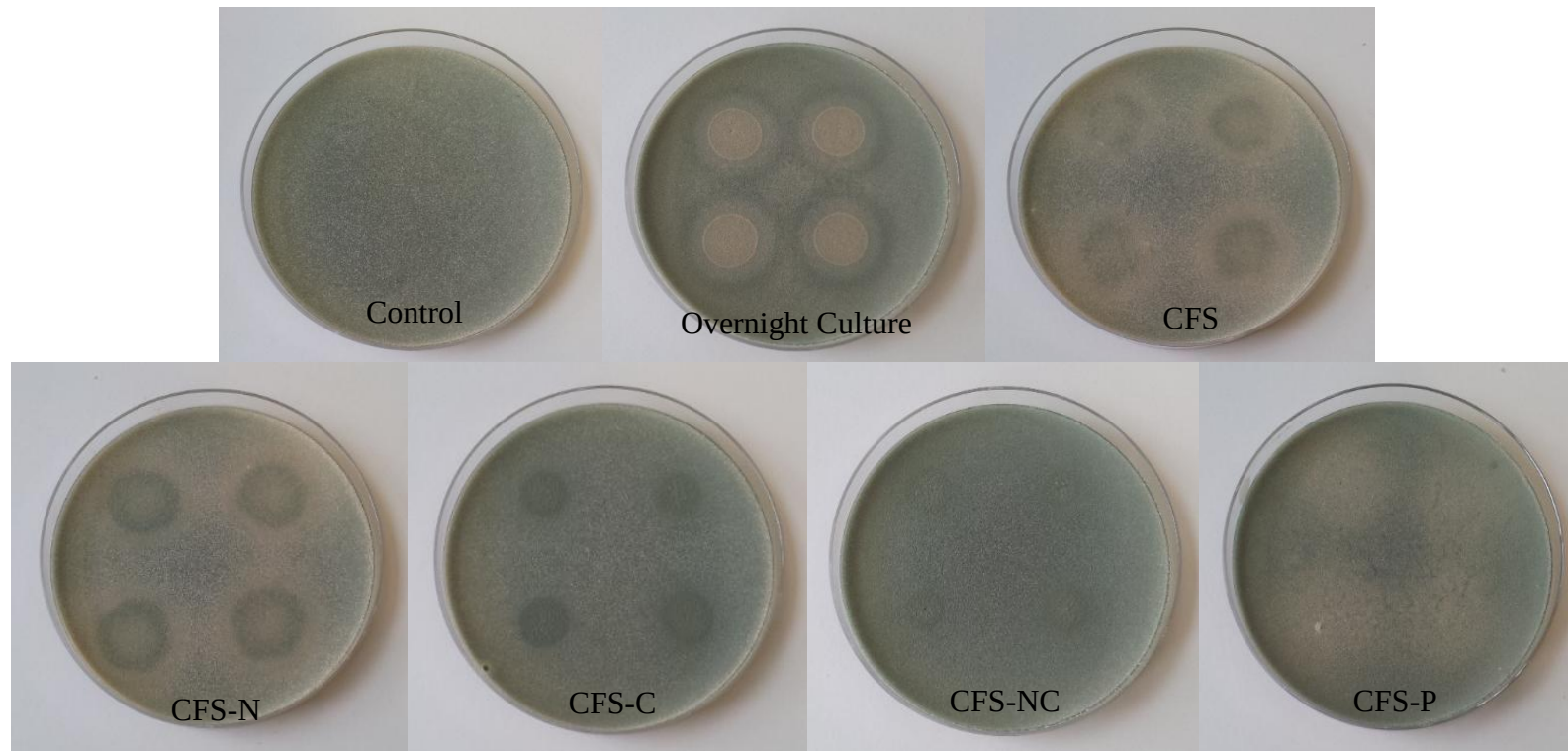


Figure 6.15. Spot test on PD agar between *Penicillium roqueforti* and cell-free supernatant (CFS) of *Staphylococcus equorum* adjusted to pH 7 (CFS-N), treated with catalase (CFS-C), adjusted to pH 7 and treated with catalase (CFS-NC) and treated with proteinase (CFS-P). Control is *P. roqueforti* only. Overnight culture is *S. equorum* with the cell.

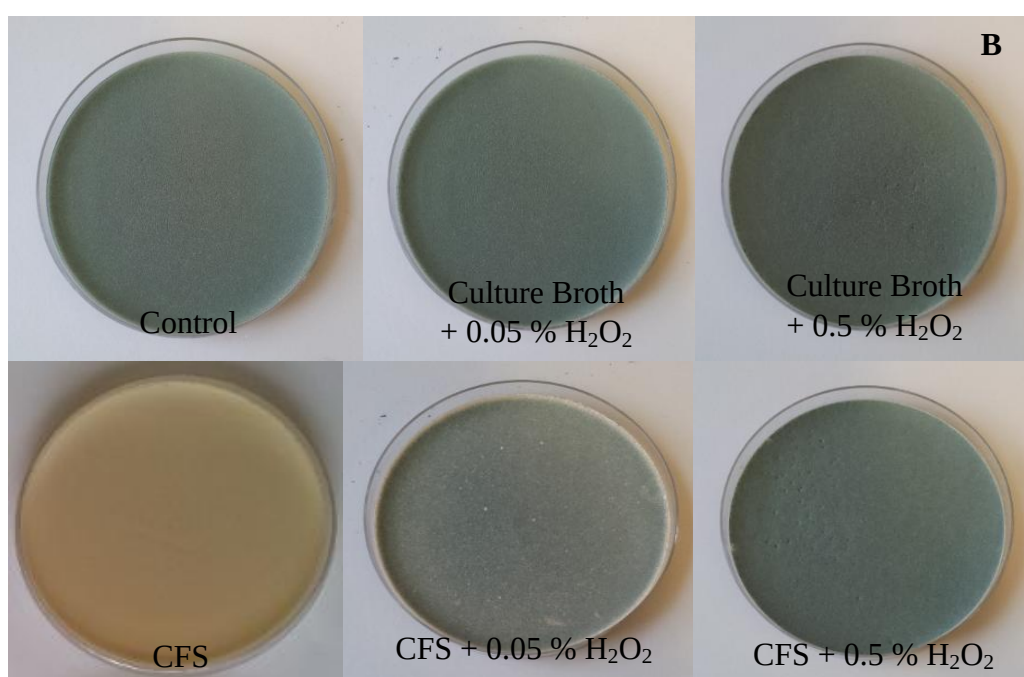
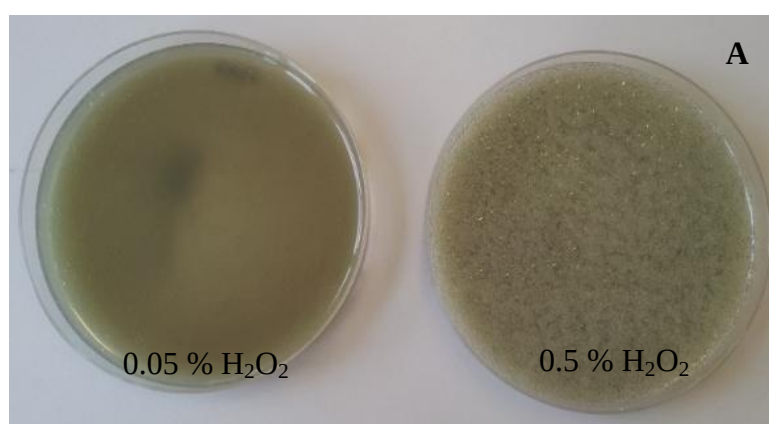


Figure 6.16. Single layer assay between *Penicillium roqueforti* and cell-free supernatant (CFS) of *Staphylococcus equorum* treated with H_2O_2 on PD agar, before incubation (A) and after incubation at 25°C for 5 days (B). Control is *P. roqueforti* only.

6.4 DISCUSSION

6.4.1. IMPACT OF *STAPHYLOCOCCUS EQUORUM* ON MICROBIAL CHARACTERISTICS

All lactic acid bacteria (MRS), *Lactococcus* (M17) and total aerobic (PCA) counts showed the expected profiles before ripening which were higher than mould (RBCA) counts showing the lactic starter cultures contributed to the fermentation. *Lactococcus* counts increased during fermentation to 10^{15} CFU/ml which is a very high number for a fermented milk sample. This number is usually for freeze-dried bacterial culture. However, this result was also shown by Whiley (2013) and Apostolou (personal communication). So, it is expected that this could be an effect of adding powdered starter as it may not entirely disperse.

Lactococcus then started to decrease at the core and crust of first week of ripening for cheeses made without *P. roqueforti* (C1, C2, C3). In cheeses made with *P. roqueforti* (C4, C5, C6), the *Lactococcus* counts decreased at the core of one week ripened C4 and at the pre rubbing-up step for C5 and C6. It is also clearly seen from Table 6.3 that the *Lactococcus* counts at the pre-salting curd was not different with the counts at the post-salting curd. These show that even salting is cited as causing a reduction in *Lactococcus* counts in Cheddar cheese (McMahon *et al.*, 2014), the reduction effect is not within 24 hours. According to Cantor *et al.* (2004), the numbers of lactococci and lactobacilli in the core decrease slowly from about 10^9 CFU/g after salting to $10^7 - 10^8$ CFU/g at the end of ripening. In the original study developing the cheese model, Whiley (2013) showed the counts of *Lactococcus* decreased at 4 days after salting in a model cheese made with *L. lactis* and *P. roqueforti*. In Stichelton study (Yunita, 2010), the counts of *Lactococcus* decreased at the core of 6-weeks-old cheese.

During ripening, a contaminant *Penicillium* appeared inevitably in model cheeses without the addition of starter *P. roqueforti* (C1, C2 and C3). It was detected from the first week of ripening in the crust and second (C1 and C2) or third (C3) week of ripening in the core as an effect of piercing which allows oxygen to enter the core part or pushes the *Penicillium* into the core of the cheeses. However, the contaminant *Penicillium* was not detected at the pre-rubbing-up stage. So, it is assumed that the spores of the contaminant *Penicillium* were dispersed in the atmosphere on dust particles and it was introduced when the model cheeses were rubbed-up. The pH of the C1, C2 and C3 samples collected at the pre-rubbing-up step was also quite close to the pH of C4 (model cheese with *Lc. lactis* and *P. roqueforti*; pH 4.55) indicating appropriate conditions were present for the *Penicillium* growth.

In model cheeses without *S. equorum* (C1 and C4), the LAB, *Lactococcus* and total aerobic counts for C1 (model cheese with *L. lactis* only) after 4 weeks of ripening were slightly lower than those of C4 (model cheese with *L. lactis* and *P. roqueforti*). This suggests that the addition of starter *P. roqueforti* could improve the LAB, *Lactococcus* and total aerobic growth, which the contaminant *Penicillium* did not. In model cheeses with *S. equorum*, it was evident that ‘blueing’ of either starter *P. roqueforti* or contaminant *Penicillium* was slower in the presence of *S. equorum* at the first week of ripening. Interestingly, according to MSA counts, *Staphylococcus* were only detected in the cheeses with *P. roqueforti* starter addition (C5 and C6) and only in the crust. This indicates that at late ripening, *S. equorum* grew together with the added *Penicillium roqueforti*, but not the contaminant *Penicillium*.

The pH in the crust of model cheeses with starter *P. roqueforti* (C4, C5 and C6) was higher than that of model cheeses without starter *P. roqueforti* (C1,

C2, C3). This was due to the increased proteolysis leading to ammonia production from amino acids by the starter *P. roqueforti*. Furthermore, at week 3, in model cheeses without starter *P. roqueforti* (C1, C2, C3), the pH of the C1-crust was slightly higher than that of C2 and C3 and in model cheeses with starter *P. roqueforti* (C4, C5 and C6), the pH of the C4-crust was also higher than that of C5 and C6. This suggests that the presence of *S. equorum* in the model cheeses C2, C3, C5 and C6 could inhibit the production of ammonia in the crust through the mould growth inhibition.

However, in the core, the mould did not blue well even though the models were pierced every week, which is more frequently than occurs with Stilton production. This was then reflected in the pH of the core staying low and the crust pH rising. There are three possibilities. First of all, the surface of the models had been engulfed by the mould so fast. Thus, after piercing the oxygen could not enter into the core of the models. Secondly, the oxygen level available in the desiccator vessel was very low and was not well circulated. Thirdly, the model cheeses were quite soft so after piercing, the holes quickly closed-up and no oxygen gets in.

6.4.2. IMPACT OF *STAPHYLOCOCCUS EQUORUM* ON PHYSICAL CHARACTERISTICS

From texture analysis, the way *S. equorum* was introduced into the models gave different results. Model cheeses with *S. equorum* added during fermentation (C2 and C5) produced softer cheeses. Conversely, the model cheese with surface-spread *S. equorum*, *L. lactis* and *P. roqueforti* (C6) had the best texture which was harder and more solid than the other models.

According to McSweeney and Fox (2004), lactate catabolism producing CO₂ and H₂O in surface mould-ripened cheeses is important for texture development. Increasing CO₂ from lactic acid catabolism and NH₃ from amino acid catabolism could then increase the pH. Interestingly, the pH of C6 model crust (7.34±0.04) was not significantly different from the pH of C4 model crust (7.64±0.10) where the two Stilton cheese starter cultures were used together and no *S. equorum*. It means that although *P. roqueforti* could not sporulate well because of the addition of *S. equorum* at early ripening and the pH was not affected, the activity of *Lactococcus* at fermentation would not be disturbed by the *S. equorum* and the presence of more oxygen at the crust also created a desired pH for mould growth. On the other hand, the presence of *S. equorum* in fermentation in models C2 and C5 could slow the *Lactococcus* activity in converting lactose to lactate.

It is noteworthy that the texture of the cheeses did not relate to the water activity (a_w). However, the a_w affects the growth rate of *P. roqueforti* (Cantor *et. al.*, 2004). The optimum condition for *P. roqueforti* to grow and sporulate is at a_w 0.96-0.98 and sporulation is inhibited at a_w 0.92-0.94. In this study, the average a_w was not significantly different between the crust and the core which was about 0.93-0.95 in the crust and 0.94-0.96 in the core. This also would explain the lack of blueing of *P. roqueforti* in the core as it was not at the right a_w besides the reason of not being enough oxygen present. In the crust, the a_w might not be at the optimum level either, but the crust is in direct contact with the oxygen so that a dense growth of *P. roqueforti* was still shown.

In addition to the model C6 cheese having a firm texture with the a_w at the crust at 0.93, the colour of this model cheese was paler than the others. This indicates *S. equorum* which was spread just before ripening completely inhibited

the sporulation of *P. roqueforti*. However, there is no publication in *S. equorum*-*P. roqueforti* interactions showing the impact of a_w on the mould sporulation. In *Candida lipolytica*-*P. roqueforti* interactions, Juszczuk *et al.* (2005) noticed at a_w 0.978, spores were still present in the presence of the yeast, but no spores was produced at a_w 0.995 and no growth at a_w 0.997. Presumably the yeast grew better inhibiting the mould. Therefore, the delayed sporulation of the mould in model C6 causing the paler colour was assumed to be because of the growth of *S. equorum* which was supported by the lower a_w .

Based on the interaction between *S. equorum* and *P. roqueforti* on PDA agar, the sporulation inhibitory effect was because of antifungal agent produced by *S. equorum*, but it was not acid, bacteriocin or H_2O_2 . Interestingly, on the SLA test, the plate containing untreated *S. equorum* CFS with higher H_2O_2 concentration was greener than the plate containing untreated *S. equorum* CFS with lower H_2O_2 concentration, indicating the growth and sporulation of *P. roqueforti* were not inhibited by H_2O_2 . Instead, the oxygen produced was needed for *P. roqueforti* sporulation. Another possibility is that the H_2O_2 was being inactivated by the PDA.

It is also interesting to note that the CFS treated with catalase started to coagulate since it was incubated at 37°C which is an optimum temperature for catalase and became a curd after the boiling step which is used to inactivate the catalase. This result was different with the CFS of *Lactobacillus plantarum* treated in the same way which remained liquid (Mugampoza, 2013). It seems that the catalase enzyme used (500 Unit/ml) was not in the right concentration. Otherwise, there may be some other factors which made the catalase precipitate as staphylococci are very well known for producing extracellular enzymes including proteases, DNases and this could be having an effect.

According to Boysen *et al.* (2000), *P. roqueforti* is resistant to lactic acid, acetic acid, carbon dioxide and several other lactic acid bacterial metabolites. However, 2-hydroxy-3-phenylpropanoic acid (phenyl lactic acid, PLA) produced by *Geotrichum candidum* has been shown to cause inhibition of *P. roqueforti* growth and sporulation (Dieuleveux *et al.*, 1998). The minimum inhibitory concentration of PLA for mould at pH 4.0 is 45 mM (Strom *et al.*, 2002). From the genus *Staphylococcus*, the macrocyclic peptide antibiotic micrococcin P₁ was reported for the first time in *S. equorum* WS 2733 (Otaka and Kaji, 1974) and it could inhibit *Listeria monocytogenes* on soft cheese (Carnio *et al.*, 2000). However, how it acts on *P. roqueforti* has not been reported yet. There might also be another metabolite produced by *S. equorum* affecting *P. roqueforti* sporulation which is associated with a specific pH. Based on SPME GCMS on model cheeses (Chapter 7), faltarinol which is a natural toxin found in carrots, was detected at retention time 27.79 (Appendix 11).

6.5 CONCLUSION

The presence of *S. equorum* in cheeses without *P. roqueforti* addition which was introduced in both ways could inhibit the surface growth of contaminant *Penicillium*. It also slowed *P. roqueforti* growth in cheeses with the added starter *P. roqueforti*. The addition of *S. equorum* in the initial process made the cheese core softer, but in the surface-smear cheeses, this resulted in a paler coloured crust and firmer textured cheese. Therefore, its presence as a smear at the beginning of ripening could have a positive effect on cheese texture. Its ability to prevent *Penicillium* surface growth could be beneficial for white cheeses where this is an undesirable flaw. However, its presence during early fermentation should be avoided if soft texture is not the desirable characteristic.

However, the potential use of *S. equorum* B2 which was isolated from the crust of commercial Stilton cheese, as an adjunct culture in blue cheese making still needs further investigation. Other *S. equorum* which were isolated from other cheeses (Chapter 5) should also be examined for comparison purposes. In addition, increasing the proportion of *S. equorum* added would be a valuable factor for further investigation.

The antifungal activity of the *S. equorum* isolate on the control of starter *P. roqueforti* sporulation was also confirmed on the laboratory media. However, the metabolites produced causing this activity has not been discovered yet. Thus further investigation is needed to determine how *S. equorum* affects *Penicillium* sporulation. Further research on finding the right quantity of catalase enzyme for analysing H₂O₂ would be beneficial as 500 U/ml produced a strange result.

CHAPTER 7

THE IMPACT OF *STAPHYLOCOCCUS EQUORUM*

ON FLAVOUR

7.1 INTRODUCTION

Starter cultures dominate the microflora in cheese making. During production, some LAB in the starter cultures start to die so that the cells release enzymes which further breakdown milk proteins, mainly casein, to small peptides and amino acids. These dead starter culture cells are an important food source for subsequent generations of non-starter lactic acid bacteria (NSLAB) (American Academy of Microbiology, 2015). Yeast and moulds growing during the ripening step also make the cheese microflora more heterogenous.

In the final product, those microorganisms become important because their metabolic activity produces compounds that affect the flavour, aroma, texture and colour of the cheese. Therefore, the sensory quality of ripened cheese is not only determined by the initial microorganisms and biochemical properties of the milk, but also their evolution during ripening. The sensory attributes of blue cheeses have been generated by a panel using Flash profiling (Gkatzionis *et al.*, 2013; Table 7.1). The flavour compounds produced in three different sites of Stilton cheese can be seen in Table 7.2 and some key important flavour compounds found in blue cheeses can be observed in Table 7.3.

Table 7.1. Sensory attributes of blue cheeses generated by the panel in Flash profile.

Attribute	Number of Judges Using The Attribute
Blue cheese/blue cheese aroma/blue cheesy	8
Cheese/cheesy	5
Musty-frusty	5
Milk/milky	5
Butter/buttery	4
Cheddar/Cheddary/Cheddar cheese	3
Stilton	3
Rancid	3
Mould/mouldy	3
Creamy	3
Mature	2
Fruity	2
Sweet/sweety	2
Nutty	2
Acid/ acidic	2
Old socks/smelly socks	2
Sharp/smooth	2
Paint	2

Source: Gkatzionis *et al.*, (2013)

Table 7.2. Flavour compounds produced in three different sites of Stilton cheese by SPME GCMS.

Compounds	LRI ^a	Blue		Outer		White	
		Mean	SD	Mean	SD	Mean	SD
Methanethiol	<500 ¹	0.35	0.18	4.1	1.13	0.16	0.04
Ethanol	<500 ¹	0.11	0.09	0.14	0.06	1.6	0.49
Acetone	<500 ¹	2.5	0.97	19	5	1.7	0.52
Unidentified 3	604	0.41	0.17	0.35	0.06	2	0.26
2-Butanone	610 ²	0.67	0.35	17	10	3.2	0.49
2-Methyl-1-propanol	634 ²	0.86	0.25	ND	-	1.5	0.23
3-methyl-butanal	659 ²	1.3	1.19	1.6	1.06	6.5	2.21
2-methyl-butanal	668 ²	0.18	0.17	0.19	0.11	0.81	0.42
2-Pentanone	688 ¹	5.2	3.67	56	42	2.1	0.77
Unidentified 4	690	49	16	39	22	28	3
2-Pentanol	698 ²	0.2	0.04	0.28	0.16	0.23	0.06
2-Hydroxy-2-butanone	709 ²	3.5	1.19	3.8	0.39	6.4	3.63
3-Methyl-1-butanol	733 ²	10	2.35	1.3	0.63	18	2
2-Methyl-1-butanol	737 ²	2.8	0.59	0.41	0.1	4.7	0.74
Dimethyl disulfide	750 ²	1.8	0.82	4.9	1.19	1.8	0.2
3-Methyl-2-pentanone	754 ²	ND	-	1.6	0.78	0.17	0.09
2-Hexanone	789 ²	0.38	0.33	2.3	1.85	0.07	0.02
Octane	800 ¹	0.51	0.38	0.78	0.46	0.22	0.09
2,4-Dimethyl-heptane	822 ²	0.95	0.59	1.2	0.81	0.25	0.09
2-Heptanone	890 ¹	23	19	116	83	4.3	1.1
α -Pinene	944 ²	0.05	0.03	1.2	0.35	0.04	0.01
3-Octanone	988 ²	ND	-	1.2	0.27	ND	-
2-Octanone	992 ²	0.35	0.37	1.2	0.68	0.05	0.02
4-Methylanisole	1030 ²	0.83	0.46	0.53	0.12	0.14	0.01
Unidentified 5	1034	0.35	0.11	0.26	0.1	0.27	0.07
Unidentified 6	1039	0.13	0.04	0.27	0.08	0.1	0.05
8-Nonen-2-one	1085 ²	ND	-	1.7	1.17	ND	-
2-Nonanone	1093 ¹	8.6	7.49	27	16	1.7	0.63
2-Undecanone	1298 ²	ND	-	0.58	0.27	ND	-

The values are relative to the peak area observed when the headspace above a 5 μ g/l, 2-nonanone solution was analysed. Each value is based on four replicates.

ND: not detected.

^aLRI: linear retention indices of the compounds relative to an alkane series. Superscripts show identification using either authentic standards¹ or retention indices from the literature and their mass spectra with the NIST mass spectral library².

Source: Gkatzionis *et al.*, (2009).

Table 7.3. Description of some key flavour components in blue cheeses.

Flavour Compound	Formula	Metabolism	Description	References
Methanethiol	CH ₄ S	Amino Acid (methionine)	Rotting cabbage, cheese, Sulphur	Neeter and De Jong, 1992
Dimethyl disulfide	C ₂ H ₆ S ₂	Amino acid (methionine)	sulfurous vegetable cabbage onion	Kubickovs and Grosch, 1997
2-Methyl-1-propanol	C ₄ H ₁₀ O	Amino acid (valine)	Ethereal winey	www.thegoodscentcompany.com
2-Methyl propanal	C ₄ H ₈ O	Amino acid (valine)	Banana, malty, chocolate-like	Neeter and De Jong, 1992
2-Methyl propanoic acid (isobutyric acid)	C ₄ H ₈ O ₂	Amino acid (valine)	Acidic, sour, cheese	www.thegoodscentcompany.com
3-Methyl-1-butanol	C ₅ H ₁₂ O	Amino acid (leucine)	Fusel, alcoholic, pungent, etherial, cognac, fruity, banana and molasses	Neeter and De Jong, 1992
3-Methyl butanal	C ₅ H ₁₀ O	Amino acid (leucine)	Malty, cheese	Curioni and Bosset, 2002
3-Methyl butanoic acid (isovaleric acid)	C ₅ H ₁₀ O ₂	Amino acid (leucine)	Cheese, acidic, sour, pungent, fruity, stinky, ripe fatty and fruity note	www.thegoodscentcompany.com
2-Methyl-1-butanol	C ₅ H ₁₂ O	Amino acid (isoleucine)	Roasted wine onion fruity	www.thegoodscentcompany.com
2-Methyl butanal	C ₅ H ₁₀ O	Amino acid (isoleucine)	Musty cocoa coffee nutty	www.thegoodscentcompany.com
2-Methyl butanoic acid	C ₅ H ₁₀ O ₂	Amino acid (isoleucine)	Pungent acid roquefort cheese	www.thegoodscentcompany.com

Continued on next page

Flavour Compound	Formula	Metabolism	Description	References
Phenylacetic acid	C ₈ H ₈ O ₂	Amino acid (phenylalanine)	Sweet, floral, honey, rose, chocolate, tobacco	www.thegoodscentscopy.com
Benzaldehyde	C ₇ H ₆ O	Amino acid (phenylalanine)	Bitter almond oil, sweet cherry	Kubickovs and Grosch, 1997
Propionic acid	C ₃ H ₆ O ₂	Sugar (lactose)	Pungent, sour milk, cheese	Curioni and Bosset, 2002
Diacetyl	C ₄ H ₆ O ₂	Sugar (lactose)	Buttery	Escamilla-Hurtado <i>et al.</i> , 1996
2-Nonanone	C ₉ H ₁₈ O	Fat (capric acid)	Fruity, sweet, waxy, soapy, cheese, green herbaceous, coconut-like	Lawlor <i>et al.</i> , 2003
2-Pentanone	C ₅ H ₁₀ O	Fat (caproic acid)	Fruit, sweet, wine, woody, banana-like	Walker and Mills, 2014
2-Hexanone	C ₆ H ₁₂ O	Fat (enanthic acid)	Fruity fungal meaty buttery	www.thegoodscentscopy.com
2-Octanone	C ₈ H ₁₆ O	Fat (pelargonic acid)	Musty, Bleu and Parmesan cheese-like with earthy and dairy nuances	www.thegoodscentscopy.com
2-Heptanone	C ₇ H ₁₄ O	Fat (caprylic acid)	Cheese, fruity, green banana with a creamy nuance	www.thegoodscentscopy.com
8-Nonen-2-one	C ₉ H ₁₆ O	Fat (linoleic or linolenic acid)	Blue-veined cheese like	Karahadian <i>et al.</i> , 1985; Urbach, 1997

Bitterness is due to the accumulation of hydrophobic peptides (methionine, lysine, valine, leucine, proline, phenylalanine, tyrosine, isoleucine and tryptophan) which are formed by the action of proteolytic enzymes on casein (Lemieux and Simard, 1992). *Enterococcus faecalis* produced more bitter flavours and acid in pasteurised-milk cheese (Centeno *et al.*, 1999) whereas *Lactobacillus plantarum* spp. produced less bitterness in semi-hard cow-milk cheese (Gomez *et al.*, 1996). A significantly milder Raclette cheese was produced using *S. equorum* subsp. *linens* RP29 isolate compared to the original starter *Staphylococcus succinus* subsp. *casei* DSM 15096^T (Place *et al.*, 2003).

According to Gkatzionis *et al.* (2014), the yeast *Yarrowia lipolytica* presented strong synergistic activity with the mould *P. roqueforti* in a dairy model system with or without the starter *Lc. lactis*. The mix of these microorganisms enhanced the production of methyl ketones (2-heptanone, 2-nonanone, acetone, 2-pentanone, 2-hexanone, 2-octanone, 2-undecanone, 8-nonen-2-one) and associated alcohols (2-pentanol and 2-heptanol) contributing to stronger blue cheese aromas. The pathway for the catabolism of fatty acids by *Penicillium* spp is shown in Figure 7.1 and examples of 2-pentanone and 2-pentanol are shown in Figure 7.2.

Study on aroma compounds of a 4-days-ripened blue cheese model using the yeast *Trichosporon beigleii* was conducted by Whiley (2013). His results showed that *P. roqueforti* with and without *L. lactis* produced high levels of ketones. *T. beigleii* inhibited the growth of *P. roqueforti* hence a low level of flavour compounds were produced. Models containing *Kluyveromyces lactis* with *P. roqueforti* produced high levels of acids and esters and lower levels of ketones. Models containing *D. hansenii* produced less ketones and more alcohols.

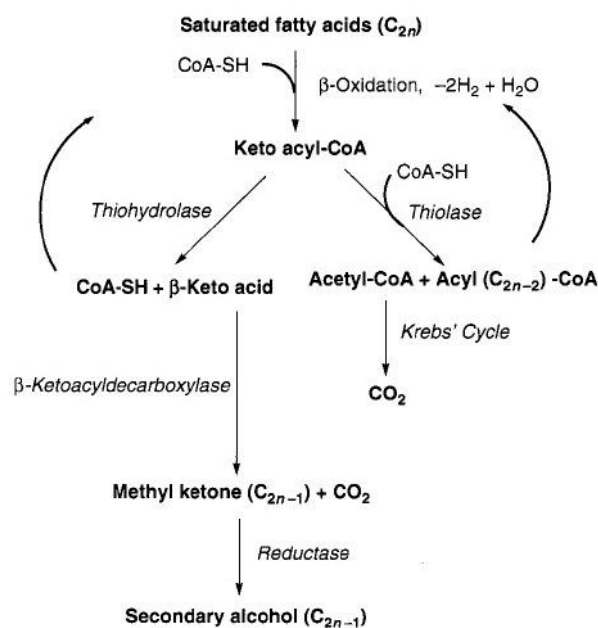


Figure 7.1. Pathway for the catabolism of fatty acids by *Penicillium* spp. (McSweeney and Sousa, 2000).

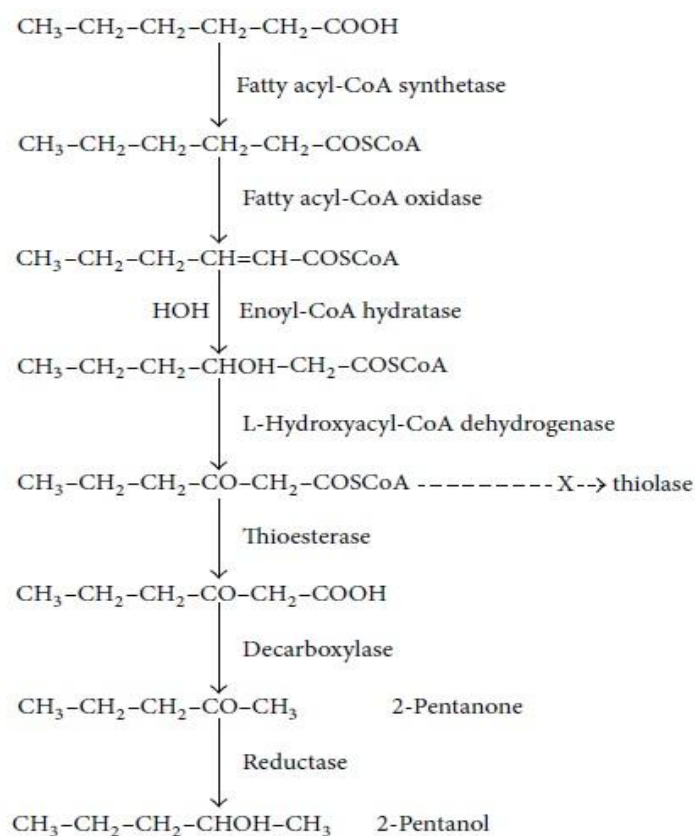


Figure 7.2. Pathway for 2-pentanone and 2-pentanol production by *Penicillium roqueforti* (Walker and Mills, 2014).

A study on aroma contribution was studied in a cheese model using Gram-negative bacteria *Microbacterium foliorum*, *Proteus vulgaris* and *Psychrobacter* sp. and deacidified by *Debaromyces hansenii* (Deetae *et al.*, 2009). The result showed that *Proteus vulgaris* produced the greatest quantities of methionine and branched chain alcohols. It also had a greater capacity for hydrolysing casein and higher deaminase activity.

The degradation of a sulfur compound L-methionine (Figure 7.3) by *S. equorum* was previously studied by Bonnarme *et al.* (2001). Their results showed that *S. equorum* had L-methionine demethylase, but was deficient in L-methionine transaminase and did not produce L-methionine deaminase. This resulted in less production of methanethiol, dimethyldisulfide and dimethyltrisulfide. These three volatile sulfur compounds are related to a desirable flavour in Cheddar cheese (Weimer *et al.*, 1999) and contributed to the strong garlic/putrid aroma of soft-smear or surface-ripened cheese (McGorin, 2011).

Søndergaard and Stahnke (2002) studied the role of *S. equorum*, *S. xylosus* and *S. carnosus* on growth and aroma production in a fermented meat model. Their results showed that *S. equorum* and *S. xylosus* did not produce 2-methyl-1-butanol. *S. xylosus* formed more diacetyl, 3-methyl-1-butanol, 2-butanone, acetoin and methyl-branched ketones than *S. carnosus* and *S. equorum*. *S. carnosus* produced more methyl-branched aldehydes, acids and esters than *S. xylosus* and *S. equorum*. *S. equorum* produced the least of the methyl-branched aldehydes. The catabolism of amino acid leucine producing 3-methyl butanal, 3-methyl-1-butanol and 3-methyl butanoic acid (isovaleric acid) is shown in Figure 7.4.

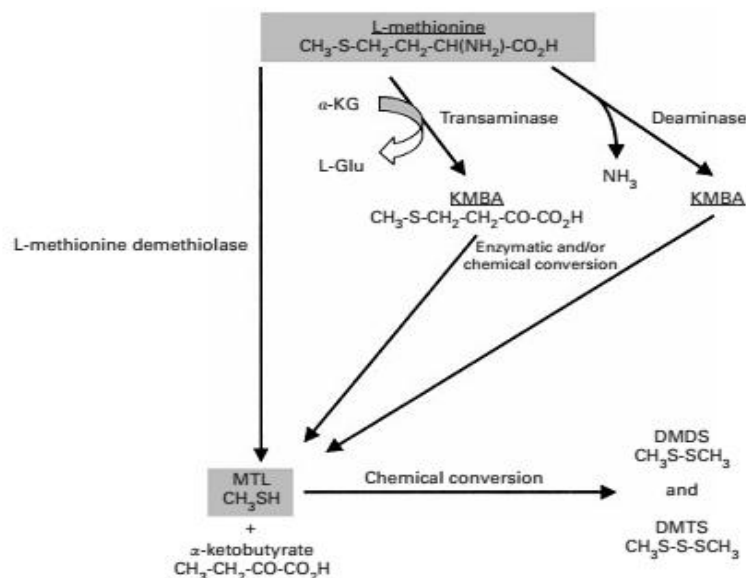


Figure 7.3. L-methionine degradation pathways (Bonnarme *et al.*, 2001). KMBA, 4-methylthio-2-oxobutyric acid; α -KG, α -ketoglutarate; L-Glu, L-glutamate; MTL, methanethiol; DMDS, dimethyldisulfide; DMTS, dimethyltrisulfide.

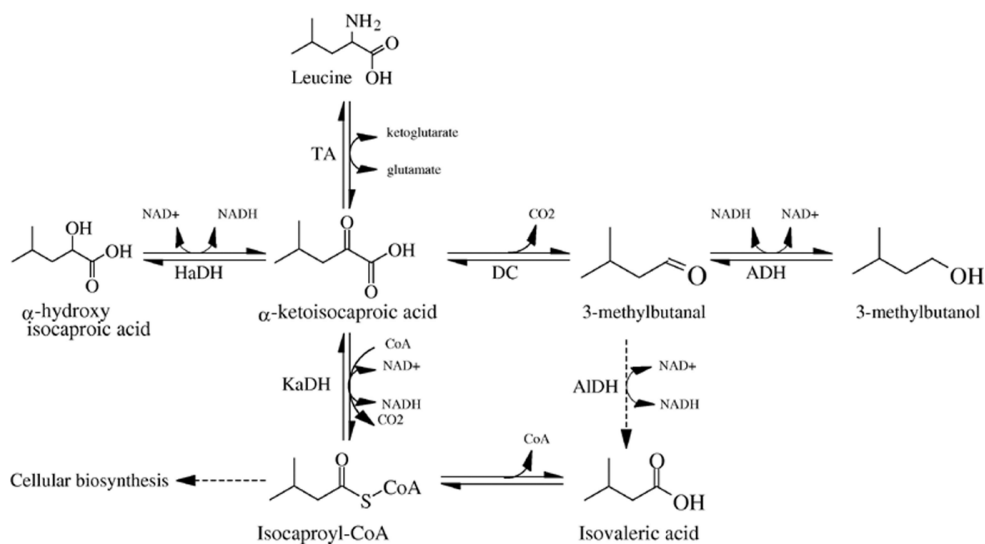


Figure 7.4. Leucine degradation pathway (Smit *et al.*, 2005). TA = transaminase, HaDH = hydroxy acid dehydrogenase, DC = keto acid decarboxylase, ADH = alcohol dehydrogenase, AIDH = aldehyde dehydrogenase and KaDH = keto acid dehydrogenase.

However, the role of *S. equorum* on aroma production in a blue cheese has not been published yet although its presence in ripened cheeses has been established. Therefore, the flavour volatiles of one month ripened blue cheese models (Chapter 6) were examined by solid phase micro extraction with GC-MS (SPME GC-MS) as used by Gkatzionis *et al.* (2009).

7.2 OBJECTIVES

The objective of this work was to investigate the role of *S. equorum* in influencing flavour characteristics of mould-ripened cheese.

7.3 RESULTS

Six model cheeses produced in relation to three experimental factors (incorporation of *S. equorum* (X), part of cheeses (Y), *P. roqueforti* addition (Z); see Table 6.2) were analysed at the end of ripening for flavour volatile production by solid phase microextraction gas chromatography-mass spectrometry (SPME GC-MS) (Section 2.13). Three technical replicates from each cheese and three replicate cheeses (biological replicates), which were made on the same occasion, were analysed. Outer crust and inner core were tested separately as different microbial counts were detected in different cheese parts (Chapter 6) as well as pH after 4 weeks of ripening.

From the SPME analysis, the chromatograms obtained were analysed by comparing the chromatogram peaks obtained with the retention indices and mass spectra published in the databases (Section 2.13) From this analysis a range of compounds were indicated which included ketones, alcohols, aldehydes and acids. The following aroma compounds: 1-heptanol, 2-heptanol, 2-nonanol, 2-ethyl-1-hexanol, hexanal, pentyl ester butanoic acid, 2-octanone, 2-decanone, 2-

undecanone, 6-methyl-5-heptene-2-one were also detected, but not in all cheeses (see Appendix 10 for detailed data). Principal component analysis (PCA) was carried out on the majority compounds detected (Figures 7.5 and 7.6) to compare the volatiles produced from the different cheeses.

Figure 7.5 shows a comparison of all aroma compounds of the six different cheese models. This shows that model cheeses containing the added starter *P. roqueforti* (C4, C5 and C6) produced high levels of ketones, acids and alcohols compared to the model cheeses with the contaminant *Penicillium* (C1, C2 and C3). PC1 (41% of the data) and PC2 (28% of the data) show that in C1, C2 and C3, there is no significant difference between flavour compounds produced in the core and the crust. In C4, C5 and C6, the crust of these three model cheeses was clustered separately from the core; this was associated with the nature of the ketones and alcohols which were produced. While the majority of ketones in the crust were 2-nonanone, 2-decanone and 2-undecanone, the majority of ketones in the core were 2-pentanone, 2-heptanone, 2-octanone and 8-nonen-2-one. The crust samples of C4, C5 and C6 also produced high levels of acids (3-methyl butanoic acid, 2-methyl propanoic acid and pentyl ester butanoic acid), associated alcohols (2-heptanol and 2 nonanol) and branched alcohols (3-methyl 1-butanol, 2-methyl 1-propanol and 2-ethyl 1-hexanol). In the core, C5 produced higher hexanal than C4 and C6 whereas C4 produced higher ethanol than C5 and C6.

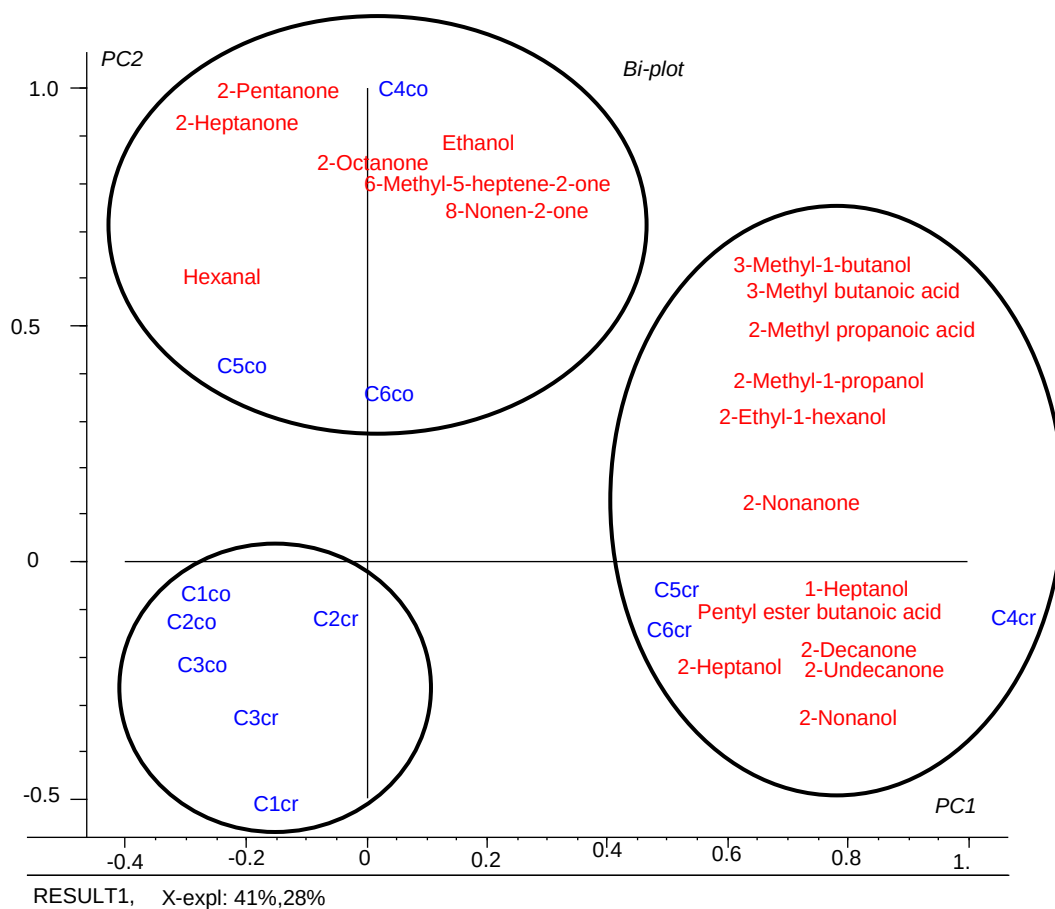


Figure 7.5. PCA plot of the aroma compounds obtained from GCMS SPME peak area data after headspace analysis of cheese models. Cheese made with: *Lc. lactis* (C1), *Lc. lactis* and incorporated *S. equorum* (C2), *Lc. lactis* and surface-spread *S. equorum* (C3), *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface-spread *S. equorum* and *P. roqueforti* (C6).
co: core and cr: crust.

Figure 7.6 focuses on the impact of *S. equorum* on flavour production in cheeses made with *P. roqueforti* (C4, C5, C6). In the core (Figure 7.6a), PC1 (66% of the data) and PC2 (34% of the data) show that the cheese with incorporated *S. equorum* (C5) was associated with acid, alcohols and ketones, whereas the control cheese with no *S. equorum* (C4) was associated with ketones and ethanol. Compared to the C4, the C5 produced high 2-decanone and 2-undecanone and cheese made with surface-spread *S. equorum* (C6) produced less flavour compounds. In the crust (Figure 7.6b), PC1 (58% of the data) and PC2 (42% of the data) show that C5 was associated with ketones and alcohols, while alcohols and acids were high in the crust of C6. However, different alcohols were detected between C5 and C6. The C5 produced high amounts of 2-heptanol, 2-nonanol and 2-ethyl-1-hexanol from fatty acid catabolism. The C6 produced high amounts of 1-heptanol, 2-methyl-1-propanol and 3-methyl-1-butanol from amino acid catabolism.

It is also interesting to note that the crust of control cheese (C4) had less ketones compared to C5 and C6 even though the *P. roqueforti* counts in C4 were higher than in C5 and C6 (Table 6.6). From Appendix 10, hexanal was the only aldehyde produced in a high amount in the core of C4 and C5 compared to C6. However, in the crust it was detected in C5 only.

As PCA analysis indicated some key differences in volatile components between the cheeses, further analysis was undertaken to determine the relative levels of specific volatiles in the different cheese models. The data were also generated from the area under the peak chromatogram similar to the data used for the PCA and the ANOVA and was performed with Design Expert (Section 2.13).

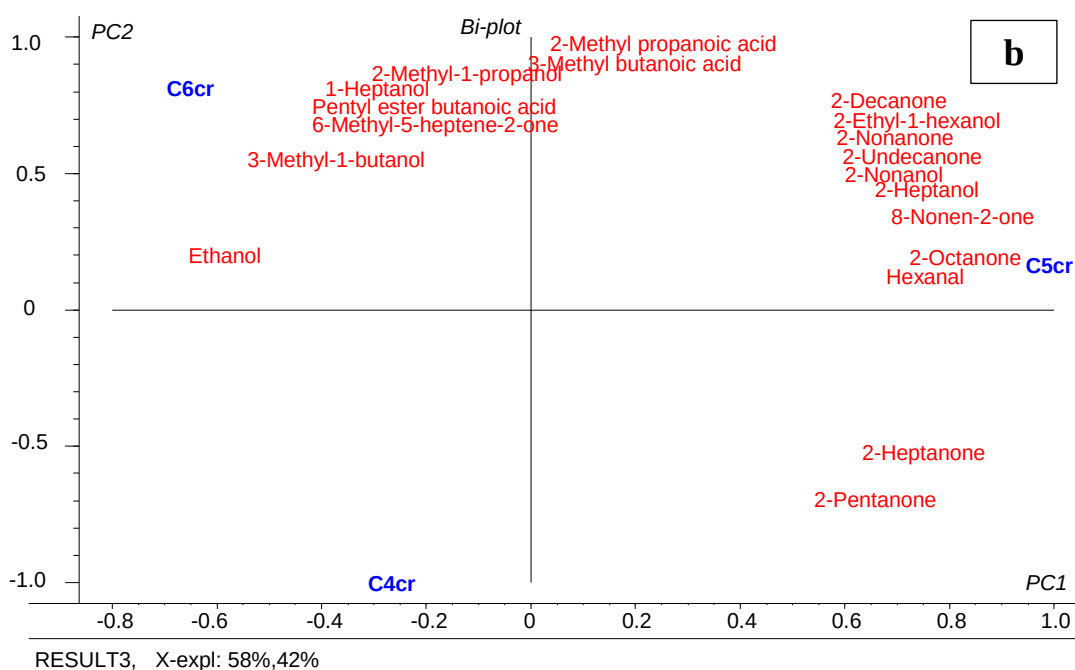
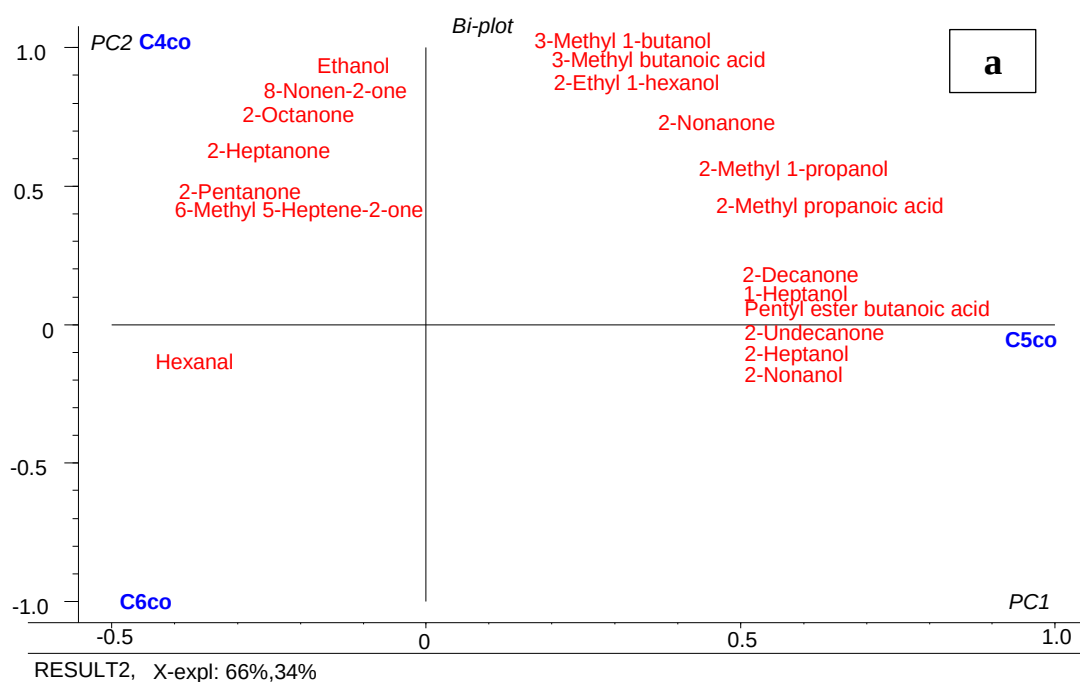


Figure 7.6. PCA plot of the aroma compounds obtained from GCMS SPME peak area data after headspace analysis of the core (a) and the crust (b) of cheese models made with starter *P. roqueforti* to show the impact of *S. equorum* inclusion. Cheese made with: *Lc. lactis* and *P. roqueforti* (C4), *Lc. lactis*, incorporated *S. equorum* and *P. roqueforti* (C5) and *Lc. lactis*, surface spread *S. equorum* and *P. roqueforti* (C6). co: core and cr: crust.

7.3.1. ALCOHOLS

Alcohols are derived from catabolism of citrate, lactose and amino acids (Figure 4.1). From SPME GCMS analysis, the major alcohols in all six model cheeses were ethanol, 3-methyl-1-butanol and 2-methyl-1-propanol, while the minor alcohols were 1-heptanol, 2-heptanol, 2-nonanol and 2-ethyl-1-hexanol (Appendix 10).

For ethanol (Figure 7.7), the incorporation of non-starter *S. equorum* (X) had no impact on the ethanol production ($P>0.05$), but parts of the cheeses (Y), the addition of starter *P. roqueforti* (Z) and its interaction (YZ) produced significantly different amount of ethanol ($P<0.05$). Therefore, Y1Z1 and Y2Z1 represents the average of C1, C2 and C3 core and crust respectively, while Y1Z2 and Y2Z2 represents the average of C4, C5, and C6 core and crust respectively. Ethanol was detected distinctly in the core of models with starter *P. roqueforti* (1.15×10^7 area units, AU).

Figure 7.8 shows how the incorporation of *S. equorum* (X), the addition of *P. roqueforti* (Z) and its interaction (XZ) significantly influences the level of 3-methyl-1-butanol in the model cheeses ($P<0.05$). However, there were no differences between the core and the crust ($P>0.05$). The core and crust data have been averaged for this experimental factor. *S. equorum* showed no effect in the absence of starter *P. roqueforti*, since *P. roqueforti* is needed to elevate the 3-methyl-1-butanol levels. When the mould was added (C4, C5, C6), these elevated levels are reduced by the surface-spread and incorporation of *S. equorum*. The latter had the greater effect such that the levels produced in cheese made with *P. roqueforti* and incorporated *S. equorum* (C5) were equivalent to those seen when the mould was not included (C2 and C3).

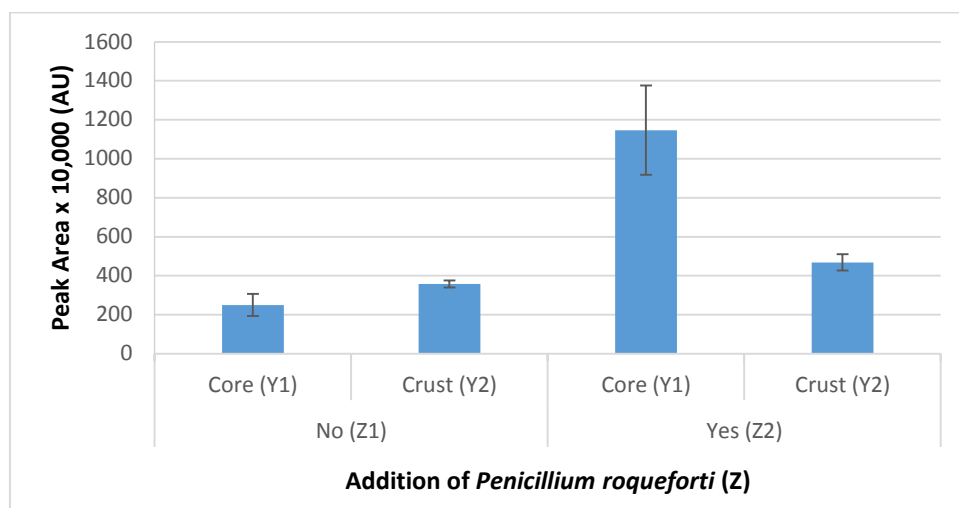


Figure 7.7. Levels of ethanol detected in the parts of model cheeses (Y) with (Z2 which is an average of models C4, C5, C6) and without (Z1 which is an average of models C1, C2, C3) the added *Penicillium roqueforti*. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

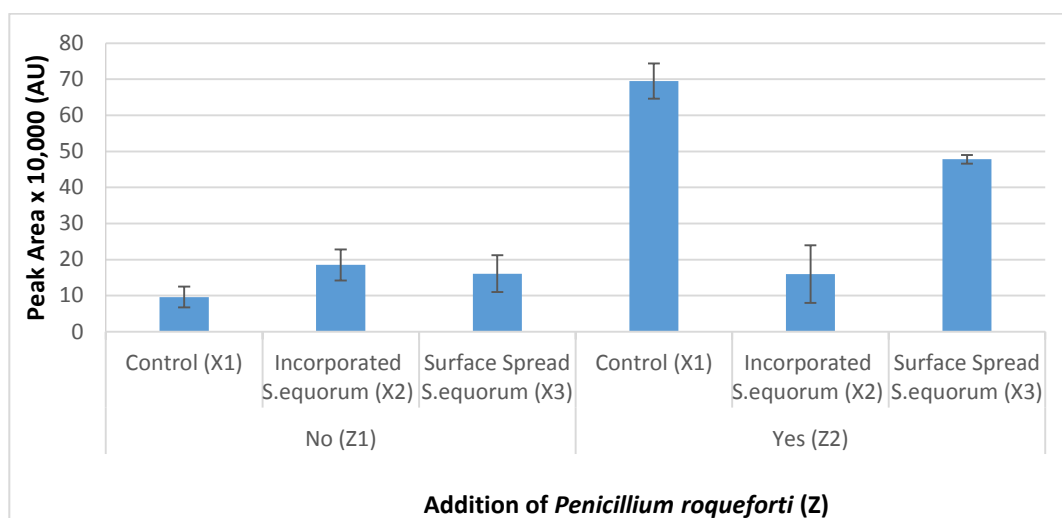


Figure 7.8. Levels of 3-methyl-1-butanol detected in the model cheeses with and without *S. equorum* (X) and the added *P. roqueforti* (Z). C1: X1Z1, C2: X2Z1, C3: X3Z1, C4: X1Z2, C5: X2Z2, C6: X3Z2. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

The trend of 2-methyl-1-propanol was similar to that of the 3-methyl-1-butanol except in this case there was a crust/core difference with higher levels of 3-methyl-1-propanol in the crust. In model cheeses without starter *P. roqueforti* (C1, C2 and C3), the level of 2-methyl 1-propanol was not significantly different either with or without *S. equorum* ($P>0.05$; Figure 7.9). In the crust of cheeses made with the addition of *P. roqueforti* (C4, C5, C6), the 2-methyl-1-propanol was produced at its highest levels in the control cheese (without *S. equorum*; C4; 6×10^4 AU; $P<0.05$) but the level was reduced in C6 (with surface spread *S. equorum*; 4×10^4 AU) and even less in C5 (with incorporated *S. equorum*; 2×10^4 AU). In the core of the control cheese (C4), a five times greater amount of this alcohol was produced than in C5 and C6. However, there was no significant difference in the production of the 2-methyl-1-propanol between cheeses with incorporated *S. equorum* (C5) and surface-spread *S. equorum* (C6) which was around 10^3 AU.

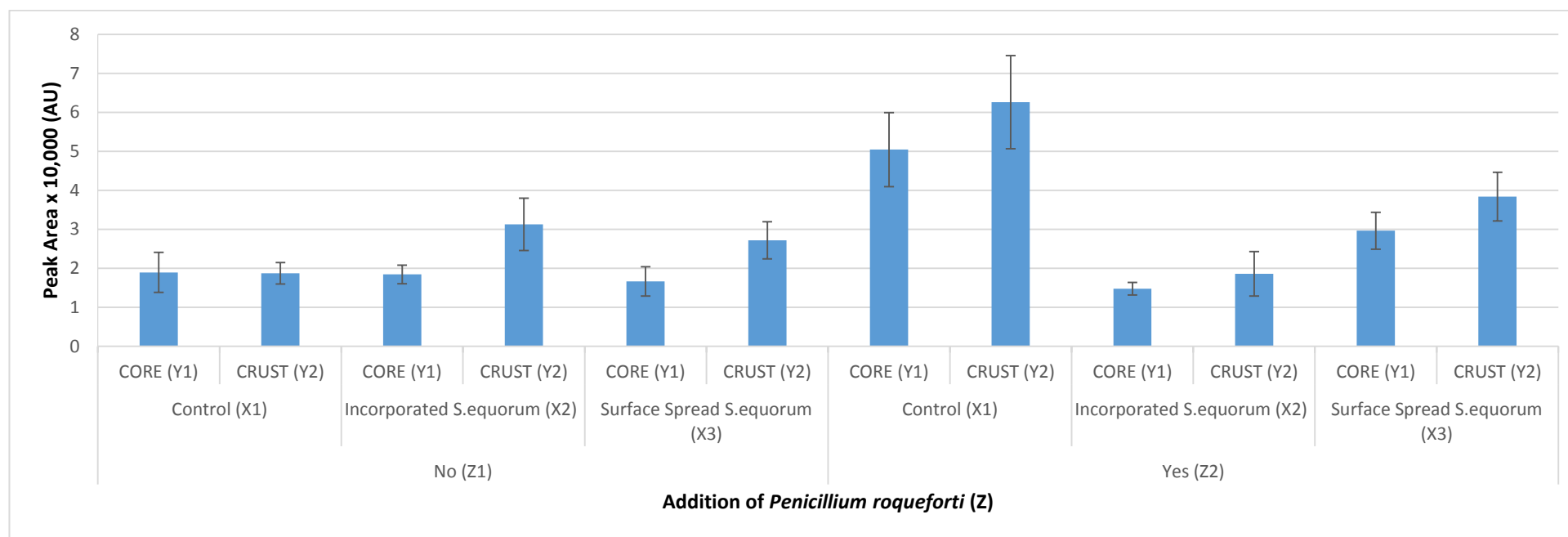


Figure 7.9. Levels of 2-methyl-1-propanol detected in the parts of model cheeses (Y) with and without *S. equorum* (X) and the added *P. roqueforti* (Z). C1: X1Z1, C2: X2Z1, C3: X3Z1, C4: X1Z2, C5: X2Z2, C6: X3Z2. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

7.3.2. ACIDS

Acids are derived from catabolism of amino acids (Figure 4.1). From SPME GCMS analysis, the dominant acids produced in the model cheeses were 3-methyl butanoic acid and 2-methyl propanoic acid in which the trends follow the trends of their respective alcohols although with fewer differences dependent on cheese part and how *S. equorum* was introduced.

Figure 7.10 shows that the addition of *P. roqueforti* (Z) increased the level of production of 3-methyl butanoic acid significantly ($P < 0.05$). The incorporation of non-starter *S. equorum* (X) and the cheese parts (Y) had no significant effect on the production of 3-methyl butanoic acid. Therefore Z1 is an average of C1, C2 and C3 and Z2 is an average of C4, C5 and C6.

The incorporation of *S. equorum* (X), the addition of *P. roqueforti* (Z) and its interaction (XZ) significantly influenced the level of 2-methyl propanoic acid in the model cheeses ($P < 0.05$; Figure 7.11). However, there was no differences in the 2-methyl propanoic acid production between different parts of the model cheeses (Y; $P > 0.05$). Therefore, each bar in the figure is an average of the core and crust. From Figure 7.11, all cheeses made without the addition of *P. roqueforti* (C1, C2 and C3) showed low production of this acid. The 2-methyl propanoic acid was highly produced in the control cheese (C4; 5×10^4 AU). The levels slightly decreased in cheese made with incorporated *S. equorum* (C5) and with surface-spread *S. equorum* (C6) although the decrease of 2-methyl propanoic acid levels were not statistically different.

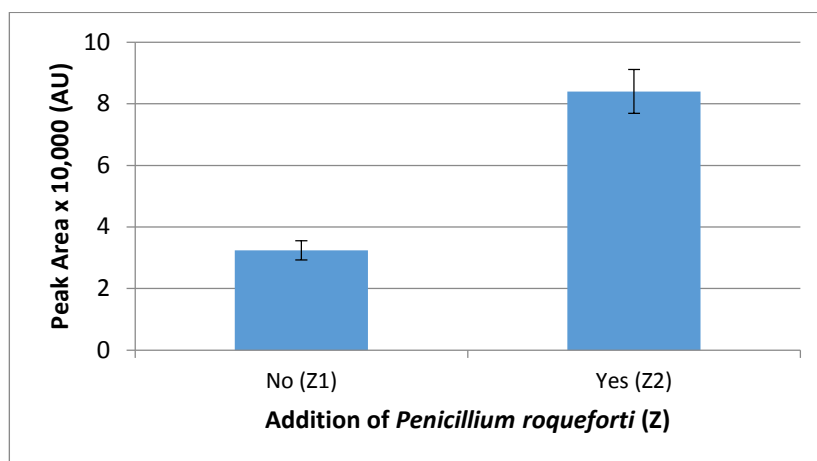


Figure 7.10. Levels of 3-methyl butanoic acid detected in the model cheeses with (Z2 which is an average of models C4, C5, C6) and without (Z1 which is an average of models C1, C2, C3) the added *P. roqueforti*. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

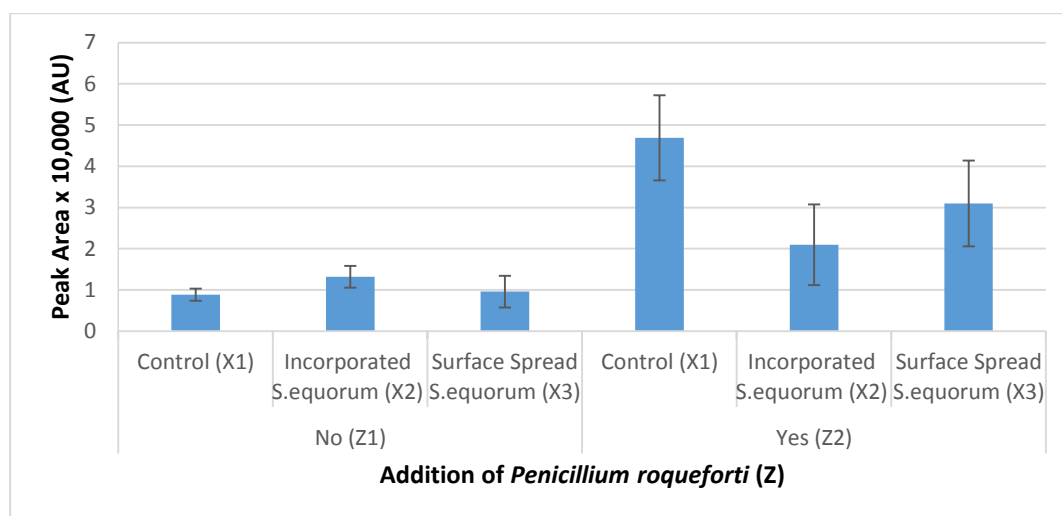


Figure 7.11. Levels of 2-methyl propanoic acid detected in the model cheeses with and without *S. equorum* (X) and the added *P. roqueforti* (Z). C1: X1Z1, C2: X2Z1, C3: X3Z1, C4: X1Z2, C5: X2Z2, C6: X3Z2. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

7.3.3. KETONES

Ketones are derived from catabolism of free fatty acids (Figure 4.1). From SPME GCMS analysis, 2-heptanone, 2-nonanone, 2-pentanone and 8-nonen-2-one were the main ketones detected in chromatographic profiles of all model cheeses.

Similar to the result for ethanol production (Figure 7.7), the addition of *S. equorum* (X) did not influence the level of 2-heptanone and 2-nonanone ($P>0.05$), but parts of the cheeses (Y), the addition of starter *P. roqueforti* (Z) and the interaction of these factors (YZ) produced significantly different levels of 2-heptanone and 2-nonanone (Figures 7.12 and 7.13; $P<0.05$). Therefore, in Figures 7.12 and 7.13, Y1Z1 and Y2Z1 represent the average of C1, C2 and C3, while Y1Z2 and Y2Z2 represent the average of C4, C5, and C6. Levels of 2-heptanone were higher in the core than the crust ($P<0.05$). This is true for the model cheeses with (C4, C5, C6) or without (C1, C2, C3) starter *P. roqueforti*, although the highest quantity of 2-heptanone was observed in the core of the model cheese with *P. roqueforti* where the sporulation of *P. roqueforti* was not observed.

For 2-nonanone, all cheeses made without the addition of *P. roqueforti* (C1, C2 and C3) showed low levels of production of this ketone, but in cheeses made with *P. roqueforti* addition (C4, C5, C6), more 2-nonanone was produced in the crust than the core. It is interesting to note that in cheeses made with the addition of *P. roqueforti*, while the crust had the highest amount of 2-nonanone (6×10^4 AU), the core had the highest amount of 2-heptanone (4×10^4 AU).

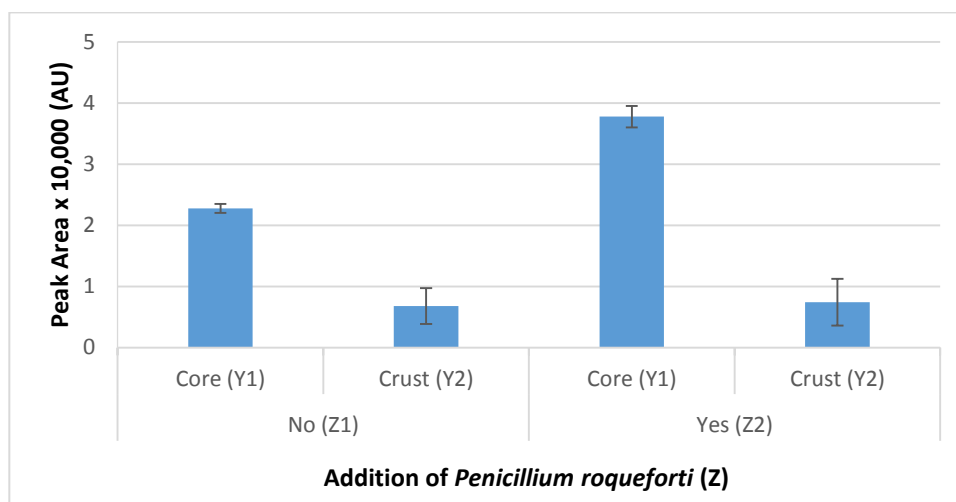


Figure 7.12. Levels of 2-heptanone detected in the parts of model cheeses (Y) with (Z2 which is an average of models C4, C5, C6) and without (Z1 which is an average of models C1, C2, C3) the added *Penicillium roqueforti*. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

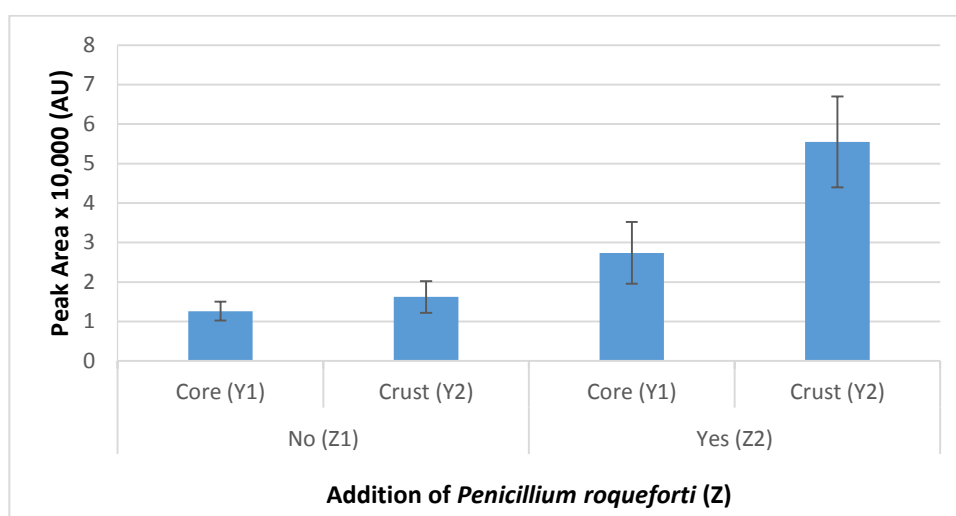


Figure 7.13. Levels of 2-nonanone detected in parts of model cheeses (Y) with (Z2 which is an average of models C4, C5, C6) and without (Z1 which is an average of models C1, C2, C3) the added *Penicillium roqueforti*. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

From Figure 7.14, in cheese made with *Lc. lactis* only (C1), the production of 2-pentanone in the core was significantly higher than that of the crust ($P < 0.05$). Differences were much greater than those seen for 2-heptanone which were greater than those seen for 2-nonanone. The presence of incorporated *S. equorum* (C2) could increase the levels of this ketone in both the crust and the core, but no different when it was surface-spread. The addition of starter *P. roqueforti* increased the 2-pentanone production. In cheeses made with *P. roqueforti* (C4, C5, C6), the addition of incorporated *S. equorum* (C5) lowered the production of 2-pentanone in the core and even lower in surface-spread *S. equorum* (C6), but had no effect in the crust. This may be due to the low levels of this compound generally associated with crust samples. Also, it is interesting to note that the core of control cheese (C4) produced the highest amount of 2-pentanone (4.01×10^5 AU), while the crust produced the lowest amount (6.6×10^3 AU).

For the 8-nonen-2-one (Figure 7.15), either in the cheese made with (C4, C5, C6) or without *P. roqueforti* (C1, C2, C3), how *S. equorum* was introduced had no influence on the production of this ketone ($P > 0.05$) which was similar to the controls. Also, either in the core or the crust, the addition of *S. equorum* did not affect the 8-nonen-2-one production. Although the variation in this compound showing from big error bars causes the differences of *P. roqueforti* addition was not significant, cheese C5 which was made with starter *P. roqueforti* and incorporated *S. equorum* showed an increased level of the 8-nonen-2-one in all cheese parts.

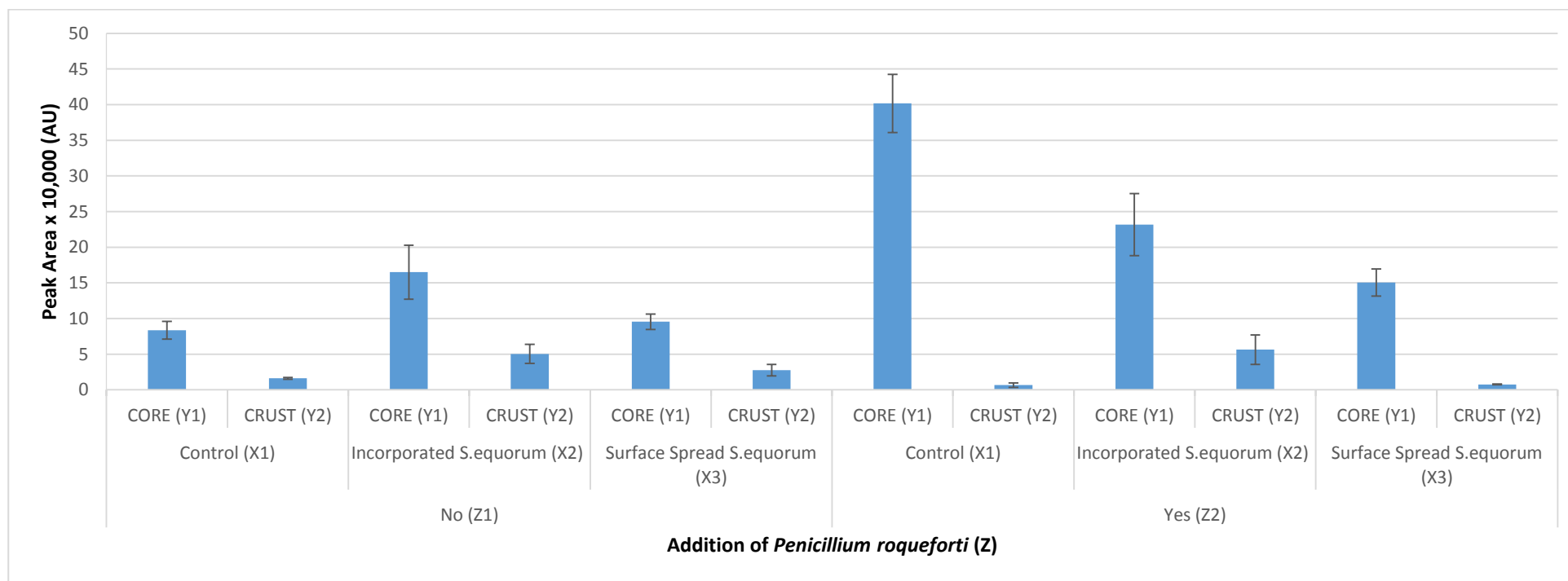


Figure 7.14. Levels of 2-pentanone detected in the parts of model cheeses (Y) with and without *S. equorum* (X) and the added *P. roqueforti* (Z). C1: X1Z1, C2: X2Z1, C3: X3Z1, C4: X1Z2, C5: X2Z2, C6: X3Z2. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

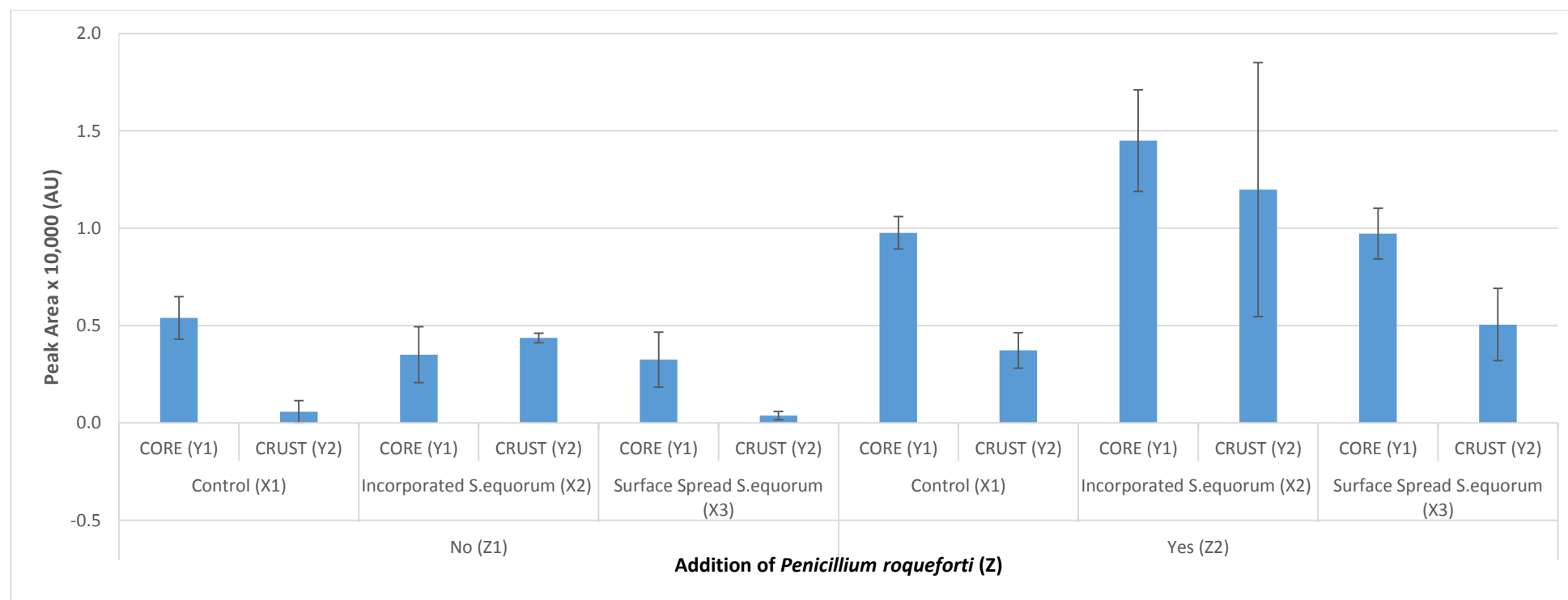


Figure 7.15. Levels of 8-nonen-2-one detected in the parts of model cheeses (Y) with and without *S. equorum* (X) and the added *P. roqueforti* (Z). C1: X1Z1, C2: X2Z1, C3: X3Z1, C4: X1Z2, C5: X2Z2, C6: X3Z2. Values are means of nine technical replicates obtained from three independent determinations $\pm$ standard errors of the means.

7.4 DISCUSSION

The major chemical groups detected in the model cheeses were alcohols, ketones and acids. From the bi-plot of PCA (principal component analysis), all model cheeses made without the addition of *P. roqueforti* (C1, C2 and C3) were not grouped with any compound confirming that the cheese with contaminated *Penicillium* would have less ketones which is a typical blue cheese aroma compound.

In the cheeses made with the addition of *P. roqueforti* (C4, C5 and C6), different alcohols, ketones, acids and aldehydes were produced in the core and crust and in different amounts. The core of these cheeses had higher levels of ketones, while the crust had higher levels of alcohols and acids. This result was different from the results of either Stilton cheese or the other studies using similar cheese models. In a commercial Stilton cheese, the blue vein and crust contained higher amounts of ketones, while the white core contained higher amounts of alcohols and aldehydes (Gkatzionis *et al.*, 2009). In a study of the impact of two different groups of yeast *D. hansenii* on flavour production, using the same cheese model used in the current study, the core and crust of a model incorporating *D. hansenii* group A was found to be associated with alcohols and acids, whereas the core of a model with *D. hansenii* group B was mostly associated with branched alcohols and aldehydes and the crust had ketones (personal communication with Constantina Apostolou). However, in a similar study with yeast *Trichosporon beigleii*, ketones were produced in the core and the crust of the model was lack of flavour compounds as the yeast inhibited the *P. roqueforti* growth (Whiley, 2013). Very high amounts of ethanol (at around 1.15×10^7 AU) were also detected in both core and crust of all model cheeses. However, Gkatzionis *et al.* (2009) detected a low level of ethanol in all parts of Stilton cheese.

Deetae *et al.* (2007) found that 3-methyl-1-butanol, 2-methyl-1-butanol, 1-butanol, 3-methyl-3-buten-1-ol and 2-methyl-1-propanol were the main alcohols produced by *S. equorum* in culture although sometimes in trace amounts. However, in the current study the 3-methyl-1-butanol and 2-methyl-1-propanol were produced in high levels in cheese made with *P. roqueforti* (C4, C5 and C6) but this was lowered by the presence of *S. equorum*, less so when this was surface-spread (C6) than when it was incorporated (C5). Thus in this combination it appears that *S. equorum* is not a key contributor to the production of alcohols and potentially through its impact on *P. roqueforti* growth may reduce their production. There is also the possibility that *S. equorum* competes with the mould for the carbon source making both alcohols decrease.

According to Gori *et al.* (2012), the 3-methyl-1-butanol and 2-methyl-1-propanol are major alcohols produced by *D. hansenii* and they give alcoholic and winey flavours in the cheese. Therefore, the reduction of both components would change the sensory characteristics of the cheese; this could be beneficial or a defect depending on the required characteristics of the cheese. However, because the alcohols are produced from the respective amino acids, the reduction of 3-methyl butanoic acid and 2-methyl propanoic acid in cheeses with either incorporated or surface-spread cheeses might be beneficial since 3-methyl butanoic acid gives a sour and stinky pungent feet odour and 2-methyl propanoic acid has a light odour of rancid butter.

Furthermore, how *S. equorum* was introduced in to the cheese showed no significant changes in the production of 2-nonanone (C₉H₁₈O) and 2-heptanone (C₇H₁₄O). However, surface-spread *S. equorum* in cheese made with *P. roqueforti* decreased the 2-pentanone (C₅H₁₀O) significantly and incorporation of *S. equorum* could slightly increase the production of 8-nonen-2-one (C₉H₁₆O). These

ketones are odour-active components and the key character of blue cheese flavour (Gallois and Langlois, 1990). Indeed, the 2-pentanone and 2-heptanone are the principal compounds in banana fruit and banana essence (Jordan *et al.*, (2001).

The levels of 2-pentanone in the model cheeses were higher than that of 2-nonanone. The 2-pentanone was easier to detect than 2-nonanone as 2-pentanone is not as fat soluble as 2-nonanone and 2-pentanone is more volatile than 2-nonanone. Therefore, it is noteworthy that the production of both ketones was related to their carbon chain length which is the 2-pentanone has a shorter carbon length than that of 2-nonanone. This is also an effect of caproic acid catabolism for 2-pentanone production and capric acid catabolism for 2-nonanone production during one month ripening.

It is interesting to note that compared to commercial Stilton cheese in which 2-heptanone was detected in a high level in the crust and 8-nonen-2-one was detected only in the crust of the cheese (Gkatzionis *et al.*, 2009), from all the model cheeses studied, high amounts of 8-nonen-2-one and 2-heptanone were shown in the core of the models. This suggests that the linoleic or linolenic acid catabolism in producing 8-nonen-2-one and the caprylic acid catabolism in producing 2-heptanone were not related to the presence of *P. roqueforti*.

Mycelium of *P. roqueforti* however could also oxidize the fatty acids, but only a low concentration of fatty acids with less than 14 carbon atoms (Lawrence and Hawke, 1968). Therefore, in the cheese models examined, the spores and/or mycelium of *P. roqueforti* might be responsible for the production of 2-pentanone, 2-nonanone and 8-nonen-2-one. However, well-distributed oxygen is needed for the *P. roqueforti* growth so that the fatty acids could be well-oxidized to increase the production of those methyl ketones in the model cheeses examined.

In addition, the sulfur-containing compounds and aldehydes which are compounds associated with protein degradation, were not major compounds in the final cheeses. That is because aldehydes are transitory compounds in cheese so that they are rapidly transformed to alcohols or to corresponding acids and sulfur-containing compounds which come from methionine degradation are the major compound produced by *Brevibacterium linens* (Molimard and Spinnler, 1996) which was not a member of the model microbial communities. However, *S. equorum* has methionine demethiolase which is important for methionine degradation, but it is deficient for methionine transaminase and the lack of methionine deaminase (Bonnarme *et al.*, 2001) which could affect the production of sulfur-containing compounds specifically dimethyldisulfide and dimethyltrisulfide.

7.5 CONCLUSIONS

Model cheeses with the contaminant *Penicillium* (C1, C2 and C3) produced low levels of all flavour compounds although they were not at the same level compared to model cheeses made with the addition of *P. roqueforti* (C4, C5 and C6). Except for 8-nonen-2-one, the presence of *S. equorum* reduced the ketones and it did not matter which way *S. equorum* was introduced. The presence of *S. equorum* also lowered the production of alcohols and acids. However, the way it was introduced gave different effects. The surface-spread *S. equorum* produced a lower amount of alcohols than the incorporated *S. equorum*. For the acids, the incorporated *S. equorum* produced lower amounts than when it was surface-spread *S. equorum*. Overall, ethanol was the component found in the greatest concentration (at around 1.15×10^7 AU) followed by 3-methyl-1-butanol

and 2-pentanone. Conversely, 8-nonen-2-one and hexanal were not the main compounds in the cheese examined.

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

Stilton cheese is a PDO product and it is well-known as the king of English cheeses. Since 1995, Stilton has been made from pasteurised cows' milk, although before the Stilton Cheesemakers' Association stated that Stilton must be made with pasteurised milk, it was made from raw milk. In March 1990, Stichelton cheese was introduced by Joe Schneider. It is as a blue-veined raw milk cheese where the production follows the Stilton making procedure.

There is much debate amongst cheese makers on the use of raw or pasteurised milks for cheese making. The risks and benefits of traditional cheeses, mainly raw milk cheeses, was discussed by Montel *et al.* (2014). For raw milk cheese makers, pasteurisation kills desirable bacteria and destroys enzymes and proteins causing a less tasty cheese; the problem of raw milk safety risks could be considered as fermentation would kill pathogenic bacteria if it is done properly. So, the main concern of traditional cheese producers is to preserve microbial diversity and exploit its benefits. Conversely, for other people, unpasteurised milk is related to health concerns caused by pathogenic bacteria. *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* and *E. coli* are the common pathogenic bacteria found in cheeses (Lafarge and Lahellec, 2000) hence pasteurisation is usually used to assure the safety of milk.

The current study continued a preliminary study on Stichelton cheese started in 2009 with the aim of identifying the microflora along the production line of Stichelton making before the role of non-starter bacteria was analysed. In

the earlier study, microflora diversity along the production process of Stichelton was examined until the point the cheese was ripened for 9 weeks but time did not allow the mature cheese to be examined. The microbial consortia of fully ripened Stichelton cheese was therefore analysed at the start of this study by both the traditional culture method and molecular approach 16S rDNA PCR DGGE in which DNA amplicons are separated from a low to high urea-formamide gradient gel by electrophoresis. Then, the separation of small PCR amplicons was distinguished by differences in their DNA sequences (Muyzer, 1999). The results showed *Lactobacillus casei/paracasei*, *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., *Alkalibacterium* sp. and *Corynebacterium casei* were detected in Stichelton; these species have not been found in Stilton cheese (Ercolini *et al.*, 2003). This shows that the bacterial composition of Stichelton was more complex than that of Stilton cheese, as has been suggested by raw milk cheese makers.

However, the culturing method has limitations for determining microbiota components as this method can only detect the microorganisms that can be cultivated *in vitro* on the media. The results on M17, MRS, Rogosa and KF Streptococcal agar also show that selective media are not always selective for a targeted microorganism. *Lactobacillus plantarum* grew on KF Streptococcal agar and M17 not only isolated *Lactococcus* but also *Kocuria* sp. grew on this medium.

The non-culture based approach is designed to overcome some of the limitations for microbiota characterisation which is evident from culture based methods. The 16S rDNA PCR DGGE method was chosen because all microbial consortia components through the cheese making could be identified although these may be in low number. However, PCR DGGE also has some limitations. First of all, it was difficult to find the right percentage combination of denaturing gradient for all DNA sequences. Some DNA sequences may be lost if a smaller

percentage range of denaturing gradient is used to separate specific bands. Secondly, because separation is based on %GC composition, the bands of different organisms with different sequences are sometimes at the same level on the DGGE gel as shown in the preliminary study (Yunita, 2010) where *Enterococcus* and *Lactococcus* banded at the same level. Thirdly, the sequencing of PCR DGGE products did not always give identifications at the species level; this was seen for *Brevibacterium* sp., *Halomonas* sp., *Acinetobacter* sp., and *Alkalibacterium* sp.

Therefore, other methods which could overcome these limitations could be applied to analyse the populations. Next generation sequencing (NGS) methods can be applied to increase a deeper description of the microbial constituents of the cheese ecosystems because NGS can be used in sequencing the total microbial nucleic acids (shotgun sequencing) and or targeted (gene-specific) sequencing (Mayo *et al.*, 2014). The NGS technique is also extremely useful compared to previous technologies being relatively low cost without the bias introduced by culture techniques, although research in these areas related to cheese is very limited (Lusk *et al.*, 2012).

Meta-omics methods can also be applied to overcome the limitations of what?. Genomic analyses can be used to predict a bacterial species potentiality. Proteomic, transcriptomic and metabolomic analyses can give much more information (the proteins of interests, transcripts synthesized and metabolites produced respectively) once the genome sequence of an organism is known (Zagorec *et al.*, 2008). Meta-omics (metagenomics and metatranscriptomics) and biochemical analyses were used to overview a surface-ripened cheese community (Dugat-Bony *et al.*, 2015). An overview of the cheese maturation process, the metabolic activities of the different community members and their possible

interactions were shown in the results. However, at the moment these methods still have limitations in particular the difficulty to detect low abundant species which could also contribute to the matrix transformation.

According to Mayo *et al.* (2014), the combination between NGS and meta-omic methods has a positive future prospect. By using these methods for future studies, the *in-situ* metabolic activities of the microbial community composition examined in this study could be analysed to get a complete overview of the cheese ripening process specifically for raw milk blue-veined cheeses, where numerous interactions between raw milk consortia, added starter cultures and community members along the process cannot be avoided. Delcenserie *et al.* (2014) characterised the microbiota composition of Herve cheese, a Belgian PDO cheese, using not only a traditional culture method, but also a metagenomics approach based on 16S rDNA pyrosequencing which could allow faster and better identification. This method showed a total of 207 different phylotypes in samples of raw or pasteurized milk Herve cheeses, showing that the biodiversity of these cheeses was high. Furthermore, a set of biomarker genes could be selected to manage the safety risks and benefits of various microorganisms which come from the farm, production or cheese makers.

Moving on to bacteria identification, other non-starter bacteria were detected along the Stichelton production even though it uses a mixed culture of *Lc. lactis* and a secondary starter *P. roqueforti* which are similar to Stilton. For example, *Lb. plantarum* was detected in the raw milk and after salting; *S. equorum* and *Enterococcus durans* were found before and after piercing; *Lb. casei/paracasei*, *E. faecalis* and *Brevibacterium* sp. were identified in the final cheese. This indicates that the raw milk alone is unlikely to be the source but also salt addition, unpreserved curd handling, piercing, ripening conditions and long-time

ripening are all contributing to the cheese microbial diversity. Montel *et al.* (2014) also said that the diversity of traditional practices in cheese making result in a wide variety of microbiota and environments which affects the microbial activities.

It was notable that *Lc. lactis* survived into the core and crust parts of the final cheese which was detected by both culturing and PCR DGGE methods. The 16S rDNA sequencing showed this was subspecies *lactis*. Whether the surviving species originated from the raw milk or was part of the added starter culture was of interest as it could demonstrate the role of raw milk flora in contributing to cheese production. From PFGE analysis of final cheese and starter culture isolates, it was shown that the isolate was not from the starter culture and so this *Lc. lactis* subsp. *lactis* which survived into the final cheese, specifically in the core part, was from the raw milk. This shows that the natural lactic flora from the raw milk could be more tolerant with the conditions during cheese making than the added starter so that these could contribute to the final flora and sensory properties and also could be a potential starter culture for Stichelton cheese production. Psoni *et al.* (2007) found wild *Lc. lactis* isolated from a raw goats' milk cheese may control the growth of *E. coli* O157:H7, *Yersinia enterocolitica*, *S. aureus*, *B. cereus*, *L. monocytogenes* and *E. faecalis* in the production of Batzos, a Greek PDO cheese demonstrating the safety of raw milk cheese. In traditional Spanish cheeses (Cabrales, Peñamellera, Genestoso, Casín), wild *Lc. lactis* subsp. *lactis* in the raw milk is used as starter culture so that no starter is added and only the natural lactic flora is used for the fermentation (Fernández *et al.*, 2011).

The addition of commercial starter culture may still be relevant in a raw milk cheese because it could stimulate the growth of the natural lactic flora in the

raw milk. Increasing the numbers of starter cells would accelerate the ripening process (Fox *et al.*, 2000). Also, according to Gutiérrez-Méndez *et al.* (2008), *Lc. lactis* isolated from raw milk dairy products or commercial starter cultures presented faster lactose fermentation and proteolytic activity than those presented by strains isolated from non-dairy sources, but because there was not a clear relationship between the source of isolation and the ability of lactococci strains to produce aroma, strains generating stronger aromas presenting more efficient amino acid catabolism is needed and one of both strains could be an option.

S. equorum was found to be present in Stichelton and has been shown to be present in Stilton cheese (Ercolini *et al.*, 2003), Tilsit cheese (Bockelmann, 2002) and Raclette cheese (Carnio *et al.*, 2000). However, the role of *S. equorum* in blue-veined cheeses has not published although its role in a semi-soft smear Tilsit cheese has been presented (Figure 5.1) by Bockelmann (2002). In the current study, the *S. equorum* was used in cheese models to determine its impact on cheese production. The *S. equorum* was introduced in two ways; it was added at early fermentation and it was smeared on the surface before ripening as PCR DGGE studies suggested this was a way it was introduced. The results showed that it survived in both conditions after the cheese was ripened for one month, although only in the crust of the cheese when made with *P. roqueforti* (C5 and C6). The presence of this bacterium in the crust of one month ripened C5 is an unexpected result as the study on the response of *S. equorum* to acid suggested *S. equorum* did not grow at pH 3 and 4 so it should be killed during fermentation. However, pH studies were done in BHI broth, not in the fermented milk. The result could be different as milk proteins may buffer more than the BHI broth so the pH effect can be negated.

The surface-spread *S. equorum* at early ripening in the blue cheese models examined also inhibited *P. roqueforti* sporulation. In blue cheese production, this could cause a defect and might be the answer to why sometimes blue cheeses fail to blue. However, the use of this bacterium might be useful for white Stilton or other white mould-ripened cheeses such as Brie and Camembert cheeses where the presence of *Penicillium* is undesirable. To do this, the bacterium could be introduced at the middle or end of the ripening step as is done with other smear-ripened or washed-rind cheeses. From a trial experiment, a model cheese made from raw milk supplied by University of Nottingham Dairy Centre showed an interesting result (Appendix 12). In particular the blue veins developed in the cheese core along the piercing line during one month's ripening validating the cheese making methods for small-scale cheese where the cheese will look like Stilton if the microflora needed was available in the milk. However, the use of *S. equorum* as an adjunct starter culture needs further analysis specifically on its role on antifungal activity causing the inhibition of *P. roqueforti* sporulation. Also, it might be useful if the colour of the model cheese were examined using a colorimeter. This could make the colour of mould defects more quantifiable as the green colour of the cheese crust and the white colour of the cheese core presented in this study were at different levels when *S. equorum* was present.

In terms of texture, the hardness of the control cheeses (14 *g* for C1 and 12 *g* for C4) was much softer than Stilton cheese (25 *g*) as an effect of proteolysis. The surface-spread *S. equorum* in C6 increased the hardness of the cheese to 16 *g*. Texture of the raw milk model cheese was even much harder than the original model (Appendix 13) as the relative humidity in the vessel was reduced to 53% using MgN_2 and 33% using $MgCl_2$ (Greenspan, 1977). In addition, a cheese ripening and drying mat which is usually used in industry was also used in this

trial experiment. This could have an effect on the texture as the whey syneresis could still take place during ripening. In the original models, aluminum foil swabbed with 70% ethanol was used as the base of cheese hoops and it resulted in whey being detained at the bottom of the cheese even though the foil was changed every week.

However, the model cheese system used in this study had some limitations showing an impact not only on the physical characteristics, but also the microbial characteristics so that was slightly different from the original Stichelton cheese. Firstly, UHT milk was chosen to control the microbiota precisely from the beginning of the process, but the cheese texture became soft although it was ripened for one month. After one month, the cheese texture was expected to be hard due to drying out and whey drainage. This affected *P. roqueforti* sporulation which was inhibited inside the model as oxygen could not enter the core part. The cheese curd was very soft as β -lactoglobulin can form a layer over the casein micelle because of the high temperatures used during the production of UHT milk; this can prevent curd formation in cheese. In the raw milk cheese trial experiment, the cheese texture was harder than the original UHT models, but it could not be concluded that this was because of the raw milk as it may have been the low relative humidity inside the ripening vessel. Re-examining the experiment in more depth is necessary. Furthermore, the size of the model cheese (85 mm in diameter and 100 mm in height) is smaller than the original size (178 mm in diameter, 203 mm in height) and the ripening process was also conducted in a small ripening vessel so that the oxygen level inside the vessel might not be enough for the cheese size. Therefore, the ratio between the cheese size and the vessel size should be calculated representing the right proportion between the real cheese and the real ripening room.

This study also showed that compared to the cheese made with *Lc. lactis* and *P. roqueforti*, the addition of *S. equorum* either incorporated or surface-spread produced more flavour compounds in the crust. In the core, less flavour compounds were detected as an effect of the failure of *P. roqueforti* to sporulate as in the case of a low oxygen level. So, increasing flavour compounds in the core of model cheese would be possible if the oxygen could be distributed into the cheese core successfully. This could be related to the texture as the softer cheese is unlikely to allow the vein piercing to successfully introduce oxygen due to vein collapse.

In the model cheeses, the production of 2-pentanone contributing to stronger blue cheese aroma was very low compared to Stilton cheese and 2-pentanol, which is a compound formed from 2-pentanone because of a reductase enzyme, was not detected either. This may be because the model cheeses were only ripened for 4 weeks whereas Stilton cheese is usually ripened for 12 weeks. So, the cheeses represent an intermediate stage of ripening before the breakdown of caproic acids is completed. Lack of sulphur-containing compounds which were detected in Stilton (Gkatzionis *et al.*, 2014) is expected because of the lack of methionine deaminase and methionine transaminase deficiency in methionine catabolism as suggested by Bonnarne *et al.* (2001). Moreover, Stilton or Stichelton cheeses have a diverse community of microorganisms, whereas in the model cheese examined, the microorganism composition used was controlled to specified species and hence indicates this diversity is important in providing the full characteristics of the cheese.

Furthermore, as the microbiology studies have shown Stichelton cheese is a complex ecosystem, and interactions between the *Lc. lactis* starter, co-starter *P. roqueforti* and non-starter *S. equorum* shown in this study are not the whole

picture of what is happening inside the real cheese. Interactions between these microorganisms with *Lb. plantarum* may be interesting to examine to see the flavour changes as *Lb. plantarum* which was detected in the raw milk and Stichelton in the PCR DGGE study could increase the production of 3-methyl-1-butanol, 2-methyl-1-propanol, 3-methyl butanal and 2-methyl butanal and acetoin when it was co-cultured with *Lc. lactis* only (Mugampoza, 2013).

The role of *Brevibacterium* sp. which was detected in the Stichelton crust after piercing was also never examined in this study. This bacterium is a major surface-ripening microorganism in the surface-ripened Irish cheeses such as Gubbeen, Durrus, Ardahan, Milleens (Mounier *et al.*, 2005) as well as French cheeses such as Limburger, Munster, and Tilsiter (Deetae *et al.*, 2007). Its role in sulfur-containing compounds production (Rattray and Fox, 1999) and its ability to produce a carotenoid pigment which gives an orange-red-brown colour have been demonstrated (Galaup *et al.*, 2015) and this would be interesting to see if the organism was combined with the tested Stichelton starter and non-starter bacteria, whether the green colour at the surface of the cheese produced by *P. roqueforti* would be reduced because of the *Brevibacterium* competing with the mould.

Finally, this study did not look at the yeast microflora during Stichelton production. Yeasts have been shown to be an important part of the flora of Stilton cheese and to influence aroma volatile production (Gkatzionis *et al.*, 2009; Gkatzionis *et al.*, 2014). Before interactions between bacteria, mould and yeasts can be done, it would be useful to look at the yeast flora along the process so important yeasts in Stichelton-making can be identified first. However, from unpublished work done by Constantina Apostolou in association with this study, the role of the yeast *D. hansenii* has been examined in a cheese model. Interesting results were shown as two different strains of this yeast, which produced identical

Terminal Restriction Fragment Length Polymorphism (TRFLP) profiles, showed different activities. While one strain was important to produce a solid texture, the second strain was important in ketone production which is typical blue cheese aroma compounds. An interesting combination would be to see the interaction between surface-spread *S. equorum* with the second strain of *D. hansenii*.

Therefore to produce a better understanding of the contribution of the microbiota in Stichelton cheese production, the interactions of the organisms examined to date with other microorganisms such as *Lb. plantarum*, *Brevibacterium* sp. and yeasts are needed. This could lead to much more controlled fermentations in the future.

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Appendix 1. Media and chemicals preparation.

a. MEDIA

1. M17 AGAR (Oxoid code CM0785)

The agar (24.1 g) was dissolved in 500 ml of RO water and autoclaved at 121°C for 15 min. The media was allowed to cool to 55°C before the addition of 25 ml of 1% (w/v) sterile lactose solution (Oxoid code LP00070). This was mixed before plating.

2. MANITOL SALT AGAR (Oxoid code CM0085)

The agar (55.5 g) was dissolved in 500 ml of RO water and autoclaved at 121°C for 15 min. The media was allowed to cool to 55°C. Then, it was poured into petri dishes and was allowed to set.

3. ROGOSA AGAR (Oxoid code CM0627)

The agar (41 g) was dissolved in 500 ml of RO water and heated by using hot stirrer plate until it dissolved completely. It then cooled in 55°C waterbath. After the temperature of media reached 55°C, glacial acetic acid (0.66 ml) was added into the agar. This was mixed before plating.

4. BAIRD PARKER AGAR (Oxoid code CM0275)

The agar (31.5 g) was dissolved in 500 ml of RO water and autoclaved at 121°C for 15 min. The media was allowed to cool to 55°C before the addition of 25 ml of Egg Yolk Tellurite Emulsion (Oxoid code SR0054). This was mixed before plating.

5. NUTRIENT AGAR (Acumedia code 7145A)

The agar (11.5 g) was dissolved in 500 ml of RO water and autoclaved at 121°C for 15 min. The media was allowed to cool to 55°C. Then, it was poured into petri dishes and was allowed to set.

6. STREPTOCOCCUS AGAR (Oxoid code CM0701)

The agar (24.1 g) was dissolved in 500 ml of RO water, autoclaved at 121°C for 15 min and cooled until 55°C. Then, 1% (w/v) sterile solution of 2,3,5-triphenyltetrazolium chloride (5 ml) was added into the media using a sterile syringe and a sterile filter. This was mixed before plating.

7. ROSE BENGAL CHLORAMPHENICOL AGAR (Oxoid code CM0549)

The agar (16 g) was dissolved in 500 ml of RO water and autoclaved at 121°C only for 5 min. The media was allowed to cool to 55°C and one vial chloramphenicol (Oxoid code SR0078H) diluted with 3 ml ethanol was added into the agar.

8. BRAIN HEART INFUSION AGAR (Oxoid code CM1135)

The Brain Heart Infusion broth (23.5 g) with 1.5% (w/v) agar (7.5 g in 500 ml RO water; Oxoid code LP0011) was autoclaved at 121°C for 15 min, cooled until 55°C and then plated.

9. MRS AGAR (Oxoid code CM0361)

The agar (31 g) was dissolved in 500 ml of RO water, autoclaved at 121°C for 15 min, cooled until 55°C and then plated.

b. CHEMICALS

1. PHOSPHATE BUFFERED SALINE (Oxoid code BR0014G)

One tablet was dissolved in 100 ml RO water by using a magnetic stirrer, autoclaved at 121°C for 15 min and stored at room temperature.

2. MAXIMUM RECOVERY DILUENT (MRD; Oxoid code CM0733)

MRD (4.75 g) was dissolved in 500 ml RO water, autoclaved at 121°C for 15 min and stored at room temperature.

3. SODIUM CHLORIDE (NaCl) 5M

NaCl (29.2 g) was dissolved in 100 ml RO water, autoclaved at 121°C for 15 min and stored at room temperature.

4. TES BUFFER (Tris Base 50 mM, EDTA 1 mM, Sucrose 8.7 %)

Tris base (0.61 g), EDTA (0.029 g) and sucrose (8.7 g) were mixed with 70 ml RO water. The pH was adjusted to 8 and distilled water was added until the final volume reached 100 ml. The mixture was autoclaved at 121°C for 15 min and stored at room temperature.

5. NTS BUFFER (Sodium Chloride (NaCl) 0.2M, Tris Base 0.1 M, SDS 2%)

NaCl (1.17 g), tris base (1.21 g) and sodium dodecyl sulfate (SDS; 2 g) were mixed with 70 ml RO water. The pH was adjusted to 8 and distilled water was added until the final volume reached 100 ml. Then, the mixture was autoclaved at 121°C for 15 min and stored at room temperature.

6. TE BUFFER (Tris Base 10 mM, EDTA 1 mM)

Tris base (0.12 g) and EDTA (0.029 g) were mixed with 70 ml RO water.

The pH was adjusted to 8 and distilled water was added until the final volume reached 100 ml. The mixture was autoclaved at 121°C for 15 min and stored at room temperature.

7. 50X TAE BUFFER

Tris base (121 g), glacial acetic acid (28.55 ml) and EDTA 0.5M (50 ml) were mixed with 400 ml RO water. The pH was adjusted to 8 and distilled water was added until the volume reached 500 ml. Finally, the mixture was autoclaved at 121°C for 15 min and stored at room temperature.

8. EDTA 0.5M

EDTA (29.26 g) was added in to 70 ml RO water and the pH value was measured. The mixture has to reach pH 8 to make the EDTA dissolve completely by addition of 5M NaOH at around 100 ml. After that, the solution was measured the volume and added water until the volume reached 200 ml. Finally, the mixture was autoclaved at 121°C for 15 min and stored at room temperature.

9. NaOH 5M

NaOH (20 g) was dissolved in 80 ml RO water and the final volume adjusted to 100 ml with RO water. The mixture was autoclaved at 121°C for 15 min and stored at room temperature.

10. 0% DENATURANT SOLUTION

The 50X TAE buffer (2 ml) and 78 ml deionised water were put in the 100 ml Duran bottle. Then, acrylamide/bis 40% (20 ml) was added. Acrylamide/bis 40% is carcinogenic so that addition of it must do in the fume hood. The mixture was sterilised by using 0.45 µm sterile filter (yellow) and sterile syringe and put into sterile dark bottle (or clear bottle covered by aluminum foil). Finally, the 0% denaturant solution was degassed for 15 min and stored at 4°C carcinogenic fridges.

11. 100% DENATURANT SOLUTION

Urea (42 g) and 50X TAE buffer (2 ml) were put in the clear bottle. Then, acrylamide/bis 40% (20 ml) and deionised formamide (40 ml) were added. The addition of these chemicals has to do in the fume hood because of carcinogenic reason. After that, the 100% denaturant solution was also degassed at the same way as the 0% denaturant solution and stored at 4°C carcinogenic fridges.

12. DGGE DYE SOLUTION

Bromophenol blue (0.05 g) and xylene cyanol (0.05 g) were mixed in the sterile universal bottle and 1X TAE buffer (10 ml) was added. This solution was kept at room temperature.

13. 2X GEL LOADING DYE

Glycerol 100% (7 ml) and sterile RO water (2.5 ml) were mixed in a sterile universal bottle. Xylene cyanol 2% (0.25 ml) and bromophenol blue 2% (0.25 ml) were added and mixed. This solution was stored at room temperature.

14. TN BUFFER (Tris-HCl 10mM, NaCl 1M)

Tris base (12.1 g) was mixed with 50 ml distilled water using stirrer on a hotplate. The pH was adjusted to 8 and distilled water was added until the volume reached 100 ml. The tris-HCl solution (5 ml) and 1M NaCl (100 ml) were mixed and distilled water was added until the volume was 500 ml. The mixture was autoclaved at 121°C for 15 min and stored at room temperature.

15. LYSIS BUFFER (Tris-HCl 6mM, EDTA 0.1mM, 1% N-Lauryl-Sarcosine Sodium)

Tris-HCl solution (3 ml), EDTA 0.1mM (Section 2.8; 0.1 ml) and 1% N-Lauryl-Sarcosine Sodium (sarcosyl; 5 g) were mixed with 400 ml distilled water. The pH was measured to 7.6 and distilled water was added until the volume was 500 ml. The mixture was autoclaved at 121°C for 15 min and stored at room temperature. Lysozyme (Sigma L6876; 10 mg/ml) was added when it was used.

16. PROTEINASE-K BUFFER

EDTA 0.5M (500 ml) and sarcosyl (5 g) were mixed and autoclaved at 121°C for 15 min and stored at room temperature. Proteinase-K (Thermo Fisher Scientific AM2548; 20 mg/ml) was added when it was used.

17. 10X TBE BUFFER

Tris base (108 g), boric acid (55 g) and EDTA 0.5M (40 ml) were mixed with 700 ml distilled water. The pH was measured to 8 and distilled water was added until the volume 1,000 ml. The mixture was autoclaved at 121°C for 15 min and stored at room temperature.

Appendix 2. The significance of PFGE clusters of *lactococcus lactis* isolates obtained from Stilton cheese and starter cluster.

a. AMOVA

Source of variation	df	SS	MS	Est. Var.	%
Among populations	4	289.086	72.272	11.248	31%
Within populations	25	621.054	24.842	24.842	69%
Total	29	910.141		36.090	100%
Stat	Value	P (rand >= data)			
PhiPT	0.312	0.002			

Probability, P (rand >= data), for PhiPT is based on permutation across the full data set.

b. Pair wise population PhiPT values from AMOVA analysis

	1	2	3	4	5	
1	0.000	0.001	0.001	0.004	0.007	1
2	0.172	0.000	0.030	0.074	0.070	2
3	0.209	0.430	0.000	0.096	0.100	3
4	0.268	0.261	0.269	0.000	0.657	4
5	0.497	0.643	0.691	0.333	0.000	5
	1	2	3	4	5	

1 – 5: clusters which were delineated at 40% similarity level.

Appendix 3. The significance of PFGE clusters of *Staphylococcus equorum* isolates obtained from Stilton, Danish Blue and Reblochon cheese with:

a. *Sma*I

1. AMOVA

Source of variation	df	SS	MS	Est. Var.	%
Among populations	4	334.731	83.683	21.222	56%
Within populations	19	313.144	16.481	16.481	44%
Total	23	647.875		37.703	100%
Stat	Value	P (rand >= data)			
PhiPT	0.563	0.001			

Probability, P (rand >= data), for PhiPT is based on permutation across the full data set.

2. Pair wise population PhiPT values from AMOVA analysis

	1	2	3	4	5	
1	0.000	0.010	0.020	0.020	0.020	1
2	0.224	0.000	0.350	0.340	0.300	2
3	0.459	0.691	0.000	0.360	0.320	3
4	0.603	0.759	0.000	0.000	0.330	4
5	0.601	0.778	0.000	0.000	0.000	5
	1	2	3	4	5	

1 – 5: clusters which were delineated at 50% similarity level.

b. *SacI*

1. AMOVA

Source of variation	df	SS	MS	Est. Var.	%
Among populations	3	240.376	80.125	13.530	46%
Within populations	18	290.992	16.166	16.166	54%
Total	21	531.368		29.696	100%
Stat	Value	P (rand >= data)			
PhiPT	0.456	0.002			

Probability, P (rand >= data), for PhiPT is based on permutation across the full data set.

2. Pair wise population PhiPT values from AMOVA analysis

	1	2	3	4	
1	0.000	0.010	0.010	0.020	1
2	0.319	0.000	0.020	0.040	2
3	0.279	0.572	0.000	0.320	3
4	0.578	0.675	0.775	0.000	4
	1	2	3	4	

1 – 4: clusters which were delineated at 50% similarity level.

c. *ApaI*

1. AMOVA

Source of variation	df	SS	MS	Est. Var.	%
Among populations	3	263.879	87.960	19.007	52%
Within populations	19	331.356	17.440	17.440	48%
Total	22	595.235		36.447	100%
Stat	Value	P (rand >= data)			
PhiPT	0.522	0.001			

Probability, P (rand >= data), for PhiPT is based on permutation across the full data set.

2. Pair wise population PhiPT values from AMOVA analysis

	1	2	3	4	
1	0.000	0.010	0.020	0.010	1
2	0.335	0.000	0.140	0.130	2
3	0.481	0.626	0.000	0.230	3
4	0.612	0.732	0.000	0.000	4
	1	2	3	4	

1 – 5: clusters which were delineated at 55% similarity level.

Appendix 4. The significant ANOVA of acid tolerance of *Staphylococcus equorum* isolates obtained from Stilton core (A), Stilton crust (B), Danish blue (C), Reblochon core (D) and Reblochon crust (E) at:

a. pH 7 – 4 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.226	4	1.806	6.621	.031
Within Groups	1.364	5	.273		
Total	8.590	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	1.01500	.52236	.402	-1.0804	3.1104
	C3	1.97000	.52236	.063	-.1254	4.0654
	D19	1.95000	.52236	.065	-.1454	4.0454
	E3	2.34500*	.52236	.032	.2496	4.4404
B2	A2	-1.01500	.52236	.402	-3.1104	1.0804
	C3	.95500	.52236	.449	-1.1404	3.0504
	D19	.93500	.52236	.466	-1.1604	3.0304
	E3	1.33000	.52236	.217	-.7654	3.4254
C3	A2	-1.97000	.52236	.063	-4.0654	.1254
	B2	-.95500	.52236	.449	-3.0504	1.1404
	D19	-.02000	.52236	1.000	-2.1154	2.0754
	E3	.37500	.52236	.943	-1.7204	2.4704
D19	A2	-1.95000	.52236	.065	-4.0454	.1454
	B2	-.93500	.52236	.466	-3.0304	1.1604
	C3	.02000	.52236	1.000	-2.0754	2.1154
	E3	.39500	.52236	.933	-1.7004	2.4904
E3	A2	-2.34500*	.52236	.032	-4.4404	-.2496
	B2	-1.33000	.52236	.217	-3.4254	.7654
	C3	-.37500	.52236	.943	-2.4704	1.7204
	D19	-.39500	.52236	.933	-2.4904	1.7004

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
E3	2	3.0050	
C3	2	3.3800	3.3800
D19	2	3.4000	3.4000
B2	2	4.3350	4.3350
A2	2		5.3500
Sig.		.217	.063

b. pH 7 – 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.956	4	3.489	8.929	.017
Within Groups	1.954	5	.391		
Total	15.910	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	1.07500	.62512	.497	-1.4327	3.5827
	C3	2.03500	.62512	.104	-.4727	4.5427
	D19	2.13500	.62512	.089	-.3727	4.6427
	E3	-.92500	.62512	.613	-3.4327	1.5827
B2	A2	-1.07500	.62512	.497	-3.5827	1.4327
	C3	.96000	.62512	.585	-1.5477	3.4677
	D19	1.06000	.62512	.508	-1.4477	3.5677
	E3	-2.00000	.62512	.110	-4.5077	.5077
C3	A2	-2.03500	.62512	.104	-4.5427	.4727
	B2	-.96000	.62512	.585	-3.4677	1.5477
	D19	.10000	.62512	1.000	-2.4077	2.6077
	E3	-2.96000*	.62512	.026	-5.4677	-.4523
D19	A2	-2.13500	.62512	.089	-4.6427	.3727
	B2	-1.06000	.62512	.508	-3.5677	1.4477
	C3	-.10000	.62512	1.000	-2.6077	2.4077
	E3	-3.06000*	.62512	.023	-5.5677	-.5523
E3	A2	.92500	.62512	.613	-1.5827	3.4327
	B2	2.00000	.62512	.110	-.5077	4.5077
	C3	2.96000*	.62512	.026	.4523	5.4677
	D19	3.06000*	.62512	.023	.5523	5.5677

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
D19	2	3.3100	
C3	2	3.4100	
B2	2	4.3700	4.3700
A2	2	5.4450	5.4450
E3	2		6.3700
Sig.		.089	.110

c. pH 7 – 24 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.464	4	10.116	15.232	.005
Within Groups	3.321	5	.664		
Total	43.784	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	1.25000	.81494	.586	-2.0191	4.5191
	C3	3.14000	.81494	.058	-.1291	6.4091
	D19	5.10000*	.81494	.008	1.8309	8.3691
	E3	-.20500	.81494	.999	-3.4741	3.0641
B2	A2	-1.25000	.81494	.586	-4.5191	2.0191
	C3	1.89000	.81494	.275	-1.3791	5.1591
	D19	3.85000*	.81494	.026	.5809	7.1191
	E3	-1.45500	.81494	.468	-4.7241	1.8141
C3	A2	-3.14000	.81494	.058	-6.4091	.1291
	B2	-1.89000	.81494	.275	-5.1591	1.3791
	D19	1.96000	.81494	.251	-1.3091	5.2291
	E3	-3.34500*	.81494	.046	-6.6141	-.0759
D19	A2	-5.10000*	.81494	.008	-8.3691	-1.8309
	B2	-3.85000*	.81494	.026	-7.1191	-.5809
	C3	-1.96000	.81494	.251	-5.2291	1.3091
	E3	-5.30500*	.81494	.007	-8.5741	-2.0359
E3	A2	.20500	.81494	.999	-3.0641	3.4741
	B2	1.45500	.81494	.468	-1.8141	4.7241
	C3	3.34500*	.81494	.046	.0759	6.6141
	D19	5.30500*	.81494	.007	2.0359	8.5741

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05		
		a	b	c
D19	2	2.4650		
C3	2	4.4250	4.4250	
B2	2		6.3150	6.3150
A2	2		7.5650	7.5650
E3	2			7.7700
Sig.		.251	.058	.468

d. pH 7 – 48 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.248	4	10.812	11.994	.009
Within Groups	4.507	5	.901		
Total	47.756	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	-1.69500	.94946	.468	-5.5038	2.1138
	C3	1.27000	.94946	.685	-2.5388	5.0788
	D19	4.48000*	.94946	.027	.6712	8.2888
	E3	-.20000	.94946	.999	-4.0088	3.6088
B2	A2	1.69500	.94946	.468	-2.1138	5.5038
	C3	2.96500	.94946	.119	-.8438	6.7738
	D19	6.17500*	.94946	.007	2.3662	9.9838
	E3	1.49500	.94946	.566	-2.3138	5.3038
C3	A2	-1.27000	.94946	.685	-5.0788	2.5388
	B2	-2.96500	.94946	.119	-6.7738	.8438
	D19	3.21000	.94946	.092	-.5988	7.0188
	E3	-1.47000	.94946	.579	-5.2788	2.3388
D19	A2	-4.48000*	.94946	.027	-8.2888	-.6712
	B2	-6.17500*	.94946	.007	-9.9838	-2.3662
	C3	-3.21000	.94946	.092	-7.0188	.5988
	E3	-4.68000*	.94946	.022	-8.4888	-.8712
E3	A2	.20000	.94946	.999	-3.6088	4.0088
	B2	-1.49500	.94946	.566	-5.3038	2.3138
	C3	1.47000	.94946	.579	-2.3388	5.2788
	D19	4.68000*	.94946	.022	.8712	8.4888

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
D19	2	3.1650	
C3	2	6.3750	6.3750
A2	2		7.6450
E3	2		7.8450
B2	2		9.3400
Sig.		.092	.119

e. pH 6 – 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.518	4	7.630	17.796	.004
Within Groups	2.144	5	.429		
Total	32.662	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	.99000	.65477	.597	-1.6366	3.6166
	C3	3.43000*	.65477	.017	.8034	6.0566
	D19	1.13000	.65477	.494	-1.4966	3.7566
	E3	-1.95500	.65477	.137	-4.5816	.6716
B2	A2	-.99000	.65477	.597	-3.6166	1.6366
	C3	2.44000	.65477	.066	-.1866	5.0666
	D19	.14000	.65477	.999	-2.4866	2.7666
	E3	-2.94500*	.65477	.032	-5.5716	-.3184
C3	A2	-3.43000*	.65477	.017	-6.0566	-.8034
	B2	-2.44000	.65477	.066	-5.0666	.1866
	D19	-2.30000	.65477	.081	-4.9266	.3266
	E3	-5.38500*	.65477	.002	-8.0116	-2.7584
D19	A2	-1.13000	.65477	.494	-3.7566	1.4966
	B2	-.14000	.65477	.999	-2.7666	2.4866
	C3	2.30000	.65477	.081	-.3266	4.9266
	E3	-3.08500*	.65477	.027	-5.7116	-.4584
E3	A2	1.95500	.65477	.137	-.6716	4.5816
	B2	2.94500*	.65477	.032	.3184	5.5716
	C3	5.38500*	.65477	.002	2.7584	8.0116
	D19	3.08500*	.65477	.027	.4584	5.7116

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05		
		a	b	c
C3	2	.0000		
D19	2	2.3000	2.3000	
B2	2	2.4400	2.4400	
A2	2		3.4300	3.4300
E3	2			5.3850
Sig.		.066	.494	.137

f. pH 6 – 24 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	54.426	4	13.607	66.151	.000
Within Groups	1.028	5	.206		
Total	55.454	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	.02000	.45353	1.000	-1.7993	1.8393
	C3	2.45000*	.45353	.015	.6307	4.2693
	D19	2.45000*	.45353	.015	.6307	4.2693
	E3	-3.92500*	.45353	.002	-5.7443	-2.1057
B2	A2	-.02000	.45353	1.000	-1.8393	1.7993
	C3	2.43000*	.45353	.016	.6107	4.2493
	D19	2.43000*	.45353	.016	.6107	4.2493
	E3	-3.94500*	.45353	.002	-5.7643	-2.1257
C3	A2	-2.45000*	.45353	.015	-4.2693	-.6307
	B2	-2.43000*	.45353	.016	-4.2493	-.6107
	D19	.00000	.45353	1.000	-1.8193	1.8193
	E3	-6.37500*	.45353	.000	-8.1943	-4.5557
D19	A2	-2.45000*	.45353	.015	-4.2693	-.6307
	B2	-2.43000*	.45353	.016	-4.2493	-.6107
	C3	.00000	.45353	1.000	-1.8193	1.8193
	E3	-6.37500*	.45353	.000	-8.1943	-4.5557
E3	A2	3.92500*	.45353	.002	2.1057	5.7443
	B2	3.94500*	.45353	.002	2.1257	5.7643
	C3	6.37500*	.45353	.000	4.5557	8.1943
	D19	6.37500*	.45353	.000	4.5557	8.1943

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05		
		a	b	c
C3	2	.0000		
D19	2	.0000		
B2	2		2.4300	
A2	2		2.4500	
E3	2			6.3750
Sig.		1.000	1.000	1.000

g. pH 6 – 48 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.331	4	16.333	156.986	.000
Within Groups	.520	5	.104		
Total	65.852	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	.00000	.32255	1.000	-1.2939	1.2939
	C3	.00000	.32255	1.000	-1.2939	1.2939
	D19	.00000	.32255	1.000	-1.2939	1.2939
	E3	-6.39000*	.32255	.000	-7.6839	-5.0961
B2	A2	.00000	.32255	1.000	-1.2939	1.2939
	C3	.00000	.32255	1.000	-1.2939	1.2939
	D19	.00000	.32255	1.000	-1.2939	1.2939
	E3	-6.39000*	.32255	.000	-7.6839	-5.0961
C3	A2	.00000	.32255	1.000	-1.2939	1.2939
	B2	.00000	.32255	1.000	-1.2939	1.2939
	D19	.00000	.32255	1.000	-1.2939	1.2939
	E3	-6.39000*	.32255	.000	-7.6839	-5.0961
D19	A2	.00000	.32255	1.000	-1.2939	1.2939
	B2	.00000	.32255	1.000	-1.2939	1.2939
	C3	.00000	.32255	1.000	-1.2939	1.2939
	E3	-6.39000*	.32255	.000	-7.6839	-5.0961
E3	A2	6.39000*	.32255	.000	5.0961	7.6839
	B2	6.39000*	.32255	.000	5.0961	7.6839
	C3	6.39000*	.32255	.000	5.0961	7.6839
	D19	6.39000*	.32255	.000	5.0961	7.6839

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
A2	2	.0000	6.3900
B2	2	.0000	
C3	2	.0000	
D19	2	.0000	
E3	2	.0000	
Sig.		1.000	1.000

h. pH 6 – 72 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80.202	4	20.051	59.223	.000
Within Groups	1.693	5	.339		
Total	81.895	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	.00000	.58186	1.000	-2.3341	2.3341
	C3	.00000	.58186	1.000	-2.3341	2.3341
	D19	.00000	.58186	1.000	-2.3341	2.3341
	E3	-7.08000*	.58186	.000	-9.4141	-4.7459
B2	A2	.00000	.58186	1.000	-2.3341	2.3341
	C3	.00000	.58186	1.000	-2.3341	2.3341
	D19	.00000	.58186	1.000	-2.3341	2.3341
	E3	-7.08000*	.58186	.000	-9.4141	-4.7459
C3	A2	.00000	.58186	1.000	-2.3341	2.3341
	B2	.00000	.58186	1.000	-2.3341	2.3341
	D19	.00000	.58186	1.000	-2.3341	2.3341
	E3	-7.08000*	.58186	.000	-9.4141	-4.7459
D19	A2	.00000	.58186	1.000	-2.3341	2.3341
	B2	.00000	.58186	1.000	-2.3341	2.3341
	C3	.00000	.58186	1.000	-2.3341	2.3341
	E3	-7.08000*	.58186	.000	-9.4141	-4.7459
E3	A2	7.08000*	.58186	.000	4.7459	9.4141
	B2	7.08000*	.58186	.000	4.7459	9.4141
	C3	7.08000*	.58186	.000	4.7459	9.4141
	D19	7.08000*	.58186	.000	4.7459	9.4141

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
A2	2	.0000	7.0800
B2	2	.0000	
C3	2	.0000	
D19	2	.0000	
E3	2	.0000	
Sig.		1.000	1.000

i. pH 5 – 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.776	4	4.444	6.473	.033
Within Groups	3.433	5	.687		
Total	21.209	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	.34500	.82860	.992	-2.9789	3.6689
	C3	3.39500*	.82860	.046	.0711	6.7189
	D19	.93000	.82860	.791	-2.3939	4.2539
	E3	-.34500	.82860	.992	-3.6689	2.9789
B2	A2	-.34500	.82860	.992	-3.6689	2.9789
	C3	3.05000	.82860	.068	-.2739	6.3739
	D19	.58500	.82860	.946	-2.7389	3.9089
	E3	-.69000	.82860	.909	-4.0139	2.6339
C3	A2	-3.39500*	.82860	.046	-6.7189	-.0711
	B2	-3.05000	.82860	.068	-6.3739	.2739
	D19	-2.46500	.82860	.139	-5.7889	.8589
	E3	-3.74000*	.82860	.032	-7.0639	-.4161
D19	A2	-.93000	.82860	.791	-4.2539	2.3939
	B2	-.58500	.82860	.946	-3.9089	2.7389
	C3	2.46500	.82860	.139	-.8589	5.7889
	E3	-1.27500	.82860	.583	-4.5989	2.0489
E3	A2	.34500	.82860	.992	-2.9789	3.6689
	B2	.69000	.82860	.909	-2.6339	4.0139
	C3	3.74000*	.82860	.032	.4161	7.0639
	D19	1.27500	.82860	.583	-2.0489	4.5989

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
C3	2	.0000	
D19	2	2.4650	2.4650
B2	2	3.0500	3.0500
A2	2		3.3950
E3	2		3.7400
Sig.		.068	.583

Appendix 5. The significant ANOVA of salt tolerance of *Staphylococcus equorum* isolates obtained from Stilton core (A), Stilton crust (B), Danish blue (C), Reblochon core (D) and Reblochon crust (E) at:

a. 0% salt – 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.131	4	3.533	8.117	.021
Within Groups	2.176	5	.435		
Total	16.307	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	2.00500	.65973	.130	-.6415	4.6515
	C3	1.41000	.65973	.331	-1.2365	4.0565
	D19	2.11000	.65973	.111	-.5365	4.7565
	E3	-.92000	.65973	.656	-3.5665	1.7265
B2	A2	-2.00500	.65973	.130	-4.6515	.6415
	C3	-.59500	.65973	.885	-3.2415	2.0515
	D19	.10500	.65973	1.000	-2.5415	2.7515
	E3	-2.92500*	.65973	.034	-5.5715	-.2785
C3	A2	-1.41000	.65973	.331	-4.0565	1.2365
	B2	.59500	.65973	.885	-2.0515	3.2415
	D19	.70000	.65973	.819	-1.9465	3.3465
	E3	-2.33000	.65973	.079	-4.9765	.3165
D19	A2	-2.11000	.65973	.111	-4.7565	.5365
	B2	-.10500	.65973	1.000	-2.7515	2.5415
	C3	-.70000	.65973	.819	-3.3465	1.9465
	E3	-3.03000*	.65973	.030	-5.6765	-.3835
E3	A2	.92000	.65973	.656	-1.7265	3.5665
	B2	2.92500*	.65973	.034	.2785	5.5715
	C3	2.33000	.65973	.079	-.3165	4.9765
	D19	3.03000*	.65973	.030	.3835	5.6765

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
D19	2	3.4150	
B2	2	3.5200	
C3	2	4.1150	4.1150
A2	2	5.5250	5.5250
E3	2		6.4450
Sig.		.111	.079

b. 0% salt – 48 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.432	4	9.858	7.451	.025
Within Groups	6.616	5	1.323		
Total	46.048	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	-1.54500	1.15027	.682	-6.1593	3.0693
	C3	1.45500	1.15027	.721	-3.1593	6.0693
	D19	4.00000	1.15027	.084	-.6143	8.6143
	E3	-.97000	1.15027	.906	-5.5843	3.6443
B2	A2	1.54500	1.15027	.682	-3.0693	6.1593
	C3	3.00000	1.15027	.203	-1.6143	7.6143
	D19	5.54500*	1.15027	.024	.9307	10.1593
	E3	.57500	1.15027	.984	-4.0393	5.1893
C3	A2	-1.45500	1.15027	.721	-6.0693	3.1593
	B2	-3.00000	1.15027	.203	-7.6143	1.6143
	D19	2.54500	1.15027	.307	-2.0693	7.1593
	E3	-2.42500	1.15027	.341	-7.0393	2.1893
D19	A2	-4.00000	1.15027	.084	-8.6143	.6143
	B2	-5.54500*	1.15027	.024	-10.1593	-.9307
	C3	-2.54500	1.15027	.307	-7.1593	2.0693
	E3	-4.97000*	1.15027	.038	-9.5843	-.3557
E3	A2	.97000	1.15027	.906	-3.6443	5.5843
	B2	-.57500	1.15027	.984	-5.1893	4.0393
	C3	2.42500	1.15027	.341	-2.1893	7.0393
	D19	4.97000*	1.15027	.038	.3557	9.5843

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
D19	2	3.8450	
C3	2	6.3900	6.3900
A2	2	7.8450	7.8450
E3	2		8.8150
B2	2		9.3900
Sig.		.084	.203

c. 5% salt – 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.784	4	3.696	12.734	.008
Within Groups	1.451	5	.290		
Total	16.236	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	2.81500*	.53876	.017	.6538	4.9762
	C3	2.22500*	.53876	.045	.0638	4.3862
	D19	2.98000*	.53876	.014	.8188	5.1412
	E3	3.47000*	.53876	.007	1.3088	5.6312
B2	A2	-2.81500*	.53876	.017	-4.9762	-.6538
	C3	-.59000	.53876	.804	-2.7512	1.5712
	D19	.16500	.53876	.997	-1.9962	2.3262
	E3	.65500	.53876	.746	-1.5062	2.8162
C3	A2	-2.22500*	.53876	.045	-4.3862	-.0638
	B2	.59000	.53876	.804	-1.5712	2.7512
	D19	.75500	.53876	.652	-1.4062	2.9162
	E3	1.24500	.53876	.277	-.9162	3.4062
D19	A2	-2.98000*	.53876	.014	-5.1412	-.8188
	B2	-.16500	.53876	.997	-2.3262	1.9962
	C3	-.75500	.53876	.652	-2.9162	1.4062
	E3	.49000	.53876	.882	-1.6712	2.6512
E3	A2	-3.47000*	.53876	.007	-5.6312	-1.3088
	B2	-.65500	.53876	.746	-2.8162	1.5062
	C3	-1.24500	.53876	.277	-3.4062	.9162
	D19	-.49000	.53876	.882	-2.6512	1.6712

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
E3	2	2.9500	6.4200
D19	2	3.4400	
B2	2	3.6050	
C3	2	4.1950	
A2	2		
Sig.		.277	1.000

d. 5% salt – 48 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.382	4	9.845	7.379	.025
Within Groups	6.671	5	1.334		
Total	46.053	9			

Tukey HSD

(I) Isolate	(J) Isolate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A2	B2	-1.53000	1.15510	.691	-6.1637	3.1037
	C3	1.45000	1.15510	.726	-3.1837	6.0837
	D19	4.02500	1.15510	.083	-.6087	8.6587
	E3	-.93000	1.15510	.918	-5.5637	3.7037
B2	A2	1.53000	1.15510	.691	-3.1037	6.1637
	C3	2.98000	1.15510	.210	-1.6537	7.6137
	D19	5.55500*	1.15510	.025	.9213	10.1887
	E3	.60000	1.15510	.981	-4.0337	5.2337
C3	A2	-1.45000	1.15510	.726	-6.0837	3.1837
	B2	-2.98000	1.15510	.210	-7.6137	1.6537
	D19	2.57500	1.15510	.301	-2.0587	7.2087
	E3	-2.38000	1.15510	.358	-7.0137	2.2537
D19	A2	-4.02500	1.15510	.083	-8.6587	.6087
	B2	-5.55500*	1.15510	.025	-10.1887	-.9213
	C3	-2.57500	1.15510	.301	-7.2087	2.0587
	E3	-4.95500*	1.15510	.039	-9.5887	-.3213
E3	A2	.93000	1.15510	.918	-3.7037	5.5637
	B2	-.60000	1.15510	.981	-5.2337	4.0337
	C3	2.38000	1.15510	.358	-2.2537	7.0137
	D19	4.95500*	1.15510	.039	.3213	9.5887

*The mean difference is significant at the 0.05 level.

Isolate	N	Subset for alpha = 0.05	
		a	b
D19	2	3.8350	
C3	2	6.4100	6.4100
A2	2	7.8600	7.8600
E3	2		8.7900
B2	2		9.3900
Sig.		.083	.210

Appendix 6. The significant ANOVA of water activity of:

a. Model cheeses between crust and core

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.003	11	.000	2.917	.014
Within Groups	.002	24	.000		
Total	.005	35			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1cr	C1co	-.01667	.00793	.627	-.0453	.0119
	C2cr	-.00667	.00793	.999	-.0353	.0219
	C2co	-.01333	.00793	.860	-.0419	.0153
	C3cr	-.00667	.00793	.999	-.0353	.0219
	C3co	-.02000	.00793	.374	-.0486	.0086
	C4cr	.01000	.00793	.977	-.0186	.0386
	C4co	-.01000	.00793	.977	-.0386	.0186
	C5cr	.00667	.00793	.999	-.0219	.0353
	C5co	.00000	.00793	1.000	-.0286	.0286
	C6cr	.00667	.00793	.999	-.0219	.0353
	C6co	-.00667	.00793	.999	-.0353	.0219
C1co	C1cr	.01667	.00793	.627	-.0119	.0453
	C2cr	.01000	.00793	.977	-.0186	.0386
	C2co	.00333	.00793	1.000	-.0253	.0319
	C3cr	.01000	.00793	.977	-.0186	.0386
	C3co	-.00333	.00793	1.000	-.0319	.0253
	C4cr	.02667	.00793	.084	-.0019	.0553
	C4co	.00667	.00793	.999	-.0219	.0353
	C5cr	.02333	.00793	.188	-.0053	.0519
	C5co	.01667	.00793	.627	-.0119	.0453
	C6cr	.02333	.00793	.188	-.0053	.0519
	C6co	.01000	.00793	.977	-.0186	.0386
C2cr	C1cr	.00667	.00793	.999	-.0219	.0353
	C1co	-.01000	.00793	.977	-.0386	.0186
	C2co	-.00667	.00793	.999	-.0353	.0219
	C3cr	.00000	.00793	1.000	-.0286	.0286
	C3co	-.01333	.00793	.860	-.0419	.0153
	C4cr	.01667	.00793	.627	-.0119	.0453
	C4co	-.00333	.00793	1.000	-.0319	.0253
	C5cr	.01333	.00793	.860	-.0153	.0419
	C5co	.00667	.00793	.999	-.0219	.0353
	C6cr	.01333	.00793	.860	-.0153	.0419
	C6co	.00000	.00793	1.000	-.0286	.0286
C2co	C1cr	.01333	.00793	.860	-.0153	.0419
	C1co	-.00333	.00793	1.000	-.0319	.0253
	C2cr	.00667	.00793	.999	-.0219	.0353
	C3co	.00667	.00793	.999	-.0219	.0353
	C3co	-.00667	.00793	.999	-.0353	.0219
	C4cr	.02333	.00793	.188	-.0053	.0519
	C4co	.00333	.00793	1.000	-.0253	.0319

	C5cr	.02000	.00793	.374	-.0086	.0486
	C5co	.01333	.00793	.860	-.0153	.0419
	C6cr	.02000	.00793	.374	-.0086	.0486
	C6co	.00667	.00793	.999	-.0219	.0353
C3cr	C1cr	.00667	.00793	.999	-.0219	.0353
	C1co	-.01000	.00793	.977	-.0386	.0186
	C2cr	.00000	.00793	1.000	-.0286	.0286
	C2co	-.00667	.00793	.999	-.0353	.0219
	C3co	-.01333	.00793	.860	-.0419	.0153
	C4cr	.01667	.00793	.627	-.0119	.0453
	C4co	-.00333	.00793	1.000	-.0319	.0253
	C5cr	.01333	.00793	.860	-.0153	.0419
	C5o	.00667	.00793	.999	-.0219	.0353
	C6cr	.01333	.00793	.860	-.0153	.0419
	C6co	.00000	.00793	1.000	-.0286	.0286
C3co	C1cr	.02000	.00793	.374	-.0086	.0486
	C1co	.00333	.00793	1.000	-.0253	.0319
	C2cr	.01333	.00793	.860	-.0153	.0419
	C2co	.00667	.00793	.999	-.0219	.0353
	C3cr	.01333	.00793	.860	-.0153	.0419
	C4cr	.03000*	.00793	.034	.0014	.0586
	C4co	.01000	.00793	.977	-.0186	.0386
	C5cr	.02667	.00793	.084	-.0019	.0553
	C5co	.02000	.00793	.374	-.0086	.0486
	C6cr	.02667	.00793	.084	-.0019	.0553
	C6co	.01333	.00793	.860	-.0153	.0419
C4cr	C1cr	-.01000	.00793	.977	-.0386	.0186
	C1co	-.02667	.00793	.084	-.0553	.0019
	C2cr	-.01667	.00793	.627	-.0453	.0119
	C2co	-.02333	.00793	.188	-.0519	.0053
	C3cr	-.01667	.00793	.627	-.0453	.0119
	C3co	-.03000*	.00793	.034	-.0586	-.0014
	C4co	-.02000	.00793	.374	-.0486	.0086
	C5cr	-.00333	.00793	1.000	-.0319	.0253
	C5co	-.01000	.00793	.977	-.0386	.0186
	C6cr	-.00333	.00793	1.000	-.0319	.0253
	C6co	-.01667	.00793	.627	-.0453	.0119
C4co	C1cr	.01000	.00793	.977	-.0186	.0386
	C1co	-.00667	.00793	.999	-.0353	.0219
	C2cr	.00333	.00793	1.000	-.0253	.0319
	C2co	-.00333	.00793	1.000	-.0319	.0253
	C3cr	.00333	.00793	1.000	-.0253	.0319
	C3co	-.01000	.00793	.977	-.0386	.0186
	C4cr	.02000	.00793	.374	-.0086	.0486
	C5co	.01667	.00793	.627	-.0119	.0453
	C5co	.01000	.00793	.977	-.0186	.0386
	C6cr	.01667	.00793	.627	-.0119	.0453
	C6co	.00333	.00793	1.000	-.0253	.0319
C5cr	C1cr	-.00667	.00793	.999	-.0353	.0219
	C1co	-.02333	.00793	.188	-.0519	.0053
	C2cr	-.01333	.00793	.860	-.0419	.0153
	C2co	-.02000	.00793	.374	-.0486	.0086
	C3cr	-.01333	.00793	.860	-.0419	.0153
	C3co	-.02667	.00793	.084	-.0553	.0019
	C4cr	.00333	.00793	1.000	-.0253	.0319
	C4co	-.01667	.00793	.627	-.0453	.0119

	C5co		-.00667	.00793	.999	-.0353	.0219
	C6cr		.00000	.00793	1.000	-.0286	.0286
	C6co		-.01333	.00793	.860	-.0419	.0153
C5co	C1cr		.00000	.00793	1.000	-.0286	.0286
	C1co		-.01667	.00793	.627	-.0453	.0119
	C2cr		-.00667	.00793	.999	-.0353	.0219
	C2co		-.01333	.00793	.860	-.0419	.0153
	C3cr		-.00667	.00793	.999	-.0353	.0219
	C3co		-.02000	.00793	.374	-.0486	.0086
	C4cr		.01000	.00793	.977	-.0186	.0386
	C4co		-.01000	.00793	.977	-.0386	.0186
	C5cr		.00667	.00793	.999	-.0219	.0353
	C6cr		.00667	.00793	.999	-.0219	.0353
	C6co		-.00667	.00793	.999	-.0353	.0219
C6cr	C1cr		-.00667	.00793	.999	-.0353	.0219
	C1cr		-.02333	.00793	.188	-.0519	.0053
	C2cr		-.01333	.00793	.860	-.0419	.0153
	C2co		-.02000	.00793	.374	-.0486	.0086
	C3cr		-.01333	.00793	.860	-.0419	.0153
	C3co		-.02667	.00793	.084	-.0553	.0019
	C4cr		.00333	.00793	1.000	-.0253	.0319
	C4co		-.01667	.00793	.627	-.0453	.0119
	C5cr		.00000	.00793	1.000	-.0286	.0286
	C5co		-.00667	.00793	.999	-.0353	.0219
	C6co		-.01333	.00793	.860	-.0419	.0153
C6co	C1cr		.00667	.00793	.999	-.0219	.0353
	C1co		-.01000	.00793	.977	-.0386	.0186
	C2cr		.00000	.00793	1.000	-.0286	.0286
	C2co		-.00667	.00793	.999	-.0353	.0219
	C3cr		.00000	.00793	1.000	-.0286	.0286
	C3co		-.01333	.00793	.860	-.0419	.0153
	C4cr		.01667	.00793	.627	-.0119	.0453
	C4cr		-.00333	.00793	1.000	-.0319	.0253
	C5cr		.01333	.00793	.860	-.0153	.0419
	C5co		.00667	.00793	.999	-.0219	.0353
	C6cr		.01333	.00793	.860	-.0153	.0419

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C4cr	3	.9300	
C5cr	3	.9333	.9333
C6cr	3	.9333	.9333
C1cr	3	.9400	.9400
C5co	3	.9400	.9400
C2cr	3	.9467	.9467
C3cr	3	.9467	.9467
C6co	3	.9467	.9467
C4co	3	.9500	.9500
C2co	3	.9533	.9533
C1co	3	.9567	.9567
C3co	3		.9600
Sig.		.084	.084

b. Model and control cheeses

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.013	5	.003	145.825	.000
Within Groups	.000	12	.000		
Total	.013	17			

Tukey HSD

(I) Cheese **	(J) Cheese **	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.01033	.00346	.092	-.0013	.0220
	3	-.04500*	.00346	.000	-.0566	-.0334
	4	.00267	.00346	.967	-.0090	.0143
	5	-.04367*	.00346	.000	-.0553	-.0320
	6	-.05700*	.00346	.000	-.0686	-.0454
2	1	-.01033	.00346	.092	-.0220	.0013
	3	-.05533*	.00346	.000	-.0670	-.0437
	4	-.00767	.00346	.298	-.0193	.0040
	5	-.05400*	.00346	.000	-.0656	-.0424
	6	-.06733*	.00346	.000	-.0790	-.0557
3	1	.04500*	.00346	.000	.0334	.0566
	2	.05533*	.00346	.000	.0437	.0670
	4	.04767*	.00346	.000	.0360	.0593
	5	.00133	.00346	.999	-.0103	.0130
	6	-.01200*	.00346	.042	-.0236	-.0004
4	1	-.00267	.00346	.967	-.0143	.0090
	2	.00767	.00346	.298	-.0040	.0193
	3	-.04767*	.00346	.000	-.0593	-.0360
	5	-.04633*	.00346	.000	-.0580	-.0347
	6	-.05967*	.00346	.000	-.0713	-.0480
5	1	.04367*	.00346	.000	.0320	.0553
	2	.05400*	.00346	.000	.0424	.0656
	3	-.00133	.00346	.999	-.0130	.0103
	4	.04633*	.00346	.000	.0347	.0580
	6	-.01333*	.00346	.022	-.0250	-.0017
6	1	.05700*	.00346	.000	.0454	.0686
	2	.06733*	.00346	.000	.0557	.0790
	3	.01200*	.00346	.042	.0004	.0236
	4	.05967*	.00346	.000	.0480	.0713
	5	.01333*	.00346	.022	.0017	.0250

*The mean difference is significant at the 0.05 level.

**1, Stilton crust; 2, Stilton core; 3, Wensleydale; 4, Danish Blue; 5, model crust; 6, model core.

Cheese	N	Subset for alpha = 0.05		
		a	b	c
Stilton core	3	.8843		
Danish Blue	3	.8920		
Stilton crust	3	.8947		
Model crust	3		.9383	
Wensleydale	3		.9397	
Model core	3			.9517
Sig.		.092	.999	1.000

Appendix 7. The significant ANOVA of pH value of:

a. Incubated milk 0 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.125	5	1.025	16.784	.000
Within Groups	.733	12	.061		
Total	5.858	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	1.19000*	.20177	.001	.5123	1.8677
	C3	.65000	.20177	.063	-.0277	1.3277
	C4	.81000*	.20177	.017	.1323	1.4877
	C5	1.16000*	.20177	.001	.4823	1.8377
	C6	-.20000	.20177	.912	-.8777	.4777
C2	C1	-1.19000*	.20177	.001	-1.8677	-.5123
	C3	-.54000	.20177	.152	-1.2177	.1377
	C4	-.38000	.20177	.455	-1.0577	.2977
	C5	-.03000	.20177	1.000	-.7077	.6477
	C6	-1.39000*	.20177	.000	-2.0677	-.7123
C3	C1	-.65000	.20177	.063	-1.3277	.0277
	C2	.54000	.20177	.152	-.1377	1.2177
	C4	.16000	.20177	.963	-.5177	.8377
	C5	.51000	.20177	.190	-.1677	1.1877
	C6	-.85000*	.20177	.012	-1.5277	-.1723
C4	C1	-.81000*	.20177	.017	-1.4877	-.1323
	C2	.38000	.20177	.455	-.2977	1.0577
	C3	-.16000	.20177	.963	-.8377	.5177
	C5	.35000	.20177	.537	-.3277	1.0277
	C6	-1.01000*	.20177	.003	-1.6877	-.3323
C5	C1	-1.16000*	.20177	.001	-1.8377	-.4823
	C2	.03000	.20177	1.000	-.6477	.7077
	C3	-.51000	.20177	.190	-1.1877	.1677
	C4	-.35000	.20177	.537	-1.0277	.3277
	C6	-1.36000*	.20177	.000	-2.0377	-.6823
C6	C1	.20000	.20177	.912	-.4777	.8777
	C2	1.39000*	.20177	.000	.7123	2.0677
	C3	.85000*	.20177	.012	.1723	1.5277
	C4	1.01000*	.20177	.003	.3323	1.6877
	C5	1.36000*	.20177	.000	.6823	2.0377

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05		
		a	b	c
C2	3	4.4900	5.0300	5.6800
C5	3	4.5200		
C4	3	4.8700		
C3	3	5.0300		
C1	3			
C6	3		5.6800	5.8800
Sig.		.152	.063	.912

b. Incubated milk 4 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.770	5	.354	5.214	.009
Within Groups	.815	12	.068		
Total	2.584	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	.89000*	.21273	.012	.1754	1.6046
	C3	.82000*	.21273	.022	.1054	1.5346
	C4	.85000*	.21273	.017	.1354	1.5646
	C5	.82000*	.21273	.022	.1054	1.5346
	C6	.54000	.21273	.187	-.1746	1.2546
C2	C1	-.89000*	.21273	.012	-1.6046	-.1754
	C3	-.07000	.21273	.999	-.7846	.6446
	C4	-.04000	.21273	1.000	-.7546	.6746
	C5	-.07000	.21273	.999	-.7846	.6446
	C6	-.35000	.21273	.587	-1.0646	.3646
C3	C1	-.82000*	.21273	.022	-1.5346	-.1054
	C2	.07000	.21273	.999	-.6446	.7846
	C4	.03000	.21273	1.000	-.6846	.7446
	C5	.00000	.21273	1.000	-.7146	.7146
	C6	-.28000	.21273	.772	-.9946	.4346
C4	C1	-.85000*	.21273	.017	-1.5646	-.1354
	C2	.04000	.21273	1.000	-.6746	.7546
	C3	-.03000	.21273	1.000	-.7446	.6846
	C5	-.03000	.21273	1.000	-.7446	.6846
	C6	-.31000	.21273	.695	-1.0246	.4046
C5	C1	-.82000*	.21273	.022	-1.5346	-.1054
	C2	.07000	.21273	.999	-.6446	.7846
	C3	.00000	.21273	1.000	-.7146	.7146
	C4	.03000	.21273	1.000	-.6846	.7446
	C6	-.28000	.21273	.772	-.9946	.4346
C6	C1	-.54000	.21273	.187	-1.2546	.1746
	C2	.35000	.21273	.587	-.3646	1.0646
	C3	.28000	.21273	.772	-.4346	.9946
	C4	.31000	.21273	.695	-.4046	1.0246
	C5	.28000	.21273	.772	-.4346	.9946

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C2	3	4.2700	4.6200 5.1600
C4	3	4.3100	
C3	3	4.3400	
C5	3	4.3400	
C6	3	4.6200	
C1	3		
Sig.		.587	.187

c. Incubated milk 8 h

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.527	5	.105	3.708	.029
Within Groups	.341	12	.028		
Total	.868	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	.42000	.13768	.083	-.0425	.8825
	C3	.49000*	.13768	.036	.0275	.9525
	C4	.47000*	.13768	.046	.0075	.9325
	C5	.46000	.13768	.052	-.0025	.9225
	C6	.30000	.13768	.314	-.1625	.7625
C2	C1	-.42000	.13768	.083	-.8825	.0425
	C3	.07000	.13768	.995	-.3925	.5325
	C4	.05000	.13768	.999	-.4125	.5125
	C5	.04000	.13768	1.000	-.4225	.5025
	C6	-.12000	.13768	.946	-.5825	.3425
C3	C1	-.49000*	.13768	.036	-.9525	-.0275
	C2	-.07000	.13768	.995	-.5325	.3925
	C4	-.02000	.13768	1.000	-.4825	.4425
	C5	-.03000	.13768	1.000	-.4925	.4325
	C6	-.19000	.13768	.738	-.6525	.2725
C4	C1	-.47000*	.13768	.046	-.9325	-.0075
	C2	-.05000	.13768	.999	-.5125	.4125
	C3	.02000	.13768	1.000	-.4425	.4825
	C5	-.01000	.13768	1.000	-.4725	.4525
	C6	-.17000	.13768	.812	-.6325	.2925
C5	C1	-.46000	.13768	.052	-.9225	.0025
	C2	-.04000	.13768	1.000	-.5025	.4225
	C3	.03000	.13768	1.000	-.4325	.4925
	C4	.01000	.13768	1.000	-.4525	.4725
	C6	-.16000	.13768	.846	-.6225	.3025
C6	C1	-.30000	.13768	.314	-.7625	.1625
	C2	.12000	.13768	.946	-.3425	.5825
	C3	.19000	.13768	.738	-.2725	.6525
	C4	.17000	.13768	.812	-.2925	.6325
	C5	.16000	.13768	.846	-.3025	.6225

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C3	3	4.1900	
C4	3	4.2100	
C5	3	4.2200	4.2200
C2	3	4.2600	4.2600
C6	3	4.3800	4.3800
C1	3		4.6800
Sig.		.738	.052

d. Pre-rubbing up

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.783	5	.557	5.029	.010
Within Groups	1.328	12	.111		
Total	4.111	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	-.05000	.27164	1.000	-.9624	.8624
	C3	.04000	.27164	1.000	-.8724	.9524
	C4	-.10000	.27164	.999	-1.0124	.8124
	C5	.92000*	.27164	.048	.0076	1.8324
	C6	.65000	.27164	.232	-.2624	1.5624
C2	C1	.05000	.27164	1.000	-.8624	.9624
	C3	.09000	.27164	.999	-.8224	1.0024
	C4	-.05000	.27164	1.000	-.9624	.8624
	C5	.97000*	.27164	.035	.0576	1.8824
	C6	.70000	.27164	.177	-.2124	1.6124
C3	C1	-.04000	.27164	1.000	-.9524	.8724
	C2	-.09000	.27164	.999	-1.0024	.8224
	C4	-.14000	.27164	.994	-1.0524	.7724
	C5	.88000	.27164	.061	-.0324	1.7924
	C6	.61000	.27164	.286	-.3024	1.5224
C4	C1	.10000	.27164	.999	-.8124	1.0124
	C2	.05000	.27164	1.000	-.8624	.9624
	C3	.14000	.27164	.994	-.7724	1.0524
	C5	1.02000*	.27164	.026	.1076	1.9324
	C6	.75000	.27164	.133	-.1624	1.6624
C5	C1	-.92000*	.27164	.048	-1.8324	-.0076
	C2	-.97000*	.27164	.035	-1.8824	-.0576
	C3	-.88000	.27164	.061	-1.7924	.0324
	C4	-1.02000*	.27164	.026	-1.9324	-.1076
	C6	-.27000	.27164	.911	-1.1824	.6424
C6	C1	-.65000	.27164	.232	-1.5624	.2624
	C2	-.70000	.27164	.177	-1.6124	.2124
	C3	-.61000	.27164	.286	-1.5224	.3024
	C4	-.75000	.27164	.133	-1.6624	.1624
	C5	.27000	.27164	.911	-.6424	1.1824

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C5	3	3.5300	
C6	3	3.8000	3.8000
C3	3	4.4100	4.4100
C1	3		4.4500
C2	3		4.5000
C4	3		4.5500
Sig.		.061	.133

e. Ripened (1 week – crust)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	52.566	5	10.513	107.716	.000
Within Groups	1.171	12	.098		
Total	53.737	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	-.01000	.25508	1.000	-.8668	.8468
	C3	.09000	.25508	.999	-.7668	.9468
	C4	-3.36000*	.25508	.000	-4.2168	-2.5032
	C5	-3.36000*	.25508	.000	-4.2168	-2.5032
	C6	-3.45000*	.25508	.000	-4.3068	-2.5932
C2	C1	.01000	.25508	1.000	-.8468	.8668
	C3	.10000	.25508	.998	-.7568	.9568
	C4	-3.35000*	.25508	.000	-4.2068	-2.4932
	C5	-3.35000*	.25508	.000	-4.2068	-2.4932
	C6	-3.44000*	.25508	.000	-4.2968	-2.5832
C3	C1	-.09000	.25508	.999	-.9468	.7668
	C2	-.10000	.25508	.998	-.9568	.7568
	C4	-3.45000*	.25508	.000	-4.3068	-2.5932
	C5	-3.45000*	.25508	.000	-4.3068	-2.5932
	C6	-3.54000*	.25508	.000	-4.3968	-2.6832
C4	C1	3.36000*	.25508	.000	2.5032	4.2168
	C2	3.35000*	.25508	.000	2.4932	4.2068
	C3	3.45000*	.25508	.000	2.5932	4.3068
	C5	.00000	.25508	1.000	-.8568	.8568
	C6	-.09000	.25508	.999	-.9468	.7668
C5	C1	3.36000*	.25508	.000	2.5032	4.2168
	C2	3.35000*	.25508	.000	2.4932	4.2068
	C3	3.45000*	.25508	.000	2.5932	4.3068
	C4	.00000	.25508	1.000	-.8568	.8568
	C6	-.09000	.25508	.999	-.9468	.7668
C6	C1	3.45000*	.25508	.000	2.5932	4.3068
	C2	3.44000*	.25508	.000	2.5832	4.2968
	C3	3.54000*	.25508	.000	2.6832	4.3968
	C4	.09000	.25508	.999	-.7668	.9468
	C5	.09000	.25508	.999	-.7668	.9468

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C3	3	3.6300	7.0800
C1	3	3.7200	
C2	3	3.7300	
C4	3		
C5	3		
C6	3		7.1700
Sig.		.998	.999

f. Ripened (2 weeks – crust)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	48.102	5	9.620	314.224	.000
Within Groups	.367	12	.031		
Total	48.470	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	.06000	.14287	.998	-.4199	.5399
	C3	-.04000	.14287	1.000	-.5199	.4399
	C4	-3.18000*	.14287	.000	-3.6599	-2.7001
	C5	-3.22000*	.14287	.000	-3.6999	-2.7401
	C6	-3.38000*	.14287	.000	-3.8599	-2.9001
C2	C1	-.06000	.14287	.998	-.5399	.4199
	C3	-.10000	.14287	.978	-.5799	.3799
	C4	-3.24000*	.14287	.000	-3.7199	-2.7601
	C5	-3.28000*	.14287	.000	-3.7599	-2.8001
	C6	-3.44000*	.14287	.000	-3.9199	-2.9601
C3	C1	.04000	.14287	1.000	-.4399	.5199
	C2	.10000	.14287	.978	-.3799	.5799
	C4	-3.14000*	.14287	.000	-3.6199	-2.6601
	C5	-3.18000*	.14287	.000	-3.6599	-2.7001
	C6	-3.34000*	.14287	.000	-3.8199	-2.8601
C4	C1	3.18000*	.14287	.000	2.7001	3.6599
	C2	3.24000*	.14287	.000	2.7601	3.7199
	C3	3.14000*	.14287	.000	2.6601	3.6199
	C5	-.04000	.14287	1.000	-.5199	.4399
	C6	-.20000	.14287	.727	-.6799	.2799
C5	C1	3.22000*	.14287	.000	2.7401	3.6999
	C2	3.28000*	.14287	.000	2.8001	3.7599
	C3	3.18000*	.14287	.000	2.7001	3.6599
	C4	.04000	.14287	1.000	-.4399	.5199
	C6	-.16000	.14287	.864	-.6399	.3199
C6	C1	3.38000*	.14287	.000	2.9001	3.8599
	C2	3.44000*	.14287	.000	2.9601	3.9199
	C3	3.34000*	.14287	.000	2.8601	3.8199
	C4	.20000	.14287	.727	-.2799	.6799
	C5	.16000	.14287	.864	-.3199	.6399

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05	
		a	b
C2	3	3.8100	7.0500
C1	3	3.8700	
C3	3	3.9100	
C4	3		
C5	3		
C6	3		7.2500
Sig.		.978	.727

g. Ripened (3 weeks – crust)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42.503	5	8.501	298.967	.000
Within Groups	.341	12	.028		
Total	42.844	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	.42000	.13768	.083	-.0425	.8825
	C3	.70000*	.13768	.003	.2375	1.1625
	C4	-2.94000*	.13768	.000	-3.4025	-2.4775
	C5	-2.51000*	.13768	.000	-2.9725	-2.0475
	C6	-2.53000*	.13768	.000	-2.9925	-2.0675
C2	C1	-.42000	.13768	.083	-.8825	.0425
	C3	.28000	.13768	.379	-.1825	.7425
	C4	-3.36000*	.13768	.000	-3.8225	-2.8975
	C5	-2.93000*	.13768	.000	-3.3925	-2.4675
	C6	-2.95000*	.13768	.000	-3.4125	-2.4875
C3	C1	-.70000*	.13768	.003	-1.1625	-.2375
	C2	-.28000	.13768	.379	-.7425	.1825
	C4	-3.64000*	.13768	.000	-4.1025	-3.1775
	C5	-3.21000*	.13768	.000	-3.6725	-2.7475
	C6	-3.23000*	.13768	.000	-3.6925	-2.7675
C4	C1	2.94000*	.13768	.000	2.4775	3.4025
	C2	3.36000*	.13768	.000	2.8975	3.8225
	C3	3.64000*	.13768	.000	3.1775	4.1025
	C5	.43000	.13768	.074	-.0325	.8925
	C6	.41000	.13768	.094	-.0525	.8725
C5	C1	2.51000*	.13768	.000	2.0475	2.9725
	C2	2.93000*	.13768	.000	2.4675	3.3925
	C3	3.21000*	.13768	.000	2.7475	3.6725
	C4	-.43000	.13768	.074	-.8925	.0325
	C6	-.02000	.13768	1.000	-.4825	.4425
C6	C1	2.53000*	.13768	.000	2.0675	2.9925
	C2	2.95000*	.13768	.000	2.4875	3.4125
	C3	3.23000*	.13768	.000	2.7675	3.6925
	C4	-.41000	.13768	.094	-.8725	.0525
	C5	.02000	.13768	1.000	-.4425	.4825

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05		
		a	b	c
C3	3	3.9500	4.2300 4.6500	7.1600 7.1800 7.5900
C2	3	4.2300		
C1	3			
C5	3			
C6	3			
C4	3			
Sig.		.379	.083	.074

h. Ripened (4 weeks – crust)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.883	5	9.177	88.222	.000
Within Groups	1.248	12	.104		
Total	47.131	17			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C1	C2	1.27000*	.26333	.004	.3855	2.1545
	C3	1.16000*	.26333	.009	.2755	2.0445
	C4	-2.49000*	.26333	.000	-3.3745	-1.6055
	C5	-2.13000*	.26333	.000	-3.0145	-1.2455
	C6	-2.19000*	.26333	.000	-3.0745	-1.3055
C2	C1	-1.27000*	.26333	.004	-2.1545	-.3855
	C3	-.11000	.26333	.998	-.9945	.7745
	C4	-3.76000*	.26333	.000	-4.6445	-2.8755
	C5	-3.40000*	.26333	.000	-4.2845	-2.5155
	C6	-3.46000*	.26333	.000	-4.3445	-2.5755
C3	C1	-1.16000*	.26333	.009	-2.0445	-.2755
	C2	.11000	.26333	.998	-.7745	.9945
	C4	-3.65000*	.26333	.000	-4.5345	-2.7655
	C5	-3.29000*	.26333	.000	-4.1745	-2.4055
	C6	-3.35000*	.26333	.000	-4.2345	-2.4655
C4	C1	2.49000*	.26333	.000	1.6055	3.3745
	C2	3.76000*	.26333	.000	2.8755	4.6445
	C3	3.65000*	.26333	.000	2.7655	4.5345
	C5	.36000	.26333	.745	-.5245	1.2445
	C6	.30000	.26333	.856	-.5845	1.1845
C5	C1	2.13000*	.26333	.000	1.2455	3.0145
	C2	3.40000*	.26333	.000	2.5155	4.2845
	C3	3.29000*	.26333	.000	2.4055	4.1745
	C4	-.36000	.26333	.745	-1.2445	.5245
	C6	-.06000	.26333	1.000	-.9445	.8245
C6	C1	2.19000*	.26333	.000	1.3055	3.0745
	C2	3.46000*	.26333	.000	2.5755	4.3445
	C3	3.35000*	.26333	.000	2.4655	4.2345
	C4	-.30000	.26333	.856	-1.1845	.5845
	C5	.06000	.26333	1.000	-.8245	.9445

*The mean difference is significant at the 0.05 level.

Cheese Model	N	Subset for alpha = 0.05		
		a	b	c
C2	3	3.8800	5.1500	7.2800
C3	3	3.9900		
C1	3			
C5	3			
C6	3			
C4	3			7.6400
Sig.		.998	1.000	.745

Appendix 8. The significance ANOVA of total cell counts of:

a. Incubated milk – 4 h (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.940 ^a	5	6.188	27.876	.000
Within Groups	1.332	6	.222		
Total	32.272	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.47115	1.000	-1.8751	1.8751
	3	.0000	.47115	1.000	-1.8751	1.8751
	4	-3.1350*	.47115	.004	-5.0101	-1.2599
	5	-3.4600*	.47115	.003	-5.3351	-1.5849
	6	-3.0050*	.47115	.005	-4.8801	-1.1299
2	1	.0000	.47115	1.000	-1.8751	1.8751
	3	.0000	.47115	1.000	-1.8751	1.8751
	4	-3.1350*	.47115	.004	-5.0101	-1.2599
	5	-3.4600*	.47115	.003	-5.3351	-1.5849
	6	-3.0050*	.47115	.005	-4.8801	-1.1299
3	1	.0000	.47115	1.000	-1.8751	1.8751
	2	.0000	.47115	1.000	-1.8751	1.8751
	4	-3.1350*	.47115	.004	-5.0101	-1.2599
	5	-3.4600*	.47115	.003	-5.3351	-1.5849
	6	-3.0050*	.47115	.005	-4.8801	-1.1299
4	1	3.1350*	.47115	.004	1.2599	5.0101
	2	3.1350*	.47115	.004	1.2599	5.0101
	3	3.1350*	.47115	.004	1.2599	5.0101
	5	-.3250	.47115	.977	-2.2001	1.5501
	6	.1300	.47115	1.000	-1.7451	2.0051
5	1	3.4600*	.47115	.003	1.5849	5.3351
	2	3.4600*	.47115	.003	1.5849	5.3351
	3	3.4600*	.47115	.003	1.5849	5.3351
	4	.3250	.47115	.977	-1.5501	2.2001
	6	.4550	.47115	.914	-1.4201	2.3301
6	1	3.0050*	.47115	.005	1.1299	4.8801
	2	3.0050*	.47115	.005	1.1299	4.8801
	3	3.0050*	.47115	.005	1.1299	4.8801
	4	-.1300	.47115	1.000	-2.0051	1.7451
	5	-.4550	.47115	.914	-2.3301	1.4201

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	
2	2	.0000	
3	2	.0000	
6	2		3.0050
4	2		3.1350
5	2		3.4600
Sig.		1.000	.914

b. Incubated milk – 8 h (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33.972	5	6.794	41.547	.000
Within Groups	.981	6	.164		
Total	34.953	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.00000	.40439	1.000	-1.6094	1.6094
	3	.00000	.40439	1.000	-1.6094	1.6094
	4	-3.17000*	.40439	.002	-4.7794	-1.5606
	5	-4.03000*	.40439	.000	-5.6394	-2.4206
	6	-2.57000*	.40439	.005	-4.1794	-.9606
2	1	.00000	.40439	1.000	-1.6094	1.6094
	3	.00000	.40439	1.000	-1.6094	1.6094
	4	-3.17000*	.40439	.002	-4.7794	-1.5606
	5	-4.03000*	.40439	.000	-5.6394	-2.4206
	6	-2.57000*	.40439	.005	-4.1794	-.9606
3	1	.00000	.40439	1.000	-1.6094	1.6094
	2	.00000	.40439	1.000	-1.6094	1.6094
	4	-3.17000*	.40439	.002	-4.7794	-1.5606
	5	-4.03000*	.40439	.000	-5.6394	-2.4206
	6	-2.57000*	.40439	.005	-4.1794	-.9606
4	1	3.17000*	.40439	.002	1.5606	4.7794
	2	3.17000*	.40439	.002	1.5606	4.7794
	3	3.17000*	.40439	.002	1.5606	4.7794
	5	-.86000	.40439	.382	-2.4694	.7494
	6	.60000	.40439	.686	-1.0094	2.2094
5	1	4.03000*	.40439	.000	2.4206	5.6394
	2	4.03000*	.40439	.000	2.4206	5.6394
	3	4.03000*	.40439	.000	2.4206	5.6394
	4	.86000	.40439	.382	-.7494	2.4694
	6	1.46000	.40439	.074	-.1494	3.0694
6	1	2.57000*	.40439	.005	.9606	4.1794
	2	2.57000*	.40439	.005	.9606	4.1794
	3	2.57000*	.40439	.005	.9606	4.1794
	4	-.60000	.40439	.686	-2.2094	1.0094
	5	-1.46000	.40439	.074	-3.0694	.1494

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	2.5700 3.1700 4.0300
2	2	.0000	
3	2	.0000	
6	2		
4	2		
5	2		
Sig.		1.000	.074

c. Incubated milk – 24 h (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.424 ^a	5	8.085	35.572	.000
Within Groups	1.364	6	.227		
Total	41.788	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.47674	1.000	-1.8974	1.8974
	3	.0000	.47674	1.000	-1.8974	1.8974
	4	-3.1200*	.47674	.005	-5.0174	-1.2226
	5	-4.4450*	.47674	.001	-6.3424	-2.5476
	6	-3.1250*	.47674	.005	-5.0224	-1.2276
2	1	.0000	.47674	1.000	-1.8974	1.8974
	3	.0000	.47674	1.000	-1.8974	1.8974
	4	-3.1200*	.47674	.005	-5.0174	-1.2226
	5	-4.4450*	.47674	.001	-6.3424	-2.5476
	6	-3.1250*	.47674	.005	-5.0224	-1.2276
3	1	.0000	.47674	1.000	-1.8974	1.8974
	2	.0000	.47674	1.000	-1.8974	1.8974
	4	-3.1200*	.47674	.005	-5.0174	-1.2226
	5	-4.4450*	.47674	.001	-6.3424	-2.5476
	6	-3.1250*	.47674	.005	-5.0224	-1.2276
4	1	3.1200*	.47674	.005	1.2226	5.0174
	2	3.1200*	.47674	.005	1.2226	5.0174
	3	3.1200*	.47674	.005	1.2226	5.0174
	5	-1.3250	.47674	.188	-3.2224	.5724
	6	-.0050	.47674	1.000	-1.9024	1.8924
5	1	4.4450*	.47674	.001	2.5476	6.3424
	2	4.4450*	.47674	.001	2.5476	6.3424
	3	4.4450*	.47674	.001	2.5476	6.3424
	4	1.3250	.47674	.188	-.5724	3.2224
	6	1.3200	.47674	.190	-.5774	3.2174
6	1	3.1250*	.47674	.005	1.2276	5.0224
	2	3.1250*	.47674	.005	1.2276	5.0224
	3	3.1250*	.47674	.005	1.2276	5.0224
	4	.0050	.47674	1.000	-1.8924	1.9024
	5	-1.3200	.47674	.190	-3.2174	.5774

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	
2	2	.0000	
3	2	.0000	
4	2		3.1200
6	2		3.1250
5	2		4.4450
Sig.		1.000	.188

d. Pre-salting curd (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	46.091 ^a	5	9.218	24.047	.001
Within Groups	2.300	6	.383		
Total	48.391	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.61915	1.000	-2.4641	2.4641
	3	.0000	.61915	1.000	-2.4641	2.4641
	4	-4.1850*	.61915	.004	-6.6491	-1.7209
	5	-3.9300*	.61915	.005	-6.3941	-1.4659
	6	-3.6000*	.61915	.009	-6.0641	-1.1359
2	1	.0000	.61915	1.000	-2.4641	2.4641
	3	.0000	.61915	1.000	-2.4641	2.4641
	4	-4.1850*	.61915	.004	-6.6491	-1.7209
	5	-3.9300*	.61915	.005	-6.3941	-1.4659
	6	-3.6000*	.61915	.009	-6.0641	-1.1359
3	1	.0000	.61915	1.000	-2.4641	2.4641
	2	.0000	.61915	1.000	-2.4641	2.4641
	4	-4.1850*	.61915	.004	-6.6491	-1.7209
	5	-3.9300*	.61915	.005	-6.3941	-1.4659
	6	-3.6000*	.61915	.009	-6.0641	-1.1359
4	1	4.1850*	.61915	.004	1.7209	6.6491
	2	4.1850*	.61915	.004	1.7209	6.6491
	3	4.1850*	.61915	.004	1.7209	6.6491
	5	.2550	.61915	.998	-2.2091	2.7191
	6	.5850	.61915	.920	-1.8791	3.0491
5	1	3.9300*	.61915	.005	1.4659	6.3941
	2	3.9300*	.61915	.005	1.4659	6.3941
	3	3.9300*	.61915	.005	1.4659	6.3941
	4	-.2550	.61915	.998	-2.7191	2.2091
	6	.3300	.61915	.992	-2.1341	2.7941
6	1	3.6000*	.61915	.009	1.1359	6.0641
	2	3.6000*	.61915	.009	1.1359	6.0641
	3	3.6000*	.61915	.009	1.1359	6.0641
	4	-.5850	.61915	.920	-3.0491	1.8791
	5	-.3300	.61915	.992	-2.7941	2.1341

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	3.6000 3.9300 4.1850
2	2	.0000	
3	2	.0000	
6	2		
5	2		
4	2		
Sig.		1.000	.920

e. Post-salting curd (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	50.149 ^a	5	10.030	19.397	.001
Within Groups	3.102	6	.517		
Total	53.252	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.71908	1.000	-2.8618	2.8618
	3	.0000	.71908	1.000	-2.8618	2.8618
	4	-4.2100*	.71908	.008	-7.0718	-1.3482
	5	-4.4100*	.71908	.007	-7.2718	-1.5482
	6	-3.5450*	.71908	.019	-6.4068	-.6832
2	1	.0000	.71908	1.000	-2.8618	2.8618
	3	.0000	.71908	1.000	-2.8618	2.8618
	4	-4.2100*	.71908	.008	-7.0718	-1.3482
	5	-4.4100*	.71908	.007	-7.2718	-1.5482
	6	-3.5450*	.71908	.019	-6.4068	-.6832
3	1	.0000	.71908	1.000	-2.8618	2.8618
	2	.0000	.71908	1.000	-2.8618	2.8618
	4	-4.2100*	.71908	.008	-7.0718	-1.3482
	5	-4.4100*	.71908	.007	-7.2718	-1.5482
	6	-3.5450*	.71908	.019	-6.4068	-.6832
4	1	4.2100*	.71908	.008	1.3482	7.0718
	2	4.2100*	.71908	.008	1.3482	7.0718
	3	4.2100*	.71908	.008	1.3482	7.0718
	5	-.2000	.71908	1.000	-3.0618	2.6618
	6	.6650	.71908	.926	-2.1968	3.5268
5	1	4.4100*	.71908	.007	1.5482	7.2718
	2	4.4100*	.71908	.007	1.5482	7.2718
	3	4.4100*	.71908	.007	1.5482	7.2718
	4	.2000	.71908	1.000	-2.6618	3.0618
	6	.8650	.71908	.822	-1.9968	3.7268
6	1	3.5450*	.71908	.019	.6832	6.4068
	2	3.5450*	.71908	.019	.6832	6.4068
	3	3.5450*	.71908	.019	.6832	6.4068
	4	-.6650	.71908	.926	-3.5268	2.1968
	5	-.8650	.71908	.822	-3.7268	1.9968

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	3.5450 4.2100 4.4100
2	2	.0000	
3	2	.0000	
6	2		
4	2		
5	2		
Sig.		1.000	.822

f. Pre-rubbing up (RBCA)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	120.061 ^a	5	24.012	51.919	.000
Within Groups	2.775	6	.462		
Total	122.836	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.68007	1.000	-2.7066	2.7066
	3	.0000	.68007	1.000	-2.7066	2.7066
	4	-5.6250*	.68007	.001	-8.3316	-2.9184
	5	-6.0650*	.68007	.001	-8.7716	-3.3584
	6	-7.1050*	.68007	.000	-9.8116	-4.3984
2	1	.0000	.68007	1.000	-2.7066	2.7066
	3	.0000	.68007	1.000	-2.7066	2.7066
	4	-5.6250*	.68007	.001	-8.3316	-2.9184
	5	-6.0650*	.68007	.001	-8.7716	-3.3584
	6	-7.1050*	.68007	.000	-9.8116	-4.3984
3	1	.0000	.68007	1.000	-2.7066	2.7066
	2	.0000	.68007	1.000	-2.7066	2.7066
	4	-5.6250*	.68007	.001	-8.3316	-2.9184
	5	-6.0650*	.68007	.001	-8.7716	-3.3584
	6	-7.1050*	.68007	.000	-9.8116	-4.3984
4	1	5.6250*	.68007	.001	2.9184	8.3316
	2	5.6250*	.68007	.001	2.9184	8.3316
	3	5.6250*	.68007	.001	2.9184	8.3316
	5	-.4400	.68007	.982	-3.1466	2.2666
	6	-1.4800	.68007	.363	-4.1866	1.2266
5	1	6.0650*	.68007	.001	3.3584	8.7716
	2	6.0650*	.68007	.001	3.3584	8.7716
	3	6.0650*	.68007	.001	3.3584	8.7716
	4	.4400	.68007	.982	-2.2666	3.1466
	6	-1.0400	.68007	.662	-3.7466	1.6666
6	1	7.1050*	.68007	.000	4.3984	9.8116
	2	7.1050*	.68007	.000	4.3984	9.8116
	3	7.1050*	.68007	.000	4.3984	9.8116
	4	1.4800	.68007	.363	-1.2266	4.1866
	5	1.0400	.68007	.662	-1.6666	3.7466

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	5.6250 6.0650 7.1050
2	2	.0000	
3	2	.0000	
4	2		
5	2		
6	2		
Sig.		1.000	.363

g. Ripened cheese (1 week – crust)

1. M17

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47.533 ^a	5	9.507	6.832	.018
Within Groups	8.349	6	1.391		
Total	55.881	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.7150	1.17959	.986	-3.9796	5.4096
	3	-2.3350	1.17959	.443	-7.0296	2.3596
	4	-4.4250	1.17959	.064	-9.1196	.2696
	5	1.4700	1.17959	.803	-3.2246	6.1646
	6	-.3700	1.17959	.999	-5.0646	4.3246
2	1	-.7150	1.17959	.986	-5.4096	3.9796
	3	-3.0500	1.17959	.233	-7.7446	1.6446
	4	-5.1400*	1.17959	.034	-9.8346	-.4454
	5	.7550	1.17959	.983	-3.9396	5.4496
	6	-1.0850	1.17959	.928	-5.7796	3.6096
3	1	2.3350	1.17959	.443	-2.3596	7.0296
	2	3.0500	1.17959	.233	-1.6446	7.7446
	4	-2.0900	1.17959	.540	-6.7846	2.6046
	5	3.8050	1.17959	.114	-.8896	8.4996
	6	1.9650	1.17959	.593	-2.7296	6.6596
4	1	4.4250	1.17959	.064	-.2696	9.1196
	2	5.1400*	1.17959	.034	-.4454	9.8346
	3	2.0900	1.17959	.540	-2.6046	6.7846
	5	5.8950*	1.17959	.018	1.2004	10.5896
	6	4.0550	1.17959	.090	-.6396	8.7496
5	1	-1.4700	1.17959	.803	-6.1646	3.2246
	2	-.7550	1.17959	.983	-5.4496	3.9396
	3	-3.8050	1.17959	.114	-8.4996	.8896
	4	-5.8950*	1.17959	.018	-10.5896	-1.2004
	6	-1.8400	1.17959	.647	-6.5346	2.8546
6	1	.3700	1.17959	.999	-4.3246	5.0646
	2	1.0850	1.17959	.928	-3.6096	5.7796
	3	-1.9650	1.17959	.593	-6.6596	2.7296
	4	-4.0550	1.17959	.090	-8.7496	.6396
	5	1.8400	1.17959	.647	-2.8546	6.5346

Cheese Model	N	Subset for alpha = 0.05	
		a	b
5	2	5.3150	
2	2	6.0700	
1	2	6.7850	6.7850
6	2	7.1550	7.1550
3	2	9.1200	9.1200
4	2		11.2100
Sig.		.114	.064

2. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.135 ^a	5	9.027	8.425	.011
Within Groups	6.429	6	1.071		
Total	51.564	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.6900	1.03512	.980	-3.4296	4.8096
	3	-1.3250	1.03512	.787	-5.4446	2.7946
	4	-4.3600*	1.03512	.039	-8.4796	-.2404
	5	1.7000	1.03512	.604	-2.4196	5.8196
	6	.1950	1.03512	1.000	-3.9246	4.3146
2	1	-.6900	1.03512	.980	-4.8096	3.4296
	3	-2.0150	1.03512	.458	-6.1346	2.1046
	4	-5.0500*	1.03512	.020	-9.1696	-.9304
	5	1.0100	1.03512	.910	-3.1096	5.1296
	6	-.4950	1.03512	.995	-4.6146	3.6246
3	1	1.3250	1.03512	.787	-2.7946	5.4446
	2	2.0150	1.03512	.458	-2.1046	6.1346
	4	-3.0350	1.03512	.158	-7.1546	1.0846
	5	3.0250	1.03512	.160	-1.0946	7.1446
	6	1.5200	1.03512	.693	-2.5996	5.6396
4	1	4.3600*	1.03512	.039	.2404	8.4796
	2	5.0500*	1.03512	.020	.9304	9.1696
	3	3.0350	1.03512	.158	-1.0846	7.1546
	5	6.0600*	1.03512	.008	1.9404	10.1796
	6	4.5550*	1.03512	.032	.4354	8.6746
5	1	-1.7000	1.03512	.604	-5.8196	2.4196
	2	-1.0100	1.03512	.910	-5.1296	3.1096
	3	-3.0250	1.03512	.160	-7.1446	1.0946
	4	-6.0600*	1.03512	.008	-10.1796	-1.9404
	6	-1.5050	1.03512	.701	-5.6246	2.6146
6	1	-.1950	1.03512	1.000	-4.3146	3.9246
	2	.4950	1.03512	.995	-3.6246	4.6146
	3	-1.5200	1.03512	.693	-5.6396	2.5996
	4	-4.5550*	1.03512	.032	-8.6746	-.4354
	5	1.5050	1.03512	.701	-2.6146	5.6246

Cheese Model	N	Subset for alpha = 0.05	
		a	b
5	2	5.0500	8.0750 11.1100
2	2	6.0600	
6	2	6.5550	
1	2	6.7500	
3	2	8.0750	
4	2		
Sig.		.160	.158

3. PCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	68.165 ^a	5	13.633	24.077	.001
Within Groups	3.397	6	.566		
Total	71.562	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.5850	.75248	.390	-1.4098	4.5798
	3	-2.4700	.75248	.107	-5.4648	.5248
	4	-3.4650*	.75248	.026	-6.4598	-.4702
	5	3.5050*	.75248	.025	.5102	6.4998
	6	1.0900	.75248	.704	-1.9048	4.0848
2	1	-1.5850	.75248	.390	-4.5798	1.4098
	3	-4.0550*	.75248	.012	-7.0498	-1.0602
	4	-5.0500*	.75248	.004	-8.0448	-2.0552
	5	1.9200	.75248	.242	-1.0748	4.9148
	6	-.4950	.75248	.981	-3.4898	2.4998
3	1	2.4700	.75248	.107	-.5248	5.4648
	2	4.0550*	.75248	.012	1.0602	7.0498
	4	-.9950	.75248	.766	-3.9898	1.9998
	5	5.9750*	.75248	.002	2.9802	8.9698
	6	3.5600*	.75248	.023	.5652	6.5548
4	1	3.4650*	.75248	.026	.4702	6.4598
	2	5.0500*	.75248	.004	2.0552	8.0448
	3	.9950	.75248	.766	-1.9998	3.9898
	5	6.9700*	.75248	.001	3.9752	9.9648
	6	4.5550*	.75248	.007	1.5602	7.5498
5	1	-3.5050*	.75248	.025	-6.4998	-.5102
	2	-1.9200	.75248	.242	-4.9148	1.0748
	3	-5.9750*	.75248	.002	-8.9698	-2.9802
	4	-6.9700*	.75248	.001	-9.9648	-3.9752
	6	-2.4150	.75248	.116	-5.4098	.5798
6	1	-1.0900	.75248	.704	-4.0848	1.9048
	2	.4950	.75248	.981	-2.4998	3.4898
	3	-3.5600*	.75248	.023	-6.5548	-.5652
	4	-4.5550*	.75248	.007	-7.5498	-1.5602
	5	2.4150	.75248	.116	-.5798	5.4098

Cheese Model	N	Subset for alpha = 0.05			
		a	b	c	d
5	2	4.1850			
2	2	6.1050	6.1050		
6	2	6.6000	6.6000		
1	2		7.6900	7.6900	
3	2			10.1600	10.1600
4	2				11.1550
Sig.		.116	.390	.107	.766

4. RBCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	54.610 ^a	5	10.922	10.716	.006
Within Groups	6.115	6	1.019		
Total	60.725	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-2.0250	1.00955	.432	-6.0429	1.9929
	3	4.6500*	1.00955	.026	.6321	8.6679
	4	-.0150	1.00955	1.000	-4.0329	4.0029
	5	1.9200	1.00955	.478	-2.0979	5.9379
	6	2.4550	1.00955	.276	-1.5629	6.4729
2	1	2.0250	1.00955	.432	-1.9929	6.0429
	3	6.6750*	1.00955	.004	2.6571	10.6929
	4	2.0100	1.00955	.438	-2.0079	6.0279
	5	3.9450	1.00955	.054	-.0729	7.9629
	6	4.4800*	1.00955	.031	.4621	8.4979
3	1	-4.6500*	1.00955	.026	-8.6679	-.6321
	2	-6.6750*	1.00955	.004	-10.6929	-2.6571
	4	-4.6650*	1.00955	.026	-8.6829	-.6471
	5	-2.7300	1.00955	.204	-6.7479	1.2879
	6	-2.1950	1.00955	.364	-6.2129	1.8229
4	1	.0150	1.00955	1.000	-4.0029	4.0329
	2	-2.0100	1.00955	.438	-6.0279	2.0079
	3	4.6650*	1.00955	.026	.6471	8.6829
	5	1.9350	1.00955	.471	-2.0829	5.9529
	6	2.4700	1.00955	.272	-1.5479	6.4879
5	1	-1.9200	1.00955	.478	-5.9379	2.0979
	2	-3.9450	1.00955	.054	-7.9629	.0729
	3	2.7300	1.00955	.204	-1.2879	6.7479
	4	-1.9350	1.00955	.471	-5.9529	2.0829
	6	.5350	1.00955	.993	-3.4829	4.5529
6	1	-2.4550	1.00955	.276	-6.4729	1.5629
	2	-4.4800*	1.00955	.031	-8.4979	-.4621
	3	2.1950	1.00955	.364	-1.8229	6.2129
	4	-2.4700	1.00955	.272	-6.4879	1.5479
	5	-.5350	1.00955	.993	-4.5529	3.4829

Cheese Model	N	Subset for alpha = 0.05		
		a	b	c
3	2	3.4350		
6	2	5.6300	5.6300	
5	2	6.1650	6.1650	6.1650
1	2		8.0850	8.0850
4	2		8.1000	8.1000
2	2			10.1100
Sig.		.204	.272	.054

5. MSA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.359 ^a	5	5.672	78.694	.000
Within Groups	.432	6	.072		
Total	28.792	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.26847	1.000	-1.0685	1.0685
	3	.0000	.26847	1.000	-1.0685	1.0685
	4	.0000	.26847	1.000	-1.0685	1.0685
	5	-4.1250*	.26847	.000	-5.1935	-3.0565
	6	.0000	.26847	1.000	-1.0685	1.0685
2	1	.0000	.26847	1.000	-1.0685	1.0685
	3	.0000	.26847	1.000	-1.0685	1.0685
	4	.0000	.26847	1.000	-1.0685	1.0685
	5	-4.1250*	.26847	.000	-5.1935	-3.0565
	6	.0000	.26847	1.000	-1.0685	1.0685
3	1	.0000	.26847	1.000	-1.0685	1.0685
	2	.0000	.26847	1.000	-1.0685	1.0685
	4	.0000	.26847	1.000	-1.0685	1.0685
	5	-4.1250*	.26847	.000	-5.1935	-3.0565
	6	.0000	.26847	1.000	-1.0685	1.0685
4	1	.0000	.26847	1.000	-1.0685	1.0685
	2	.0000	.26847	1.000	-1.0685	1.0685
	3	.0000	.26847	1.000	-1.0685	1.0685
	5	-4.1250*	.26847	.000	-5.1935	-3.0565
	6	.0000	.26847	1.000	-1.0685	1.0685
5	1	4.1250*	.26847	.000	3.0565	5.1935
	2	4.1250*	.26847	.000	3.0565	5.1935
	3	4.1250*	.26847	.000	3.0565	5.1935
	4	4.1250*	.26847	.000	3.0565	5.1935
	6	4.1250*	.26847	.000	3.0565	5.1935
6	1	.0000	.26847	1.000	-1.0685	1.0685
	2	.0000	.26847	1.000	-1.0685	1.0685
	3	.0000	.26847	1.000	-1.0685	1.0685
	4	.0000	.26847	1.000	-1.0685	1.0685
	5	-4.1250*	.26847	.000	-5.1935	-3.0565

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	4.1250
2	2	.0000	
3	2	.0000	
4	2	.0000	
6	2	.0000	
5	2		
Sig.		1.000	1.000

h. Ripened cheese (1 week – core)

1. M17

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.003 ^a	5	6.001	10.127	.007
Within Groups	3.555	6	.593		
Total	33.558	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.2600	.76976	.607	-1.8035	4.3235
	3	-2.7850	.76976	.074	-5.8485	.2785
	4	-1.9000	.76976	.265	-4.9635	1.1635
	5	1.5250	.76976	.443	-1.5385	4.5885
	6	.3500	.76976	.996	-2.7135	3.4135
2	1	-1.2600	.76976	.607	-4.3235	1.8035
	3	-4.0450*	.76976	.014	-7.1085	-.9815
	4	-3.1600*	.76976	.044	-6.2235	-.0965
	5	.2650	.76976	.999	-2.7985	3.3285
	6	-.9100	.76976	.831	-3.9735	2.1535
3	1	2.7850	.76976	.074	-.2785	5.8485
	2	4.0450*	.76976	.014	.9815	7.1085
	4	.8850	.76976	.845	-2.1785	3.9485
	5	4.3100*	.76976	.010	1.2465	7.3735
	6	3.1350*	.76976	.045	.0715	6.1985
4	1	1.9000	.76976	.265	-1.1635	4.9635
	2	3.1600*	.76976	.044	.0965	6.2235
	3	-.8850	.76976	.845	-3.9485	2.1785
	5	3.4250*	.76976	.031	.3615	6.4885
	6	2.2500	.76976	.160	-.8135	5.3135
5	1	-1.5250	.76976	.443	-4.5885	1.5385
	2	-.2650	.76976	.999	-3.3285	2.7985
	3	-4.3100*	.76976	.010	-7.3735	-1.2465
	4	-3.4250*	.76976	.031	-6.4885	-.3615
	6	-1.1750	.76976	.664	-4.2385	1.8885
6	1	-.3500	.76976	.996	-3.4135	2.7135
	2	.9100	.76976	.831	-2.1535	3.9735
	3	-3.1350*	.76976	.045	-6.1985	-.0715
	4	-2.2500	.76976	.160	-5.3135	.8135
	5	1.1750	.76976	.664	-1.8885	4.2385

Cheese Model	N	Subset for alpha = 0.05		
		a	b	c
5	2	4.7800		
2	2	5.0450		
6	2	5.9550	5.9550	
1	2	6.3050	6.3050	6.3050
4	2		8.2050	8.2050
3	2			9.0900
Sig.		.443	.160	.074

2. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.929 ^a	5	5.386	7.345	.015
Within Groups	4.400	6	.733		
Total	31.329	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.9750	.85632	.850	-2.4330	4.3830
	3	-2.9600	.85632	.088	-6.3680	.4480
	4	-2.0350	.85632	.293	-5.4430	1.3730
	5	.9450	.85632	.864	-2.4630	4.3530
	6	.2400	.85632	1.000	-3.1680	3.6480
2	1	-.9750	.85632	.850	-4.3830	2.4330
	3	-3.9350*	.85632	.027	-7.3430	-.5270
	4	-3.0100	.85632	.083	-6.4180	.3980
	5	-.0300	.85632	1.000	-3.4380	3.3780
	6	-.7350	.85632	.944	-4.1430	2.6730
3	1	2.9600	.85632	.088	-.4480	6.3680
	2	3.9350*	.85632	.027	.5270	7.3430
	4	.9250	.85632	.873	-2.4830	4.3330
	5	3.9050*	.85632	.028	.4970	7.3130
	6	3.2000	.85632	.065	-.2080	6.6080
4	1	2.0350	.85632	.293	-1.3730	5.4430
	2	3.0100	.85632	.083	-.3980	6.4180
	3	-.9250	.85632	.873	-4.3330	2.4830
	5	2.9800	.85632	.086	-.4280	6.3880
	6	2.2750	.85632	.215	-1.1330	5.6830
5	1	-.9450	.85632	.864	-4.3530	2.4630
	2	.0300	.85632	1.000	-3.3780	3.4380
	3	-3.9050*	.85632	.028	-7.3130	-.4970
	4	-2.9800	.85632	.086	-6.3880	.4280
	6	-.7050	.85632	.952	-4.1130	2.7030
6	1	-.2400	.85632	1.000	-3.6480	3.1680
	2	.7350	.85632	.944	-2.6730	4.1430
	3	-3.2000	.85632	.065	-6.6080	.2080
	4	-2.2750	.85632	.215	-5.6830	1.1330
	5	.7050	.85632	.952	-2.7030	4.1130

Cheese Model	N	Subset for alpha = 0.05	
		a	b
2	2	5.1300	
5	2	5.1600	
6	2	5.8650	5.8650
1	2	6.1050	6.1050
4	2	8.1400	8.1400
3	2		9.0650
Sig.		.083	.065

3. RBCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73.019 ^a	5	14.604	28.707	.000
Within Groups	3.052	6	.509		
Total	76.071	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.71324	1.000	-2.8386	2.8386
	3	.0000	.71324	1.000	-2.8386	2.8386
	4	-5.0900*	.71324	.003	-7.9286	-2.2514
	5	-5.0950*	.71324	.003	-7.9336	-2.2564
	6	-4.5800*	.71324	.005	-7.4186	-1.7414
2	1	.0000	.71324	1.000	-2.8386	2.8386
	3	.0000	.71324	1.000	-2.8386	2.8386
	4	-5.0900*	.71324	.003	-7.9286	-2.2514
	5	-5.0950*	.71324	.003	-7.9336	-2.2564
	6	-4.5800*	.71324	.005	-7.4186	-1.7414
3	1	.0000	.71324	1.000	-2.8386	2.8386
	2	.0000	.71324	1.000	-2.8386	2.8386
	4	-5.0900*	.71324	.003	-7.9286	-2.2514
	5	-5.0950*	.71324	.003	-7.9336	-2.2564
	6	-4.5800*	.71324	.005	-7.4186	-1.7414
4	1	5.0900*	.71324	.003	2.2514	7.9286
	2	5.0900*	.71324	.003	2.2514	7.9286
	3	5.0900*	.71324	.003	2.2514	7.9286
	5	-.0050	.71324	1.000	-2.8436	2.8336
	6	.5100	.71324	.973	-2.3286	3.3486
5	1	5.0950*	.71324	.003	2.2564	7.9336
	2	5.0950*	.71324	.003	2.2564	7.9336
	3	5.0950*	.71324	.003	2.2564	7.9336
	4	.0050	.71324	1.000	-2.8336	2.8436
	6	.5150	.71324	.972	-2.3236	3.3536
6	1	4.5800*	.71324	.005	1.7414	7.4186
	2	4.5800*	.71324	.005	1.7414	7.4186
	3	4.5800*	.71324	.005	1.7414	7.4186
	4	-.5100	.71324	.973	-3.3486	2.3286
	5	-.5150	.71324	.972	-3.3536	2.3236

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	4.5800 5.0900 5.0950
2	2	.0000	
3	2	.0000	
6	2		
4	2		
5	2		
Sig.		1.000	.972

i. Ripened cheese (2 weeks – crust)

1. M17

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41.289 ^a	5	8.258	17.273	.002
Within Groups	2.869	6	.478		
Total	44.157	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.2150	.69144	.547	-1.5368	3.9668
	3	1.2650	.69144	.512	-1.4868	4.0168
	4	-3.8650*	.69144	.010	-6.6168	-1.1132
	5	1.2600	.69144	.515	-1.4918	4.0118
	6	-1.1450	.69144	.598	-3.8968	1.6068
2	1	-1.2150	.69144	.547	-3.9668	1.5368
	3	.0500	.69144	1.000	-2.7018	2.8018
	4	-5.0800*	.69144	.003	-7.8318	-2.3282
	5	.0450	.69144	1.000	-2.7068	2.7968
	6	-2.3600	.69144	.092	-5.1118	.3918
3	1	-1.2650	.69144	.512	-4.0168	1.4868
	2	-.0500	.69144	1.000	-2.8018	2.7018
	4	-5.1300*	.69144	.002	-7.8818	-2.3782
	5	-.0050	.69144	1.000	-2.7568	2.7468
	6	-2.4100	.69144	.085	-5.1618	.3418
4	1	3.8650*	.69144	.010	1.1132	6.6168
	2	5.0800*	.69144	.003	2.3282	7.8318
	3	5.1300*	.69144	.002	2.3782	7.8818
	5	5.1250*	.69144	.002	2.3732	7.8768
	6	2.7200	.69144	.053	-.0318	5.4718
5	1	-1.2600	.69144	.515	-4.0118	1.4918
	2	-.0450	.69144	1.000	-2.7968	2.7068
	3	.0050	.69144	1.000	-2.7468	2.7568
	4	-5.1250*	.69144	.002	-7.8768	-2.3732
	6	-2.4050	.69144	.086	-5.1568	.3468
6	1	1.1450	.69144	.598	-1.6068	3.8968
	2	2.3600	.69144	.092	-.3918	5.1118
	3	2.4100	.69144	.085	-.3418	5.1618
	4	-2.7200	.69144	.053	-5.4718	.0318
	5	2.4050	.69144	.086	-.3468	5.1568

Cheese Model	N	Subset for alpha = 0.05	
		a	b
3	2	5.0100	7.4200 10.1400
5	2	5.0150	
2	2	5.0600	
1	2	6.2750	
6	2	7.4200	
4	2		
Sig.		.085	.053

2. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	31.549 ^a	5	6.310	8.553	.011
Within Groups	4.426	6	.738		
Total	35.975	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.2800	.85892	.682	-2.1384	4.6984
	3	1.3200	.85892	.658	-2.0984	4.7384
	4	-2.7250	.85892	.121	-6.1434	.6934
	5	2.2500	.85892	.224	-1.1684	5.6684
	6	-.5600	.85892	.982	-3.9784	2.8584
2	1	-1.2800	.85892	.682	-4.6984	2.1384
	3	.0400	.85892	1.000	-3.3784	3.4584
	4	-4.0050*	.85892	.025	-7.4234	-.5866
	5	.9700	.85892	.854	-2.4484	4.3884
	6	-1.8400	.85892	.376	-5.2584	1.5784
3	1	-1.3200	.85892	.658	-4.7384	2.0984
	2	-.0400	.85892	1.000	-3.4584	3.3784
	4	-4.0450*	.85892	.024	-7.4634	-.6266
	5	.9300	.85892	.872	-2.4884	4.3484
	6	-1.8800	.85892	.358	-5.2984	1.5384
4	1	2.7250	.85892	.121	-.6934	6.1434
	2	4.0050*	.85892	.025	.5866	7.4234
	3	4.0450*	.85892	.024	.6266	7.4634
	5	4.9750*	.85892	.009	1.5566	8.3934
	6	2.1650	.85892	.250	-1.2534	5.5834
5	1	-2.2500	.85892	.224	-5.6684	1.1684
	2	-.9700	.85892	.854	-4.3884	2.4484
	3	-.9300	.85892	.872	-4.3484	2.4884
	4	-4.9750*	.85892	.009	-8.3934	-1.5566
	6	-2.8100	.85892	.108	-6.2284	.6084
6	1	.5600	.85892	.982	-2.8584	3.9784
	2	1.8400	.85892	.376	-1.5784	5.2584
	3	1.8800	.85892	.358	-1.5384	5.2984
	4	-2.1650	.85892	.250	-5.5834	1.2534
	5	2.8100	.85892	.108	-.6084	6.2284

Cheese Model	N	Subset for alpha = 0.05	
		a	b
5	2	4.0850	6.3350 6.8950 9.0600
3	2	5.0150	
2	2	5.0550	
1	2	6.3350	
6	2	6.8950	
4	2		9.0600
Sig.		.108	.121

j. Ripened cheese (2 week – core)

1. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.910 ^a	5	2.982	4.792	.041
Within Groups	3.734	6	.622		
Total	18.644	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.6700	.78886	.946	-2.4695	3.8095
	3	.1300	.78886	1.000	-3.0095	3.2695
	4	-1.8150	.78886	.318	-4.9545	1.3245
	5	1.7250	.78886	.359	-1.4145	4.8645
	6	-.8500	.78886	.874	-3.9895	2.2895
2	1	-.6700	.78886	.946	-3.8095	2.4695
	3	-.5400	.78886	.977	-3.6795	2.5995
	4	-2.4850	.78886	.124	-5.6245	.6545
	5	1.0550	.78886	.759	-2.0845	4.1945
	6	-1.5200	.78886	.467	-4.6595	1.6195
3	1	-.1300	.78886	1.000	-3.2695	3.0095
	2	.5400	.78886	.977	-2.5995	3.6795
	4	-1.9450	.78886	.266	-5.0845	1.1945
	5	1.5950	.78886	.425	-1.5445	4.7345
	6	-.9800	.78886	.804	-4.1195	2.1595
4	1	1.8150	.78886	.318	-1.3245	4.9545
	2	2.4850	.78886	.124	-.6545	5.6245
	3	1.9450	.78886	.266	-1.1945	5.0845
	5	3.5400*	.78886	.030	.4005	6.6795
	6	.9650	.78886	.813	-2.1745	4.1045
5	1	-1.7250	.78886	.359	-4.8645	1.4145
	2	-1.0550	.78886	.759	-4.1945	2.0845
	3	-1.5950	.78886	.425	-4.7345	1.5445
	4	-3.5400*	.78886	.030	-6.6795	-.4005
	6	-2.5750	.78886	.109	-5.7145	.5645
6	1	.8500	.78886	.874	-2.2895	3.9895
	2	1.5200	.78886	.467	-1.6195	4.6595
	3	.9800	.78886	.804	-2.1595	4.1195
	4	-.9650	.78886	.813	-4.1045	2.1745
	5	2.5750	.78886	.109	-.5645	5.7145

Cheese Model	N	Subset for alpha = 0.05	
		a	b
5	2	3.5000	
2	2	4.5550	4.5550
3	2	5.0950	5.0950
1	2	5.2250	5.2250
6	2	6.0750	6.0750
4	2		7.0400
Sig.		.109	.124

2. RBCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.279 ^a	5	8.656	5.902	.026
Within Groups	8.800	6	1.467		
Total	52.079	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.4850	1.21107	.998	-5.3049	4.3349
	3	3.5500	1.21107	.158	-1.2699	8.3699
	4	-2.0450	1.21107	.581	-6.8649	2.7749
	5	-2.0500	1.21107	.579	-6.8699	2.7699
	6	-1.0750	1.21107	.936	-5.8949	3.7449
2	1	.4850	1.21107	.998	-4.3349	5.3049
	3	4.0350	1.21107	.101	-.7849	8.8549
	4	-1.5600	1.21107	.783	-6.3799	3.2599
	5	-1.5650	1.21107	.781	-6.3849	3.2549
	6	-.5900	1.21107	.995	-5.4099	4.2299
3	1	-3.5500	1.21107	.158	-8.3699	1.2699
	2	-4.0350	1.21107	.101	-8.8549	.7849
	4	-5.5950*	1.21107	.026	-10.4149	-.7751
	5	-5.6000*	1.21107	.026	-10.4199	-.7801
	6	-4.6250	1.21107	.059	-9.4449	.1949
4	1	2.0450	1.21107	.581	-2.7749	6.8649
	2	1.5600	1.21107	.783	-3.2599	6.3799
	3	5.5950*	1.21107	.026	.7751	10.4149
	5	-.0050	1.21107	1.000	-4.8249	4.8149
	6	.9700	1.21107	.957	-3.8499	5.7899
5	1	2.0500	1.21107	.579	-2.7699	6.8699
	2	1.5650	1.21107	.781	-3.2549	6.3849
	3	5.6000*	1.21107	.026	.7801	10.4199
	4	.0050	1.21107	1.000	-4.8149	4.8249
	6	.9750	1.21107	.956	-3.8449	5.7949
6	1	1.0750	1.21107	.936	-3.7449	5.8949
	2	.5900	1.21107	.995	-4.2299	5.4099
	3	4.6250	1.21107	.059	-.1949	9.4449
	4	-.9700	1.21107	.957	-5.7899	3.8499
	5	-.9750	1.21107	.956	-5.7949	3.8449

Cheese Model	N	Subset for alpha = 0.05	
		a	b
3	2	.0000	
1	2	3.5500	3.5500
2	2	4.0350	4.0350
6	2	4.6250	4.6250
4	2		5.5950
5	2		5.6000
Sig.		.059	.579

k. Ripened cheese (3 week – crust)

1. M17

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.765 ^a	5	6.553	6.532	.020
Within Groups	6.019	6	1.003		
Total	38.784	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.0250	1.00162	1.000	-4.0113	3.9613
	3	1.0300	1.00162	.893	-2.9563	5.0163
	4	-3.9750	1.00162	.051	-7.9613	.0113
	5	.4050	1.00162	.998	-3.5813	4.3913
	6	.3850	1.00162	.998	-3.6013	4.3713
2	1	.0250	1.00162	1.000	-3.9613	4.0113
	3	1.0550	1.00162	.883	-2.9313	5.0413
	4	-3.9500	1.00162	.052	-7.9363	.0363
	5	.4300	1.00162	.997	-3.5563	4.4163
	6	.4100	1.00162	.998	-3.5763	4.3963
3	1	-1.0300	1.00162	.893	-5.0163	2.9563
	2	-1.0550	1.00162	.883	-5.0413	2.9313
	4	-5.0050*	1.00162	.018	-8.9913	-1.0187
	5	-.6250	1.00162	.985	-4.6113	3.3613
	6	-.6450	1.00162	.982	-4.6313	3.3413
4	1	3.9750	1.00162	.051	-.0113	7.9613
	2	3.9500	1.00162	.052	-.0363	7.9363
	3	5.0050*	1.00162	.018	1.0187	8.9913
	5	4.3800*	1.00162	.033	.3937	8.3663
	6	4.3600*	1.00162	.034	.3737	8.3463
5	1	-.4050	1.00162	.998	-4.3913	3.5813
	2	-.4300	1.00162	.997	-4.4163	3.5563
	3	.6250	1.00162	.985	-3.3613	4.6113
	4	-4.3800*	1.00162	.033	-8.3663	-.3937
	6	-.0200	1.00162	1.000	-4.0063	3.9663
6	1	-.3850	1.00162	.998	-4.3713	3.6013
	2	-.4100	1.00162	.998	-4.3963	3.5763
	3	.6450	1.00162	.982	-3.3413	4.6313
	4	-4.3600*	1.00162	.034	-8.3463	-.3737
	5	.0200	1.00162	1.000	-3.9663	4.0063

Cheese Model	N	Subset for alpha = 0.05	
		a	b
3	2	4.0200	5.0500 5.0750 9.0250
5	2	4.6450	
6	2	4.6650	
1	2	5.0500	
2	2	5.0750	
4	2		
Sig.		.883	.051

2. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.420 ^a	5	4.284	6.751	.019
Within Groups	3.807	6	.635		
Total	25.227	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1.0150	.79660	.790	-4.1853	2.1553
	3	-.0250	.79660	1.000	-3.1953	3.1453
	4	-3.8000*	.79660	.022	-6.9703	-.6297
	5	-.0550	.79660	1.000	-3.2253	3.1153
	6	-.7950	.79660	.903	-3.9653	2.3753
2	1	1.0150	.79660	.790	-2.1553	4.1853
	3	.9900	.79660	.804	-2.1803	4.1603
	4	-2.7850	.79660	.084	-5.9553	.3853
	5	.9600	.79660	.821	-2.2103	4.1303
	6	.2200	.79660	1.000	-2.9503	3.3903
3	1	.0250	.79660	1.000	-3.1453	3.1953
	2	-.9900	.79660	.804	-4.1603	2.1803
	4	-3.7750*	.79660	.023	-6.9453	-.6047
	5	-.0300	.79660	1.000	-3.2003	3.1403
	6	-.7700	.79660	.913	-3.9403	2.4003
4	1	3.8000*	.79660	.022	.6297	6.9703
	2	2.7850	.79660	.084	-.3853	5.9553
	3	3.7750*	.79660	.023	.6047	6.9453
	5	3.7450*	.79660	.024	.5747	6.9153
	6	3.0050	.79660	.062	-.1653	6.1753
5	1	.0550	.79660	1.000	-3.1153	3.2253
	2	-.9600	.79660	.821	-4.1303	2.2103
	3	.0300	.79660	1.000	-3.1403	3.2003
	4	-3.7450*	.79660	.024	-6.9153	-.5747
	6	-.7400	.79660	.925	-3.9103	2.4303
6	1	.7950	.79660	.903	-2.3753	3.9653
	2	-.2200	.79660	1.000	-3.3903	2.9503
	3	.7700	.79660	.913	-2.4003	3.9403
	4	-3.0050	.79660	.062	-6.1753	.1653
	5	.7400	.79660	.925	-2.4303	3.9103

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	4.0300	
3	2	4.0550	
5	2	4.0850	
6	2	4.8250	4.8250
2	2	5.0450	5.0450
4	2		7.8300
Sig.		.790	.062

3. RBCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.189 ^a	5	4.838	5.085	.036
Within Groups	5.709	6	.951		
Total	29.898	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	1.1550	.97542	.830	-2.7270	5.0370
	3	1.0600	.97542	.871	-2.8220	4.9420
	4	-2.9350	.97542	.145	-6.8170	.9470
	5	-1.3800	.97542	.721	-5.2620	2.5020
	6	-.4000	.97542	.998	-4.2820	3.4820
2	1	-1.1550	.97542	.830	-5.0370	2.7270
	3	-.0950	.97542	1.000	-3.9770	3.7870
	4	-4.0900*	.97542	.040	-7.9720	-.2080
	5	-2.5350	.97542	.230	-6.4170	1.3470
	6	-1.5550	.97542	.629	-5.4370	2.3270
3	1	-1.0600	.97542	.871	-4.9420	2.8220
	2	.0950	.97542	1.000	-3.7870	3.9770
	4	-3.9950*	.97542	.044	-7.8770	-.1130
	5	-2.4400	.97542	.256	-6.3220	1.4420
	6	-1.4600	.97542	.679	-5.3420	2.4220
4	1	2.9350	.97542	.145	-.9470	6.8170
	2	4.0900*	.97542	.040	.2080	7.9720
	3	3.9950*	.97542	.044	.1130	7.8770
	5	1.5550	.97542	.629	-2.3270	5.4370
	6	2.5350	.97542	.230	-1.3470	6.4170
5	1	1.3800	.97542	.721	-2.5020	5.2620
	2	2.5350	.97542	.230	-1.3470	6.4170
	3	2.4400	.97542	.256	-1.4420	6.3220
	4	-1.5550	.97542	.629	-5.4370	2.3270
	6	.9800	.97542	.901	-2.9020	4.8620
6	1	.4000	.97542	.998	-3.4820	4.2820
	2	1.5550	.97542	.629	-2.3270	5.4370
	3	1.4600	.97542	.679	-2.4220	5.3420
	4	-2.5350	.97542	.230	-6.4170	1.3470
	5	-.9800	.97542	.901	-4.8620	2.9020

Cheese Model	N	Subset for alpha = 0.05	
		a	b
2	2	7.0250	
3	2	7.1200	
1	2	8.1800	8.1800
6	2	8.5800	8.5800
5	2	9.5600	9.5600
4	2		11.1150
Sig.		.230	.145

I. Ripened cheese (4 week – crust)

1. MRS

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25.773 ^a	5	5.155	7.008	.017
Within Groups	4.413	6	.736		
Total	30.186	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0450	.85763	1.000	-3.3682	3.4582
	3	.0050	.85763	1.000	-3.4082	3.4182
	4	-3.3150	.85763	.056	-6.7282	.0982
	5	.7000	.85763	.954	-2.7132	4.1132
	6	-2.3200	.85763	.204	-5.7332	1.0932
2	1	-.0450	.85763	1.000	-3.4582	3.3682
	3	-.0400	.85763	1.000	-3.4532	3.3732
	4	-3.3600	.85763	.053	-6.7732	.0532
	5	.6550	.85763	.965	-2.7582	4.0682
	6	-2.3650	.85763	.192	-5.7782	1.0482
3	1	-.0050	.85763	1.000	-3.4182	3.4082
	2	.0400	.85763	1.000	-3.3732	3.4532
	4	-3.3200	.85763	.056	-6.7332	.0932
	5	.6950	.85763	.955	-2.7182	4.1082
	6	-2.3250	.85763	.203	-5.7382	1.0882
4	1	3.3150	.85763	.056	-.0982	6.7282
	2	3.3600	.85763	.053	-.0532	6.7732
	3	3.3200	.85763	.056	-.0932	6.7332
	5	4.0150*	.85763	.024	.6018	7.4282
	6	.9950	.85763	.841	-2.4182	4.4082
5	1	-.7000	.85763	.954	-4.1132	2.7132
	2	-.6550	.85763	.965	-4.0682	2.7582
	3	-.6950	.85763	.955	-4.1082	2.7182
	4	-4.0150*	.85763	.024	-7.4282	-.6018
	6	-3.0200	.85763	.082	-6.4332	.3932
6	1	2.3200	.85763	.204	-1.0932	5.7332
	2	2.3650	.85763	.192	-1.0482	5.7782
	3	2.3250	.85763	.203	-1.0882	5.7382
	4	-.9950	.85763	.841	-4.4082	2.4182
	5	3.0200	.85763	.082	-.3932	6.4332

Cheese Model	N	Subset for alpha = 0.05	
		a	b
5	2	3.3750	
2	2	4.0300	4.0300
3	2	4.0700	4.0700
1	2	4.0750	4.0750
6	2	6.3950	6.3950
4	2		7.3900
Sig.		.082	.053

2. RBCA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.990 ^a	5	4.398	5.625	.029
Within Groups	4.691	6	.782		
Total	26.681	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-1.4100	.88424	.629	-4.9291	2.1091
	3	-1.3600	.88424	.658	-4.8791	2.1591
	4	-4.3700*	.88424	.019	-7.8891	-.8509
	5	-2.4250	.88424	.196	-5.9441	1.0941
	6	-2.6700	.88424	.143	-6.1891	.8491
2	1	1.4100	.88424	.629	-2.1091	4.9291
	3	.0500	.88424	1.000	-3.4691	3.5691
	4	-2.9600	.88424	.099	-6.4791	.5591
	5	-1.0150	.88424	.846	-4.5341	2.5041
	6	-1.2600	.88424	.716	-4.7791	2.2591
3	1	1.3600	.88424	.658	-2.1591	4.8791
	2	-.0500	.88424	1.000	-3.5691	3.4691
	4	-3.0100	.88424	.093	-6.5291	.5091
	5	-1.0650	.88424	.822	-4.5841	2.4541
	6	-1.3100	.88424	.687	-4.8291	2.2091
4	1	4.3700*	.88424	.019	.8509	7.8891
	2	2.9600	.88424	.099	-.5591	6.4791
	3	3.0100	.88424	.093	-.5091	6.5291
	5	1.9450	.88424	.354	-1.5741	5.4641
	6	1.7000	.88424	.469	-1.8191	5.2191
5	1	2.4250	.88424	.196	-1.0941	5.9441
	2	1.0150	.88424	.846	-2.5041	4.5341
	3	1.0650	.88424	.822	-2.4541	4.5841
	4	-1.9450	.88424	.354	-5.4641	1.5741
	6	-.2450	.88424	1.000	-3.7641	3.2741
6	1	2.6700	.88424	.143	-.8491	6.1891
	2	1.2600	.88424	.716	-2.2591	4.7791
	3	1.3100	.88424	.687	-2.2091	4.8291
	4	-1.7000	.88424	.469	-5.2191	1.8191
	5	.2450	.88424	1.000	-3.2741	3.7641

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	7.6800	
3	2	9.0400	9.0400
2	2	9.0900	9.0900
5	2	10.1050	10.1050
6	2	10.3500	10.3500
4	2		12.0500
Sig.		.143	.093

3. MSA

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	66.162 ^a	5	13.232	17.983	.001
Within Groups	4.415	6	.736		
Total	70.577	11			

Tukey HSD

(I) Cheese Model	(J) Cheese Model	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.0000	.85780	1.000	-3.4139	3.4139
	3	.0000	.85780	1.000	-3.4139	3.4139
	4	.0000	.85780	1.000	-3.4139	3.4139
	5	-5.1750*	.85780	.007	-8.5889	-1.7611
	6	-4.7750*	.85780	.011	-8.1889	-1.3611
2	1	.0000	.85780	1.000	-3.4139	3.4139
	3	.0000	.85780	1.000	-3.4139	3.4139
	4	.0000	.85780	1.000	-3.4139	3.4139
	5	-5.1750*	.85780	.007	-8.5889	-1.7611
	6	-4.7750*	.85780	.011	-8.1889	-1.3611
3	1	.0000	.85780	1.000	-3.4139	3.4139
	2	.0000	.85780	1.000	-3.4139	3.4139
	4	.0000	.85780	1.000	-3.4139	3.4139
	5	-5.1750*	.85780	.007	-8.5889	-1.7611
	6	-4.7750*	.85780	.011	-8.1889	-1.3611
4	1	.0000	.85780	1.000	-3.4139	3.4139
	2	.0000	.85780	1.000	-3.4139	3.4139
	3	.0000	.85780	1.000	-3.4139	3.4139
	5	-5.1750*	.85780	.007	-8.5889	-1.7611
	6	-4.7750*	.85780	.011	-8.1889	-1.3611
5	1	5.1750*	.85780	.007	1.7611	8.5889
	2	5.1750*	.85780	.007	1.7611	8.5889
	3	5.1750*	.85780	.007	1.7611	8.5889
	4	5.1750*	.85780	.007	1.7611	8.5889
	6	.4000	.85780	.996	-3.0139	3.8139
6	1	4.7750*	.85780	.011	1.3611	8.1889
	2	4.7750*	.85780	.011	1.3611	8.1889
	3	4.7750*	.85780	.011	1.3611	8.1889
	4	4.7750*	.85780	.011	1.3611	8.1889
	5	-.4000	.85780	.996	-3.8139	3.0139

Cheese Model	N	Subset for alpha = 0.05	
		a	b
1	2	.0000	4.7750 5.1750
2	2	.0000	
3	2	.0000	
4	2	.0000	
6	2		
5	2		
Sig.		1.000	.996

Appendix 9. List of compounds detected from headspace samples of model cheeses by SPME GCMS.

COMPOUNDS	Retention Time (min)	Key Ion (m/z)
Ethanol	4.97	45
2-Pentanone	5.91	86
Hexanal	8.47	100
2-methyl 1-propanol	8.91	74
2-Heptanone	11.07	114
3-methyl 1-butanol	11.98	70
Pentyl ester butanoic acid	12.99	89
2-Octanone	13.74	71
2-Heptanol	14.59	83
6-methyl 5-heptene-2-one	15.17	108
2-Nonanone	16.41	142
8-Nonen-2-one	17.78	58
1-Heptanol	17.93	70
2-ethyl 1-hexanol	18.71	83
2-Decanone	18.86	71
2-Nonanol	19.3	69
2-methyl propanoic acid	21.11	88
2-Undecanone	21.24	85
3-methyl butanoic acid	23.26	87
methoxy-phenyl-oxime	24.26	151
1,3-diethoxy-1,1,3,3-tetramethyl Disiloxane	24.98	177
1-bromo-1-chloromethyl-1-silacyclohexane	25.95	179
d-Mannose	26.81	147
1,5-bis-trimethylsilyl-3-methylene-1,5-Heptadiene	27.11	73
Falcarinol	27.79	131
3-benzyloxy-2-fluoro- β -hydroxy- Phenethylamine	28.1	122
2-[(trimethylsilyl)oxy]-1- [[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- 9,12,15-Octadecatrienoic acid	28.62	73
1-Pentamethyldisilyloxytridecane	30.15	147

Appendix 10. Average SPME GCMS signal intensities/peak areas (AU) and standard deviations (SD) for main compounds detected from headspace samples of model cheeses.

Flavour Compounds			C1		C2		C3		C4		C5		C6	
			Core	Crust	Core	Crust	Core	Crust	Core	Crust	Core	Crust	Core	Crust
Major Compounds (Peak Area x 10,000 AU)	Ethanol	Mean	205.01	349.88	363.32	330.92	181.34	393.00	1285.31	548.82	698.90	410.91	1456.34	444.98
		SD	50.29	64.45	189.73	269.34	62.03	304.65	447.16	176.59	520.18	519.80	664.39	103.23
	2-Pentanone	Mean	8.36	1.62	16.50	5.04	9.56	2.77	40.18	0.66	23.16	5.63	15.05	0.77
		SD	2.15	0.23	6.57	2.32	1.86	1.40	7.06	0.53	7.53	3.59	3.31	0.08
	2-Methyl-1-Propanol	Mean	1.89	1.87	1.84	3.13	1.66	2.72	5.04	6.26	1.47	1.86	2.96	3.84
		SD	0.89	0.47	0.42	1.16	0.65	0.83	1.65	2.07	0.28	0.98	0.82	1.08
	2-Heptanone	Mean	2.43	0.18	2.19	1.20	2.22	0.67	4.10	0.35	3.72	1.51	3.50	0.37
		SD	0.43	0.05	0.92	0.67	0.39	0.46	0.68	0.34	1.81	1.17	0.17	0.19
	3-Methyl-1-Butanol	Mean	12.52	6.72	14.17	22.85	10.98	21.22	64.59	74.35	23.93	8.01	49.00	46.61
		SD	7.65	1.31	3.28	2.64	3.40	0.47	3.73	8.91	0.42	3.46	5.30	6.63
	2-Nonanone	Mean	1.67	1.80	1.29	2.21	0.84	0.85	2.57	4.83	4.16	7.80	1.48	4.01
		SD	1.06	0.54	0.87	0.74	0.58	0.16	0.71	1.12	1.32	5.72	0.30	1.88
	8-Nonen-2-one	Mean	0.54	0.06	0.35	0.44	0.32	0.04	0.98	0.37	1.45	1.20	0.97	0.51
		SD	0.19	0.10	0.25	0.04	0.24	0.04	0.14	0.16	0.45	1.13	0.23	0.32
	2-Methyl Propanoic Acid	Mean	1.24	0.53	1.52	1.12	1.28	0.64	4.38	5.00	1.25	2.94	2.45	3.75
		SD	0.20	0.32	0.75	0.17	1.06	0.28	1.00	2.58	0.60	2.79	1.49	2.13
	3-Methyl Butanoic Acid	Mean	4.33	2.54	3.00	3.87	2.36	3.33	8.45	9.16	5.24	7.88	10.12	9.52
		SD	2.78	0.78	1.74	0.99	1.57	2.07	0.94	3.53	2.56	6.24	5.27	4.71

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Flavour Compounds			C1		C2		C3		C4		C5		C6	
			Core	Crust	Core	Crust	Core	Crust	Core	Crust	Core	Crust	Core	Crust
Minor Compounds (Peak Area x 100 AU)	2-Heptanol	Mean	4.88	33.22	2.22	26.95	0.00	13.79	11.34	65.12	7.96	160.74	0.00	50.09
		SD	5.61	8.97	3.84	33.43	0.00	15.30	8.96	35.64	6.37	87.14	0.00	14.81
	2-Undecanone	Mean	4.99	47.40	3.14	13.82	0.90	0.00	4.18	271.62	8.54	233.56	0.00	106.24
		SD	4.39	33.34	5.43	17.52	1.57	0.00	7.24	42.19	5.86	97.14	0.00	28.15
	2-Nonanol	Mean	2.28	140.84	0.75	72.11	0.00	26.89	0.00	342.58	2.01	439.70	0.00	195.33
		SD	3.96	38.49	1.29	112.88	0.00	25.40	0.00	157.07	1.86	246.79	0.00	15.56
	Hexanal	Mean	3.90	1.45	4.67	4.12	4.04	4.34	7.77	0.00	6.41	6.60	1.58	0.00
		SD	0.64	1.37	1.26	4.53	3.54	1.71	1.40	0.00	6.41	8.99	2.73	0.00
	Pentyl Ester Butanoic Acid	Mean	0.53	0.84	0.00	6.50	0.00	3.63	4.33	29.25	0.73	0.00	1.09	19.98
		SD	0.92	1.46	0.00	5.12	0.00	3.69	6.73	12.75	1.27	0.00	0.94	5.68
	2-Octanone	Mean	3.02	0.00	0.23	5.31	1.39	0.00	9.98	0.00	7.20	6.18	1.15	0.93
		SD	3.97	0.00	0.40	9.20	1.43	0.00	2.40	0.00	3.15	8.21	1.18	1.62
	6-Methyl-5-Heptene-2-one	Mean	1.43	0.26	1.13	0.47	0.00	0.33	3.02	0.66	1.10	0.00	0.27	1.19
		SD	1.50	0.45	1.18	0.42	0.00	0.57	0.57	1.15	1.08	0.00	0.47	0.47
	1-Heptanol	Mean	2.32	0.00	0.00	3.78	0.00	2.21	5.00	50.95	3.52	2.28	1.74	30.75
		SD	2.87	0.00	0.00	6.55	0.00	3.83	8.66	21.97	3.18	3.96	1.59	4.65
	2-Ethyl-1-Hexanol	Mean	0.00	0.00	1.38	4.85	3.82	2.52	4.25	6.59	1.54	5.23	5.57	3.36
		SD	0.00	0.00	2.39	6.71	4.55	2.58	3.96	5.60	2.66	7.81	2.10	3.58
	2-Decanone	Mean	0.69	2.64	0.00	0.51	0.00	0.00	0.00	11.69	1.65	16.22	0.00	8.08
		SD	1.20	3.29	0.00	0.89	0.00	0.00	0.00	1.10	1.69	12.38	0.00	3.81

Appendix 11. ANOVA of main flavour compounds:

a. Ethanol

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	349.75
Model	3	4.41E+06	1.47E+06	12.01	< 0.0001	Mean	555.73
Y	1	7.33E+05	7.33E+05	5.99	0.0201	C.V.	62.94
Z	1	2.28E+06	2.28E+06	18.66	0.0001	PRESS	4.95E+06
YZ	1	1.39E+06	1.39E+06	11.38	0.002	R-Squared	0.5296
Residual	32	3.91E+06	1.22E+05			Adj R-Squared	0.4855
Lack of Fit	8	1.04E+06	1.30E+05	1.09	0.4038	Pred R-Squared	0.4047
Pure Error	24	2.87E+06	1.20E+05			Adeq Precision	7.694
Corrected Total	35	8.32E+06					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	555.73	1	58.29	436.99	674.46	
Y	-142.64	1	58.29	-261.38	-23.91	1
Z	251.82	1	58.29	133.08	370.55	1
YZ	-196.66	1	58.29	-315.4	-77.93	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	2186.2	1146.85	1039.35	0.111	3.152	0.31	3.736 *	33
2	420.47	357.93	62.53	0.111	0.19	0.001	0.187	4
3	1533.42	1146.85	386.57	0.111	1.172	0.043	1.179	21
4	404.34	468.24	-63.9	0.111	-0.194	0.001	-0.191	24
5	1553.41	1146.85	406.56	0.111	1.233	0.048	1.243	23
6	1296.08	1146.85	149.23	0.111	0.453	0.006	0.447	35
7	234.85	249.89	-15.04	0.111	-0.046	0	-0.045	3
8	146.95	249.89	-102.94	0.111	-0.312	0.003	-0.308	5
9	1010.27	468.24	542.04	0.111	1.644	0.084	1.691	28
10	1210.79	1146.85	63.94	0.111	0.194	0.001	0.191	29
11	347.57	249.89	97.68	0.111	0.296	0.003	0.292	7
12	93.06	357.93	-264.88	0.111	-0.803	0.02	-0.799	8
13	560.43	249.89	310.54	0.111	0.942	0.028	0.94	11
14	335.01	357.93	-22.92	0.111	-0.07	0	-0.068	6
15	496.45	468.24	28.21	0.111	0.086	0	0.084	22
16	64.32	357.93	-293.61	0.111	-0.89	0.025	-0.887	14
17	448.75	357.93	90.81	0.111	0.275	0.002	0.271	16
18	250.25	249.89	0.36	0.111	0.001	0	0.001	17
19	623.38	357.93	265.44	0.111	0.805	0.02	0.8	12
20	83.62	468.24	-384.62	0.111	-1.166	0.043	-1.173	30
21	715.12	1146.85	-431.73	0.111	-1.309	0.054	-1.325	25

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22	531.14	468.24	62.9	0.111	0.191	0.001	0.188	36
23	138.84	468.24	-329.4	0.111	-0.999	0.031	-0.999	26
24	294.17	357.93	-63.76	0.111	-0.193	0.001	-0.19	2
25	129.94	249.89	-119.95	0.111	-0.364	0.004	-0.359	13
26	886.73	1146.85	-260.12	0.111	-0.789	0.019	-0.784	31
27	276.32	357.93	-81.61	0.111	-0.248	0.002	-0.244	10
28	233.23	249.89	-16.66	0.111	-0.051	0	-0.05	1
29	181.95	249.89	-67.94	0.111	-0.206	0.001	-0.203	9
30	163.85	249.89	-86.04	0.111	-0.261	0.002	-0.257	15
31	665.93	357.93	308	0.111	0.934	0.027	0.932	18
32	330.57	468.24	-137.67	0.111	-0.417	0.005	-0.412	34
33	745.68	468.24	277.44	0.111	0.841	0.022	0.837	20
34	769.1	1146.85	-377.75	0.111	-1.146	0.041	-1.151	19
35	473.24	468.24	5	0.111	0.015	0	0.015	32
36	170.81	1146.85	-976.04	0.111	-2.96	0.274	-3.419	27

*Case(s) with |Outlier T| > 3.50

b. 3-Methyl-1-Butanol

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	6.8
Model	5	16867.03	3373.41	72.97	< 0.0001	Mean	29.58
X	2	3086.23	1543.11	33.38	< 0.0001	C.V.	22.99
Z	1	7922.97	7922.97	171.37	< 0.0001	PRESS	1997.27
XZ	2	5857.83	2928.92	63.35	< 0.0001	R-Squared	0.924
Residual	30	1386.99	46.23			Adj R-Squared	0.9114
Lack of Fit	6	852.26	142.04	6.38	0.0004	Pred R-Squared	0.8906
Pure Error	24	534.73	22.28			Adeq Precision	21.562
Corrected Total	35	18254.02					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	29.58	1	1.13	27.26	31.89	
X[1]	9.96	1	1.6	6.69	13.24	
X[2]	-12.34	1	1.6	-15.61	-9.07	
Z	14.84	1	1.13	12.52	17.15	1
X[1]Z	15.09	1	1.6	11.82	18.36	
X[2]Z	-16.11	1	1.6	-19.38	-12.83	

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	44.52	47.8	-3.28	0.167	-0.528	0.009	-0.522	33
2	8.22	9.62	-1.4	0.167	-0.226	0.002	-0.222	4
3	63.11	69.47	-6.36	0.167	-1.025	0.035	-1.026	21
4	70.7	69.47	1.23	0.167	0.198	0.001	0.195	24
5	61.83	69.47	-7.64	0.167	-1.231	0.051	-1.242	23
6	54.86	47.8	7.05	0.167	1.136	0.043	1.142	35
7	9.11	9.62	-0.5	0.167	-0.081	0	-0.08	3
8	7.15	9.62	-2.47	0.167	-0.397	0.005	-0.392	5
9	4.45	15.97	-11.52	0.167	-1.855	0.115	-1.939	28
10	23.53	15.97	7.56	0.167	1.218	0.049	1.229	29
11	10.61	18.51	-7.9	0.167	-1.272	0.054	-1.286	7
12	25.83	18.51	7.32	0.167	1.18	0.046	1.188	8
13	17.07	18.51	-1.44	0.167	-0.232	0.002	-0.228	11
14	6.16	9.62	-3.45	0.167	-0.556	0.01	-0.55	6
15	67.84	69.47	-1.63	0.167	-0.262	0.002	-0.258	22
16	20.7	16.1	4.6	0.167	0.741	0.018	0.735	14
17	21.35	16.1	5.25	0.167	0.846	0.024	0.842	16
18	12.44	16.1	-3.67	0.167	-0.591	0.012	-0.584	17
19	20.81	18.51	2.3	0.167	0.371	0.005	0.365	12
20	8.22	15.97	-7.75	0.167	-1.249	0.052	-1.261	30
21	23.89	15.97	7.92	0.167	1.275	0.054	1.289	25

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22	53.61	47.8	5.81	0.167	0.936	0.029	0.934	36
23	11.36	15.97	-4.61	0.167	-0.743	0.018	-0.737	26
24	5.77	9.62	-3.84	0.167	-0.619	0.013	-0.613	2
25	7.1	16.1	-9.01	0.167	-1.451	0.07	-1.479	13
26	47.61	47.8	-0.19	0.167	-0.031	0	-0.031	31
27	21.91	18.51	3.4	0.167	0.547	0.01	0.541	10
28	21.28	9.62	11.66	0.167	1.879	0.118	1.967	1
29	14.83	18.51	-3.68	0.167	-0.593	0.012	-0.587	9
30	13.41	16.1	-2.69	0.167	-0.434	0.006	-0.428	15
31	21.62	16.1	5.52	0.167	0.889	0.026	0.886	18
32	40.42	47.8	-7.38	0.167	-1.189	0.047	-1.197	34
33	84.5	69.47	15.04	0.167	2.422	0.196	2.655	20
34	68.83	69.47	-0.63	0.167	-0.102	0	-0.101	19
35	45.79	47.8	-2.01	0.167	-0.324	0.003	-0.319	32
36	24.37	15.97	8.4	0.167	1.353	0.061	1.373	27

c. 2-Methyl-1-Propanol

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	1
Model	6	72.09	12.02	12.01	< 0.0001	Mean	2.88
X	2	17.31	8.66	8.65	0.0011	C.V.	34.74
Y	1	5.74	5.74	5.74	0.0233	PRESS	44.72
Z	1	17.32	17.32	17.31	0.0003	R-Squared	0.713
XZ	2	31.71	15.86	15.85	< 0.0001	Adj R-Squared	0.6536
Residual	29	29.02	1			Pred R-Squared	0.5577
Lack of Fit	5	2	0.4	0.35	0.8743	Adeq Precision	10.849
Pure Error	24	27.02	1.13				
Corrected Total	35	101.11					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.88	1	0.17	2.54	3.22	
X[1]	0.89	1	0.24	0.41	1.37	
X[2]	-0.8	1	0.24	-1.29	-0.32	
Y	0.4	1	0.17	0.058	0.74	1
Z	0.69	1	0.17	0.35	1.03	1
X[1]Z	1.19	1	0.24	0.71	1.67	
X[2]Z	-1.1	1	0.24	-1.58	-0.62	

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	2.77	3	-0.23	0.194	-0.256	0.002	-0.252	33
2	1.58	2.28	-0.7	0.194	-0.783	0.021	-0.778	4
3	6.56	5.25	1.3	0.194	1.453	0.073	1.483	21
4	6.32	6.05	0.26	0.194	0.293	0.003	0.288	24
5	3.29	5.25	-1.96	0.194	-2.186	0.165	-2.35	23
6	3.86	3	0.86	0.194	0.955	0.031	0.954	35
7	1.15	1.48	-0.33	0.194	-0.368	0.005	-0.362	3
8	1.65	1.48	0.17	0.194	0.186	0.001	0.183	5
9	2.67	2.07	0.6	0.194	0.667	0.015	0.661	28
10	1.16	1.27	-0.11	0.194	-0.123	0.001	-0.121	29
11	1.93	2.08	-0.15	0.194	-0.168	0.001	-0.165	7
12	2.01	2.88	-0.88	0.194	-0.979	0.033	-0.978	8
13	1.39	2.08	-0.7	0.194	-0.777	0.021	-0.771	11
14	2.42	2.28	0.14	0.194	0.153	0.001	0.15	6
15	4.17	6.05	-1.89	0.194	-2.1	0.152	-2.241	22
16	1.76	2.59	-0.83	0.194	-0.921	0.029	-0.919	14
17	3.2	2.59	0.61	0.194	0.678	0.016	0.672	16
18	1.7	1.79	-0.092	0.194	-0.103	0	-0.101	17
19	4.32	2.88	1.44	0.194	1.599	0.088	1.645	12
20	0.76	2.07	-1.3	0.194	-1.451	0.073	-1.481	30
21	1.62	1.27	0.35	0.194	0.394	0.005	0.388	25

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22	5.08	3.8	1.28	0.194	1.429	0.07	1.457	36
23	2.15	2.07	0.081	0.194	0.09	0	0.089	26
24	1.62	2.28	-0.67	0.194	-0.742	0.019	-0.736	2
25	1	1.79	-0.79	0.194	-0.885	0.027	-0.882	13
26	2.26	3	-0.74	0.194	-0.828	0.024	-0.823	31
27	3.06	2.88	0.17	0.194	0.193	0.001	0.19	10
28	2.88	1.48	1.4	0.194	1.554	0.083	1.595	1
29	2.2	2.08	0.12	0.194	0.131	0.001	0.129	9
30	2.3	1.79	0.51	0.194	0.564	0.011	0.557	15
31	3.19	2.59	0.6	0.194	0.667	0.015	0.661	18
32	3.11	3.8	-0.69	0.194	-0.766	0.02	-0.761	34
33	8.3	6.05	2.25	0.194	2.507	0.217	2.784	20
34	5.28	5.25	0.029	0.194	0.033	0	0.032	19
35	3.32	3.8	-0.48	0.194	-0.535	0.01	-0.528	32
36	1.65	1.27	0.38	0.194	0.423	0.006	0.417	27

d. 3-Methyl Butanoic Acid

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	3.02
Model	1	239.56	239.56	26.3	< 0.0001	Mean	5.82
Z	1	239.56	239.56	26.3	< 0.0001	C.V.	51.87
Residual	34	309.7	9.11			PRESS	347.21
Lack of Fit	10	54.04	5.4	0.51	0.8682	R-Squared	0.4362
Pure Error	24	255.66	10.65			Adj R-Squared	0.4196
Corrected Total	35	549.26				Pred R-Squared	0.3679
						Adeq Precision	7.253

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.82	1	0.5	4.8	6.84	
Z	2.58	1	0.5	1.56	3.6	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	7.85	8.4	-0.55	0.056	-0.188	0.001	-0.185	33
2	2.51	3.24	-0.73	0.056	-0.248	0.002	-0.245	4
3	9.06	8.4	0.66	0.056	0.225	0.001	0.222	21
4	12.97	8.4	4.57	0.056	1.557	0.071	1.592	24
5	8.93	8.4	0.53	0.056	0.182	0.001	0.179	23

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6	16.15	8.4	7.75	0.056	2.643	0.205	2.921	35
7	4.58	3.24	1.34	0.056	0.457	0.006	0.452	3
8	1.44	3.24	-1.8	0.056	-0.614	0.011	-0.608	5
9	14.7	8.4	6.3	0.056	2.149	0.136	2.278	28
10	5.43	8.4	-2.97	0.056	-1.012	0.03	-1.012	29
11	1.8	3.24	-1.44	0.056	-0.492	0.007	-0.486	7
12	5.01	3.24	1.77	0.056	0.604	0.011	0.599	8
13	2.2	3.24	-1.04	0.056	-0.353	0.004	-0.349	11
14	1.77	3.24	-1.47	0.056	-0.501	0.007	-0.495	6
15	8.52	8.4	0.12	0.056	0.041	0	0.04	22
16	1.35	3.24	-1.89	0.056	-0.645	0.012	-0.639	14
17	5.48	3.24	2.24	0.056	0.765	0.017	0.76	16
18	3.63	3.24	0.4	0.056	0.135	0.001	0.133	17
19	3.2	3.24	-0.039	0.056	-0.013	0	-0.013	12
20	2.46	8.4	-5.93	0.056	-2.023	0.12	-2.125	30
21	2.59	8.4	-5.81	0.056	-1.981	0.115	-2.075	25
22	13.18	8.4	4.78	0.056	1.631	0.078	1.673	36
23	6.49	8.4	-1.91	0.056	-0.651	0.012	-0.645	26
24	3.34	3.24	0.099	0.056	0.034	0	0.033	2
25	0.6	3.24	-2.64	0.056	-0.899	0.024	-0.897	13
26	6.38	8.4	-2.02	0.056	-0.689	0.014	-0.684	31
27	3.41	3.24	0.17	0.056	0.058	0	0.057	10
28	6.99	3.24	3.75	0.056	1.278	0.048	1.29	1

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29	4.99	3.24	1.75	0.056	0.597	0.01	0.591	9
30	2.84	3.24	-0.4	0.056	-0.136	0.001	-0.134	15
31	3.16	3.24	-0.079	0.056	-0.027	0	-0.026	18
32	4.22	8.4	-4.18	0.056	-1.426	0.06	-1.449	34
33	6	8.4	-2.4	0.056	-0.817	0.02	-0.813	20
34	7.37	8.4	-1.03	0.056	-0.35	0.004	-0.346	19
35	11.18	8.4	2.78	0.056	0.947	0.026	0.946	32
36	7.7	8.4	-0.7	0.056	-0.237	0.002	-0.234	27

e. 2-Methyl Propanoic Acid

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	1.39
Model	5	66.31	13.26	6.85	0.0002	Mean	2.18
X	2	7.37	3.68	1.9	0.1667	C.V.	63.96
Z	1	45.14	45.14	23.32	< 0.0001	PRESS	83.63
XZ	2	13.81	6.9	3.57	0.0408	R-Squared	0.5331
Residual	30	58.08	1.94			Adj R-Squared	0.4553
Lack of Fit	6	9.05	1.51	0.74	0.6237	Pred R-Squared	0.3277
Pure Error	24	124.39	2.04			Adeq Precision	6.698
Corrected Total	35	49.02					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.18	1	0.23	1.7	2.65	
X[1]	0.61	1	0.33	-0.058	1.28	
X[2]	-0.47	1	0.33	-1.14	0.2	
Z	1.12	1	0.23	0.65	1.59	1
X[1]Z	0.78	1	0.33	0.11	1.45	
X[2]Z	-0.73	1	0.33	-1.4	-0.062	

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	1.86	3.1	-1.24	0.167	-0.976	0.032	-0.976	33
2	0.26	0.89	-0.63	0.167	-0.495	0.008	-0.488	4
3	4.93	4.69	0.24	0.167	0.193	0.001	0.19	21
4	7.51	4.69	2.82	0.167	2.22	0.164	2.387	24
5	4.98	4.69	0.29	0.167	0.227	0.002	0.223	23
6	4.14	3.1	1.04	0.167	0.819	0.022	0.815	35
7	1.22	0.89	0.33	0.167	0.263	0.002	0.259	3
8	1.45	0.89	0.56	0.167	0.444	0.007	0.437	5
9	6.15	2.1	4.05	0.167	3.188	0.339	3.854 *	28
10	1.5	2.1	-0.6	0.167	-0.469	0.007	-0.463	29
11	0.66	1.32	-0.66	0.167	-0.523	0.009	-0.517	7
12	1.3	1.32	-0.02	0.167	-0.016	0	-0.016	8
13	2.01	1.32	0.69	0.167	0.541	0.01	0.535	11
14	0.44	0.89	-0.44	0.167	-0.349	0.004	-0.344	6
15	5.14	4.69	0.45	0.167	0.354	0.004	0.348	22
16	0.41	0.96	-0.55	0.167	-0.434	0.006	-0.428	14
17	0.95	0.96	-8.49E-03	0.167	-0.007	0	-0.007	16
18	1.23	0.96	0.27	0.167	0.211	0.001	0.208	17
19	0.97	1.32	-0.35	0.167	-0.276	0.003	-0.272	12
20	1.01	2.1	-1.09	0.167	-0.858	0.025	-0.854	30
21	0.56	2.1	-1.53	0.167	-1.208	0.049	-1.218	25

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22	5.66	3.1	2.56	0.167	2.018	0.136	2.135	36
23	1.68	2.1	-0.42	0.167	-0.33	0.004	-0.325	26
24	0.88	0.89	-5.27E-04	0.167	0	0	0	2
25	0.25	0.96	-0.71	0.167	-0.558	0.01	-0.552	13
26	1.34	3.1	-1.76	0.167	-1.386	0.064	-1.408	31
27	1.09	1.32	-0.23	0.167	-0.182	0.001	-0.179	10
28	1.06	0.89	0.17	0.167	0.137	0.001	0.135	1
29	1.9	1.32	0.58	0.167	0.456	0.007	0.45	9
30	2.36	0.96	1.4	0.167	1.104	0.041	1.108	15
31	0.56	0.96	-0.4	0.167	-0.316	0.003	-0.311	18
32	1.46	3.1	-1.64	0.167	-1.29	0.055	-1.305	34
33	2.35	4.69	-2.34	0.167	-1.846	0.114	-1.928	20
34	3.23	4.69	-1.46	0.167	-1.147	0.044	-1.153	19
35	4.13	3.1	1.03	0.167	0.814	0.022	0.809	32
36	1.69	2.1	-0.41	0.167	-0.323	0.003	-0.318	27

*Case(s) with |Outlier T| > 3.50

f. 2-Heptanone

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	0.77
Model	3	58.34	19.45	32.65	< 0.0001	Mean	1.87
Y	1	48.21	48.21	80.95	< 0.0001	C.V.	41.27
Z	1	5.47	5.47	9.19	0.0048	PRESS	24.12
YZ	1	4.65	4.65	7.82	0.0087	R-Squared	0.7538
Residual	32	19.06	0.6			Adj R-Squared	0.7307
Lack of Fit	8	4.84	0.61	1.02	0.4466	Pred R-Squared	0.6884
Pure Error	24	14.22	0.59			Adeq Precision	12.029
Corrected Total	35	77.4					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.87	1	0.13	1.61	2.13	
Y	-1.16	1	0.13	-1.42	-0.9	1
Z	0.39	1	0.13	0.13	0.65	1
YZ	-0.36	1	0.13	-0.62	-0.098	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	3.37	3.78	-0.41	0.111	-0.564	0.01	-0.558	33
2	0.22	0.68	-0.47	0.111	-0.64	0.013	-0.634	4
3	3.54	3.78	-0.23	0.111	-0.322	0.003	-0.317	21
4	0.73	0.74	-0.015	0.111	-0.021	0	-0.021	24
5	4.85	3.78	1.07	0.111	1.477	0.068	1.506	23
6	3.45	3.78	-0.33	0.111	-0.455	0.006	-0.449	35
7	2.74	2.28	0.46	0.111	0.631	0.012	0.625	3
8	2.61	2.28	0.33	0.111	0.453	0.006	0.447	5
9	0.63	0.74	-0.11	0.111	-0.153	0.001	-0.151	28
10	3.54	3.78	-0.24	0.111	-0.331	0.003	-0.326	29
11	1.54	2.28	-0.74	0.111	-1.02	0.033	-1.021	7
12	1.71	0.68	1.03	0.111	1.412	0.062	1.435	8
13	3.24	2.28	0.97	0.111	1.326	0.055	1.343	11
14	0.13	0.68	-0.55	0.111	-0.76	0.018	-0.754	6
15	0.09	0.74	-0.65	0.111	-0.898	0.025	-0.895	22
16	1.2	0.68	0.51	0.111	0.706	0.016	0.7	14
17	0.38	0.68	-0.3	0.111	-0.409	0.005	-0.404	16
18	2.4	2.28	0.12	0.111	0.165	0.001	0.162	17
19	0.45	0.68	-0.24	0.111	-0.325	0.003	-0.32	12
20	1.06	0.74	0.32	0.111	0.439	0.006	0.433	30
21	5.62	3.78	1.84	0.111	2.535	0.201	2.791	25

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22	0.55	0.74	-0.19	0.111	-0.263	0.002	-0.259	36
23	2.83	0.74	2.09	0.111	2.872	0.258	3.281	26
24	0.2	0.68	-0.49	0.111	-0.668	0.014	-0.662	2
25	1.77	2.28	-0.5	0.111	-0.693	0.015	-0.687	13
26	3.7	3.78	-0.078	0.111	-0.107	0	-0.105	31
27	1.44	0.68	0.76	0.111	1.04	0.034	1.041	10
28	1.93	2.28	-0.34	0.111	-0.472	0.007	-0.466	1
29	1.79	2.28	-0.49	0.111	-0.67	0.014	-0.664	9
30	2.48	2.28	0.2	0.111	0.28	0.002	0.275	15
31	0.42	0.68	-0.26	0.111	-0.356	0.004	-0.351	18
32	0.17	0.74	-0.57	0.111	-0.79	0.02	-0.785	34
33	0.23	0.74	-0.52	0.111	-0.71	0.016	-0.704	20
34	3.91	3.78	0.13	0.111	0.184	0.001	0.181	19
35	0.4	0.74	-0.35	0.111	-0.476	0.007	-0.47	32
36	2.02	3.78	-1.76	0.111	-2.418	0.183	-2.633	27

g. 2-Nonanone

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	2.05
Model	2	88.33	44.16	10.54	0.0003	Mean	2.79
Y	1	22.63	22.63	5.4	0.0264	C.V.	73.26
Z	1	65.7	65.7	15.69	0.0004	PRESS	164.49
Residual	33	138.22	4.19			R-Squared	0.3899
Lack of Fit	9	52.27	5.81	1.62	0.1652	Adj R-Squared	0.3529
Pure Error	24	85.95	3.58			Pred R-Squared	0.2739
Corrected Total	35	226.55				Adeq Precision	7.257

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.79	1	0.34	2.1	3.49	
Y	0.79	1	0.34	0.099	1.49	1
Z	1.35	1	0.34	0.66	2.04	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	1.14	3.35	-2.21	0.083	-1.127	0.039	-1.132	33
2	2.23	2.24	-3.72E-03	0.083	-0.002	0	-0.002	4
3	2.27	3.35	-1.08	0.083	-0.553	0.009	-0.547	21
4	5.98	4.94	1.05	0.083	0.534	0.009	0.528	24
5	3.39	3.35	0.037	0.083	0.019	0	0.019	23
6	1.69	3.35	-1.66	0.083	-0.847	0.022	-0.843	35
7	1.14	0.65	0.49	0.083	0.249	0.002	0.245	3
8	0.97	0.65	0.32	0.083	0.163	0.001	0.16	5
9	3.23	4.94	-1.71	0.083	-0.872	0.023	-0.869	28
10	3	3.35	-0.35	0.083	-0.178	0.001	-0.176	29
11	0.47	0.65	-0.17	0.083	-0.089	0	-0.088	7
12	2.07	2.24	-0.16	0.083	-0.082	0	-0.081	8
13	2.2	0.65	1.55	0.083	0.794	0.019	0.789	11
14	1.2	2.24	-1.03	0.083	-0.528	0.008	-0.522	6
15	4.78	4.94	-0.16	0.083	-0.082	0	-0.081	22
16	0.67	2.24	-1.56	0.083	-0.797	0.019	-0.792	14
17	0.97	2.24	-1.26	0.083	-0.644	0.013	-0.638	16
18	1.2	0.65	0.55	0.083	0.283	0.002	0.279	17
19	3.01	2.24	0.78	0.083	0.397	0.005	0.392	12
20	5.97	4.94	1.03	0.083	0.525	0.008	0.52	30
21	5.6	3.35	2.25	0.083	1.149	0.04	1.155	25

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22	4.86	4.94	-0.081	0.083	-0.041	0	-0.041	36
23	14.22	4.94	9.28	0.083	4.737	0.68	8.245 *	26
24	1.97	2.24	-0.26	0.083	-0.135	0.001	-0.133	2
25	0.16	0.65	-0.49	0.083	-0.249	0.002	-0.245	13
26	1.6	3.35	-1.75	0.083	-0.892	0.024	-0.889	31
27	1.55	2.24	-0.69	0.083	-0.352	0.004	-0.347	10
28	2.89	0.65	2.24	0.083	1.143	0.04	1.149	1
29	1.18	0.65	0.53	0.083	0.272	0.002	0.268	9
30	1.14	0.65	0.49	0.083	0.251	0.002	0.247	15
31	0.92	2.24	-1.32	0.083	-0.674	0.014	-0.668	18
32	1.86	4.94	-3.08	0.083	-1.571	0.075	-1.608	34
33	3.74	4.94	-1.2	0.083	-0.612	0.011	-0.606	20
34	2.06	3.35	-1.29	0.083	-0.659	0.013	-0.653	19
35	5.33	4.94	0.39	0.083	0.198	0.001	0.196	32
36	3.89	3.35	0.53	0.083	0.272	0.002	0.268	27

*Case(s) with |Outlier T| > 3.50

h. 2-Pentanone

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	5.25
Model	9	4119.45	457.72	16.59	< 0.0001	Mean	10.78
X	2	251.73	125.86	4.56	0.02	C.V.	48.75
Y	1	2319.63	2319.63	84.07	< 0.0001	PRESS	1375.4
Z	1	432.69	432.69	15.68	0.0005	R-Squared	0.8517
XY	2	248.55	124.28	4.5	0.0209	Adj R-Squared	0.8003
XZ	2	329.96	164.98	5.98	0.0073	Pred R-Squared	0.7156
YZ	1	536.9	536.9	19.46	0.0002	Adeq Precision	13.925
Residual	26	717.42	27.59				
Lack of Fit	2	338.61	169.3	10.73	0.0005		
Pure Error	24	378.81	15.78				
Corrected Total	35	4836.87					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	10.78	1	0.88	8.98	12.57	
X[1]	1.93	1	1.24	-0.61	4.48	
X[2]	1.81	1	1.24	-0.74	4.35	
Yt	-8.03	1	0.88	-9.83	-6.23	1
Z	3.47	1	0.88	1.67	5.27	1
X[1]Y	-3.54	1	1.24	-6.08	-0.99	

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X[2]Y	0.78	1	1.24	-1.77	3.32	
X[1]Z	4.25	1	1.24	1.7	6.79	
X[2]Z	-1.65	1	1.24	-4.2	0.89	
YZ	-3.86	1	0.88	-5.66	-2.06	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	11.65	17.04	-5.39	0.278	-1.207	0.056	-1.219	33
2	1.62	-2.71	4.33	0.278	0.97	0.036	0.969	4
3	40.96	35.84	5.11	0.278	1.145	0.05	1.152	21
4	1.27	4.99	-3.72	0.278	-0.834	0.027	-0.829	24
5	46.82	35.84	10.97	0.278	2.458	0.232	2.751	23
6	15.25	17.04	-1.79	0.278	-0.4	0.006	-0.394	35
7	9.81	12.69	-2.88	0.278	-0.645	0.016	-0.638	3
8	9.37	12.69	-3.32	0.278	-0.744	0.021	-0.737	5
9	3.28	3.29	-3.60E-03	0.278	-0.001	0	-0.001	28
10	14.54	25.51	-10.97	0.278	-2.458	0.232	-2.751	29
11	12.63	14.16	-1.53	0.278	-0.342	0.005	-0.336	7
12	7.12	7.38	-0.27	0.278	-0.06	0	-0.059	8
13	24.09	14.16	9.93	0.278	2.225	0.19	2.425	11
14	1.4	-2.71	4.11	0.278	0.92	0.033	0.917	6
15	0.42	4.99	-4.57	0.278	-1.024	0.04	-1.025	22
16	4.31	4.76	-0.45	0.278	-0.1	0	-0.098	14

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17	1.58	4.76	-3.18	0.278	-0.711	0.019	-0.704	16
18	11.17	7.57	3.6	0.278	0.806	0.025	0.8	17
19	2.54	7.38	-4.85	0.278	-1.086	0.045	-1.089	12
20	3.85	3.29	0.56	0.278	0.125	0.001	0.122	30
21	28.41	25.51	2.9	0.278	0.65	0.016	0.642	25
22	0.7	-1.22	1.93	0.278	0.432	0.007	0.425	36
23	9.77	3.29	6.48	0.278	1.452	0.081	1.485	26
24	1.85	-2.71	4.56	0.278	1.021	0.04	1.022	2
25	9.99	7.57	2.42	0.278	0.542	0.011	0.535	13
26	18.25	17.04	1.21	0.278	0.272	0.003	0.267	31
27	5.46	7.38	-1.92	0.278	-0.431	0.007	-0.424	10
28	5.9	12.69	-6.8	0.278	-1.523	0.089	-1.564	1
29	12.78	14.16	-1.37	0.278	-0.307	0.004	-0.302	9
30	7.52	7.57	-0.056	0.278	-0.012	0	-0.012	15
31	2.42	4.76	-2.34	0.278	-0.524	0.011	-0.516	18
32	0.73	-1.22	1.95	0.278	0.438	0.007	0.431	34
33	0.29	4.99	-4.71	0.278	-1.054	0.043	-1.057	20
34	32.75	35.84	-3.09	0.278	-0.692	0.018	-0.685	19
35	0.86	-1.22	2.08	0.278	0.466	0.008	0.459	32
36	26.55	25.51	1.04	0.278	0.232	0.002	0.228	27

i. 8-Nonen-2-one

Source	DF	Sum of Square	Mean Square	F	Prob > F	Std. Dev.	0.39
Model	4	5.67	1.42	9.49	< 0.0001	Mean	0.6
X	2	1.19	0.6	3.99	0.0287	C.V.	64.25
Y	1	1.01	1.01	6.73	0.0144	PRESS	6.25
Z	1	3.47	3.47	23.25	< 0.0001	R-Squared	0.5505
Residual	31	4.63	0.15			Adj R-Squared	0.4925
Lack of Fit	7	0.93	0.13	0.86	0.5524	Pred R-Squared	0.3938
Pure Error	24	3.71	0.15			Adeq Precision	9.4
Corrected Total	35	10.31					

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.6	1	0.064	0.47	0.73	
X[1]	-0.12	1	0.091	-0.3	0.071	
X[2]	0.26	1	0.091	0.071	0.44	
Y	-0.17	1	0.064	-0.3	-0.036	1
Z	0.31	1	0.064	0.18	0.44	1

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	0.75	0.94	-0.19	0.139	-0.524	0.009	-0.517	33
2	0.17	8.58E-03	0.16	0.139	0.455	0.007	0.449	4
3	0.89	0.96	-0.07	0.139	-0.194	0.001	-0.191	21
4	0.5	0.63	-0.13	0.139	-0.368	0.004	-0.363	24
5	1.14	0.96	0.18	0.139	0.497	0.008	0.491	23
6	0.96	0.94	0.027	0.139	0.076	0	0.074	35
7	0.49	0.34	0.15	0.139	0.404	0.005	0.399	3
8	0.38	0.34	0.039	0.139	0.108	0	0.106	5
9	0.17	1	-0.83	0.139	-2.312	0.172	-2.5	28
10	1.23	1.34	-0.1	0.139	-0.286	0.003	-0.282	29
11	0.079	0.72	-0.64	0.139	-1.773	0.101	-1.84	7
12	0.42	0.38	0.038	0.139	0.106	0	0.104	8
13	0.57	0.72	-0.14	0.139	-0.403	0.005	-0.398	11
14	0	8.58E-03	-8.58E-03	0.139	-0.024	0	-0.024	6
15	0.42	0.63	-0.21	0.139	-0.576	0.011	-0.57	22
16	0.072	-0.018	0.09	0.139	0.251	0.002	0.247	14
17	0	-0.018	0.018	0.139	0.049	0	0.049	16
18	0.42	0.32	0.11	0.139	0.297	0.003	0.293	17
19	0.41	0.38	0.024	0.139	0.068	0	0.067	12
20	1.01	1	9.95E-03	0.139	0.028	0	0.027	30
21	1.97	1.34	0.63	0.139	1.764	0.1	1.83	25

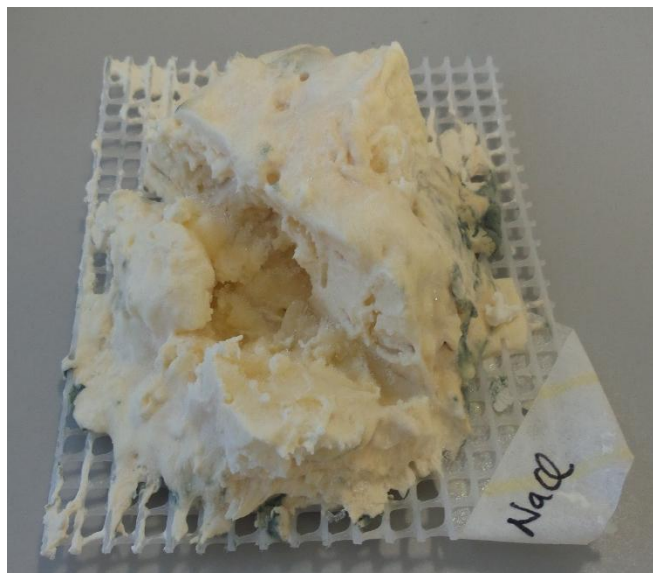
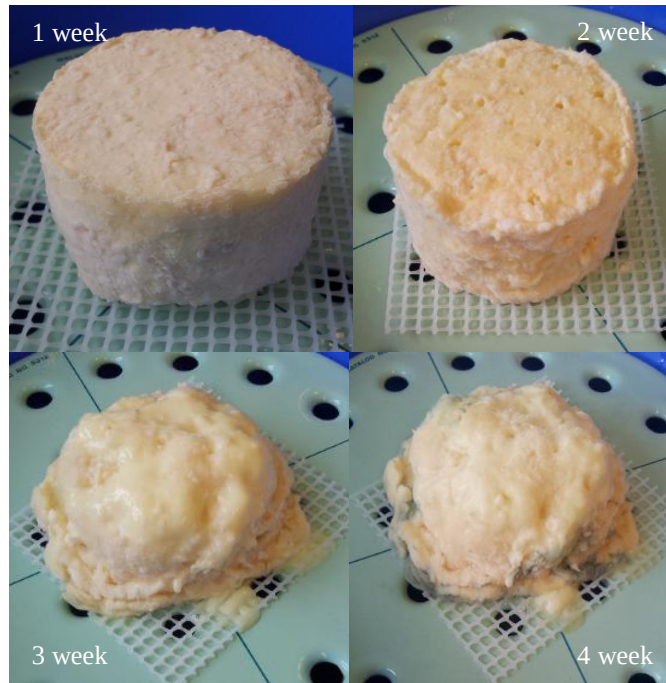
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22	0.45	0.6	-0.15	0.139	-0.424	0.006	-0.418	36
23	2.41	1	1.41	0.139	3.925	0.497	5.445 *	26
24	0	8.58E-03	-8.58E-03	0.139	-0.024	0	-0.024	2
25	0.047	0.32	-0.27	0.139	-0.75	0.018	-0.745	13
26	1.2	0.94	0.26	0.139	0.737	0.018	0.732	31
27	0.49	0.38	0.1	0.139	0.291	0.003	0.286	10
28	0.75	0.34	0.41	0.139	1.132	0.041	1.138	1
29	0.4	0.72	-0.31	0.139	-0.876	0.025	-0.872	9
30	0.5	0.32	0.19	0.139	0.523	0.009	0.517	15
31	0.041	-0.018	0.059	0.139	0.165	0.001	0.162	18
32	0.21	0.6	-0.39	0.139	-1.087	0.038	-1.091	34
33	0.2	0.63	-0.43	0.139	-1.209	0.047	-1.218	20
34	0.89	0.96	-0.072	0.139	-0.201	0.001	-0.198	19
35	0.85	0.6	0.25	0.139	0.687	0.015	0.681	32
36	1.15	1.34	-0.19	0.139	-0.532	0.009	-0.526	27

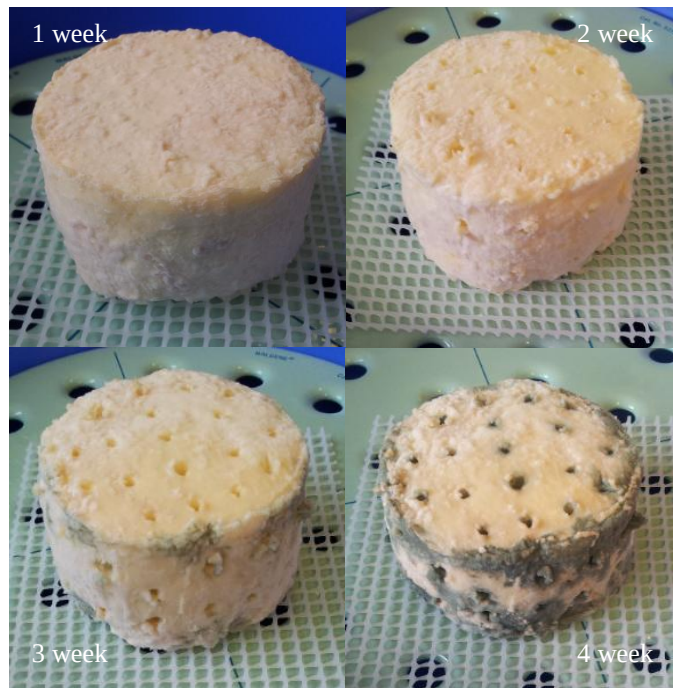
*Case(s) with |Outlier T| > 3.50

Appendix 12. The picture of trial cheese made from raw milk and starter *Lc. lactis* and *P. roqueforti* and the core appearance after 1 month ripened at:

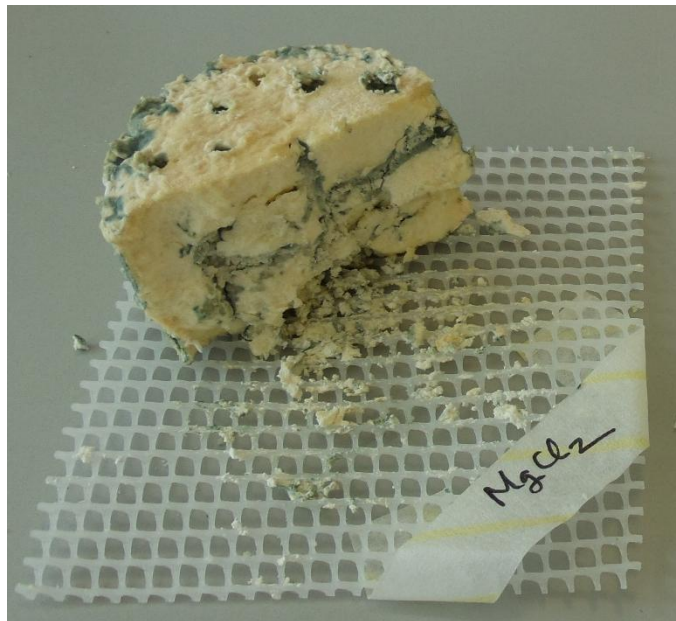
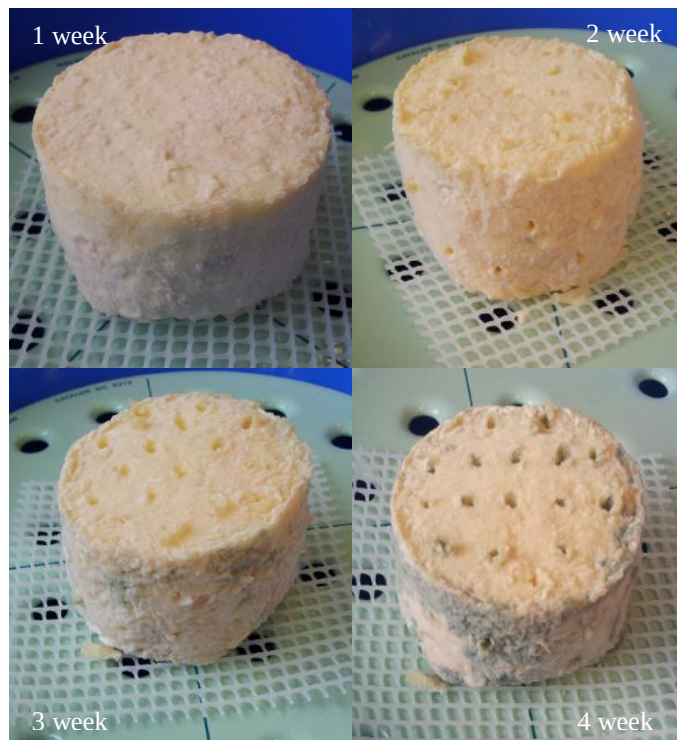
a. Relative Humidity (75%; NaCl)



b. Relative Humidity (53%; MgN₂)



c. Relative Humidity (33%; MgCl_2)



Appendix 13. The hardness of trial cheese made from raw milk compared to control cheeses and model cheese C6.

