

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Presence of coagulase-negative *Staphylococcus* in dairy products

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Science at Massey University, Palmerston North, New Zealand

Jacinda Aplin

2014

Abstract

Species within the genus *Staphylococcus* produce various virulence factors, including staphylococcal enterotoxins. Because of their production of enterotoxins, *Staphylococcus* is the third most common pathogen responsible for outbreaks of food poisoning worldwide. Whereas *Staphylococcus aureus*, a coagulase-positive *Staphylococcus*, is the leading cause of these outbreaks, coagulase-negative staphylococci (CNS) species are also present in food and are able to produce enterotoxins. More specifically, such CNS species have been linked with dairy-related food poisoning outbreaks. However, to date, no research investigating CNS species in New Zealand and their presence in food has been reported. This study therefore sets out to isolate, identify and characterise CNS from New Zealand milk and dairy products, and to evaluate their toxin-producing potential. The results from this study showed that MALDI-TOF MS is a rapid, reliable and accurate method for identifying CNS definitively to the genus level and, on most occasions, to the species level in dairy products and is therefore a potential alternative to the traditional phenotypic, such as commercial identification kits, and genotypic, such as sequencing, methods that are currently used. Of the 42 isolates analysed, none of the CNS isolates tested produced enterotoxin *in vitro*, however, 2 isolates were found to possess an enterotoxin gene. This shows a low propensity for CNS isolates in New Zealand dairy products to be a food safety risk.

Acknowledgements

I would firstly like to thank my supervisors, Dr Denise Lindsay and Dr Jon Palmer, who allowed me to really take charge of this project from the outset, all the while patiently giving me advice, feedback, encouragement, support and their expertise every step of the way.

Secondly, I would like to thank my manager, Dr Steve Holroyd, for supporting me in completing my PGDipSc and MSc over the last three years and allowing me the time to complete my laboratory work. Also to my subsequent manager, Dr Joanna Shepherd, for allowing time to complete my laboratory work as part of the PGDipSc project.

I would also like to thank Fonterra for financially supporting me through my studies over the last 3 years.

I would like to thank the Process Technical Microbiologists, Fonterra, located around New Zealand, for providing the dairy samples, and Analytical Services, Fonterra, for providing the Baird–Parker plate samples for this project.

Finally, I would like to thank Dr Susan Paulin, Institute of Environmental Science and Research Ltd (ESR) for providing the enterotoxin producing *S. aureus* species for this project.

Abbreviations

BHI	brain heart infusion broth
bp	base pair
BP	Baird–Parker agar
CFU/mL	colony forming units per millilitres
CNS	coagulase-negative <i>Staphylococcus</i> /staphylococci
CPS	coagulase-positive <i>Staphylococcus</i> /staphylococci
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
kb	kilobase
kDa	kilodalton
MALDI–TOF MS	matrix-assisted laser desorption ionisation–time of flight mass spectrometry
MSP	main spectrum
m/z	mass to charge ratio
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
<i>rpoB</i>	a gene that encodes the β -subunit of RNA polymerase
SAGs	superantigens
SE(s)	staphylococcal enterotoxin(s)
SEL(s)	staphylococcal-enterotoxin-like superantigens
<i>sodA</i>	a gene that encodes the manganese-dependent superoxide dismutase
subsp.	subspecies
spp.	species
TSA	tryptone soy agar
TSB	tryptone soy broth
TSST-1	toxic shock syndrome toxin 1
<i>tuf</i>	a gene that encodes the elongation factor Tu

Table of Contents

Abstract	ii
Acknowledgements	iii
Abbreviations	iv
List of Figures	vii
List of Tables	ix
CHAPTER ONE: INTRODUCTION	1
1.1 The Genus <i>Staphylococcus</i>	1
1.2 Coagulase-negative <i>Staphylococcus</i> (CNS)	2
1.3 Staphylococcal Enterotoxins (SEs)	2
1.4 Presence in Food	3
1.5 Enterotoxigenic Potential of CNS Species in Food	6
1.6 New Zealand	7
1.7 Protein Profiling – A Novel and Rapid Method for Identifying CNS and their Enterotoxins	9
1.8 Aims of this Investigation	12
CHAPTER TWO: MATERIALS AND METHODS	13
2.1 General Requirements	13
2.2 General Methods	15
2.2.1 Collection of samples and bacterial isolation	15
2.2.2 Positive and negative controls	18
2.2.3 Phenotypic identification	18
2.2.4 MALDI–TOF MS analysis	22
2.2.5 Identification by 16S rDNA sequencing	26
2.2.6 Immunoassay test	28
2.2.7 SE PCR	30
2.2.8 SE multiplex PCR	34
2.2.9 Interpretation of resulting identities	35
CHAPTER THREE: RESULTS AND DISCUSSION	36
3.1 Product Analysis	36
3.2 Identification of Coagulase-negative Species	36
3.2.1 Identification of CNS isolates using the BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI–TOF MS	36
3.2.2 Definitive identification of seven CNS isolates using 16S rDNA sequencing	41

3.2.3 Comparison of MALDI-TOF MS identification and 16S rDNA sequencing	42
3.2.4 Overall examination of identification results	45
3.3 Staphylococcal Enterotoxin	48
3.3.1 Immunoassay test results	48
3.3.2 SE gene identification of seven isolates	48
3.3.3 SE gene identification using multiplex PCR	48
3.3.4 Overall examination of enterotoxin results	53
CHAPTER FOUR: SUMMARISING DISCUSSION AND CONCLUSION	55
4.1.1 Identification of coagulase negative species	55
4.1.2 Staphylococcal enterotoxin potential of CNS species	56
4.2 Conclusions	58
4.3 Limitations of the present study	59
4.4 Future Work	59
CHAPTER FIVE: LIMITATIONS	56
REFERENCES	60

List of Figures

Fig. 1. Baird-Parker agar plate showing typical <i>Staphylococcus aureus</i> colonies (2A) with a visible clearing or halo around the black colony (2B, indicated with arrows).....	16
Fig. 2. Baird-Parker agar plate showing non-typical black colonies (<i>Staphylococcus epidermidis</i>) with no visible clearing or halo.....	17
Fig. 3. Two Biochemical identification test kits used to identify the 42 <i>Staphylococcus</i> species. The BBL™ Crystal™ Gram Positive identification system (A, B), and the Remel RapID™ Staph Plus identification system (C, D).....	19
Fig. 4. The BBL™ Crystal™ Gram positive identification system showing a typical result for a <i>S. aureus</i> species (A) and a coagulase negative species (<i>S. saprophyticus</i> , B).....	20
Fig. 5. Remel RapID™ Staph Plus identification system showing a typical result for a <i>S. aureus</i> species (A) and a coagulase negative species (<i>S. saprophyticus</i> , B).....	21
Fig. 6. The basic principle of Matrix assisted Laser Desorption Ionisation –time of flight mass spectrometry (MALDI-TOF MS).....	23
Fig. 7. The laboratory bench Microflex LT MALDI-TOF MS set-up (A) and the target plate (B) used for placing samples for analysis.....	25
Fig. 8. The Implen NanoPhotometer® P300 used to quantify DNA of the 7 isolates sequenced using 16S rDNA.....	27
Fig. 9. The complete Ridascreen® SET A, B, C, D, E immunoassay test kit.....	29
Fig. 10. The Techne TC-512 thermal cycler used to generate PCR products for SE PCR, multiplex PCR and 16S rDNA sequencing.....	31
Fig. 11. The Invitrogen E-Gel® system used for SE PCR analysis.....	32
Fig. 12. The UVitec Essential V2 used to photograph the gels generated using both the E-Gel system for the SE PCR results and the traditional method for the multiplex PCR results.....	33
Fig. 13. An MSP dendrogram generated by the Bruker Daltonics MALDI Biotyper 3.0 Offline Classification software for seven CNS isolates, for which their identification did not agree among the three methods BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI–TOF MS. Isolate identifications based on 16S rDNA sequencing are as follows: isolates 1 and 2 – <i>S. epidermidis</i> , isolates 3, 4 and 7 – <i>S. pasteurii</i> , isolate 5 – <i>S. saprophyticus</i> , isolate 6 – <i>S. scuii</i> subsp. <i>sciuri</i>	43
Fig. 14. A phylogenetic tree generated by MegAlign using Clustal V alignment of the seven CNS isolates for which the identification did not agree among the three methods (BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI–TOF MS). Bootstrap values > 70% are shown. Isolate identifications based on 16S rDNA sequencing are as follows: isolates 1 and 2 – <i>S. epidermidis</i> , isolates 3, 4 and 7 – <i>S. pasteurii</i> , isolate 5 – <i>S. saprophyticus</i> , isolate 6 – <i>S. scuii</i> subsp. <i>sciuri</i>	44
Fig. 15. Gel electrophoresis of CNS isolates 1-14 screened for SEA-SEE genes using multiplex PCR.....	50
Fig. 16. Gel electrophoresis of CNS isolates 15-28 screened for SEA-SEE genes using multiplex PCR.....	51

Fig. 17. Gel electrophoresis of CNS isolates 29-42 screened for SEA-SEE genes using multiplex PCR.....52

List of Tables

Table 1 Identification of 42 CNS isolates using three methods, i.e. the BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI-TOF MS.....	38
--	----

Chapter One: Introduction

1.1 The Genus *Staphylococcus*

Staphylococcus species are non-motile, non-sporeforming, encapsulated, Gram-positive, catalase-positive, facultatively anaerobic, chemorganotrophic cocci with a respiratory and fermentative metabolism at an optimal temperature of 37°C (Le Loir *et al.* 2003). Staphylococcal cells form spherical single cells, paired cocci, short chains or grape-like clusters (Le Loir *et al.* 2003) and produce opaque white to cream coloured colonies that are 2–3 mm in diameter on uncrowded areas of blood agar.

The *Staphylococcus* genus is widely distributed in nature, and is found living on the skin and mucous membranes of warm-blooded mammals and in environmental sources, i.e. soil, sand, air and water, and is also isolated from protein-based foods, i.e. meat, cheese and milk (Irlinger 2008; Even *et al.* 2010).

Within the genus *Staphylococcus*, there are 42 known species and 24 subspecies (Vasconcelos & Cunha 2010). Various phenotypic and genotypic characteristics, including nuclease production, antibiotic resistance, i.e. methicillin or novobiocin, and DNA similarity, are often used to classify *Staphylococcus* species. However, the classification of the 42 known *Staphylococcus* species is predominantly based on the production of the enzyme, coagulase, into two distinct groups: coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS).

Staphylococcus species possess numerous virulence factors, including lipases, proteases, coagulase, haemolysins (α , β , γ and δ), deoxyribonuclease (DNase), leukocidin, exfoliative toxins A and B, toxic shock syndrome toxin 1 (TSST-1) and a family of emetic pyrogenic superantigens (SAGs) (Bartolomeoli *et al.* 2009). The ability to produce coagulase is typically associated with another virulence factor, the production of enterotoxins. However, CNS isolates, on occasion, have also been shown to produce enterotoxins (Zell *et al.* 2008).

1.2 Coagulase-negative *Staphylococcus* (CNS)

Species of CNS are commonly associated with the cutaneous system (skin and mucosal membranes) of the human body (Vasconcelos & Cunha 2010). Therefore, CNS isolates are frequently encountered in human clinical samples (Gaillot *et al.* 2000). However, these coagulase-negative species are regarded as either a contaminant or a natural part of the human microbiota (Kloos & Bannerman 1994; Çitak *et al.* 2003) and not the primary pathogen causing an illness.

CNS strains can also be used in the food processing industry, contributing to flavour, aroma formation and pigmentation/colour functionalities, particularly in fermented foods (e.g. cheeses and sausages), and include the non-enterotoxin-producing *Staphylococcus carnosus* and *Staphylococcus xylosus* (Irlinger 2008; Coton *et al.* 2010; Even *et al.* 2010; Podkowik *et al.* 2013). The use of these strains of staphylococci in food processing is largely historical; for example, for over 60 years, *S. carnosus* has been used alone or in combination with other microorganisms (pediococci or lactobacilli) as a starter culture for the production of raw sausage (Zell *et al.* 2008). The true extent of the potential and actual virulence of CNS species has not been fully investigated. Therefore, CNS has largely been overlooked as a potential human pathogen, both clinically and in the food industry.

1.3 Staphylococcal Enterotoxins (SEs)

SEs are heat-stable low molecular weight (~ 22–28 kDa) extracellular proteins that belong to a large family of staphylococcal and streptococcal pyrogenic toxic SAGs (Argudín *et al.* 2010). SEs can cause toxic-shock-like syndromes and have been implicated in food poisoning incidences (Balaban & Rasooly, 2000). Therefore, SEs can function as both potent gastrointestinal toxins and SAGs (Balaban & Rasooly, 2000).

Enterotoxins are produced throughout the logarithmic growth phase or during the transition from the exponential phase to the stationary phase (Derzelle *et al.* 2009;

Argudín *et al.* 2010). These toxins are toxic in high nanogram to low microgram quantities (Larkin *et al.* 2009; Argudín *et al.* 2010) and are resistant to conditions that easily destroy the bacteria that produce them, i.e. heat treatment, low pH and proteolytic enzymes. As a result, they retain their activity in the digestive tract after ingestion (Argudín *et al.* 2010). SEs are produced when the water activity is > 0.86 and the temperature is between 10 and 46°C (Colombari *et al.* 2007). The optimum temperature range for the production of the maximum level of toxin is 40–45°C (Colombari *et al.* 2007).

SEs are named according to their emetic activities, i.e. those that induce emesis following oral administration in a primate model (Le Loir *et al.* 2003; Derzelle *et al.* 2009; Argudín *et al.* 2010; Podkowik *et al.* 2013). These include SEA, SEB, SEC (C₁, C₂, C₃, ovine and bovine variants), SED, SEE, SEG, SEH, SEI, SER, SES and SET (Balaban & Rasooly 2000; Derzelle *et al.* 2009; Vasconcelos & Cunha 2010). Enterotoxins that do not have emetic properties or have not yet been tested are designated as staphylococcal-enterotoxin-like superantigen (SEL) proteins and include SELJ, SELK, SELL, SELM, SELN, SELO, SELP, SELQ, SELU and SELV (Derzelle *et al.* 2009; Argudín *et al.* 2010; Vasconcelos & Cunha 2010). The toxic shock staphylococcal toxin, TSST-1, initially designated as SEF, lacks emetic activity and is therefore not classified as a true enterotoxin (Argudín *et al.* 2010).

1.4 Presence in Food

Staphylococcus is the third most common pathogen responsible for outbreaks of food poisoning worldwide (Atanassova *et al.* 2001; Veras *et al.* 2008; Podkowik *et al.* 2013). The main sources of staphylococcal food contamination are:

- improper storage and handling of food contaminated with staphylococci (Udo *et al.* 1999; Atanassova *et al.* 2001; Le Loir *et al.* 2003; Veras *et al.* 2008; Bartolomeoli *et al.* 2009; Argudín *et al.* 2010);
- the use of birds and mammals, i.e. cattle, sheep and goats, that either are non-symptomatic staphylococci carriers or are actively infected (i.e. mastitis)

and their products in the manufacture of food (Atanassova *et al.* 2001; Le Loir *et al.* 2003; Bartolomeoli *et al.* 2009; Hennekinne *et al.* 2009; Argudín *et al.* 2010).

As staphylococcal species are a common commensal of the skin and mucous membranes, i.e. the nose and hands of humans, food handlers can transmit staphylococci via manual contact or through respiratory secretions (Argudín *et al.* 2010; Pinchuk *et al.* 2010). Transmission of staphylococci can occur during the manufacture or cooking of foods (Hennekinne *et al.* 2009). Therefore, this type of transmission is largely associated with cooked or processed foods (Argudín *et al.* 2010).

Le Loir *et al.* (2003) noted that, in all food poisoning cases related to staphylococci, the food or one of the ingredients was contaminated with an SE-producing strain and was exposed to temperatures that allow the bacteria to grow for an extended period of time. Exposure to these inappropriate temperatures is a result of the refrigeration process failing or because a growth-permissive temperature is required during processing, e.g. cheesemaking (Le Loir *et al.* 2003).

Dairy animals with subclinical staphylococcal mastitis are a primary source of contamination, which can result in large numbers being shed into dairy-derived products or ingredients (Bartolomeoli *et al.* 2009; Hennekinne *et al.* 2009; Argudín *et al.* 2010). Staphylococci can also be transferred to foods via air, dust and food contact surfaces (Argudín *et al.* 2010). SEs can be produced by staphylococci in milk or milk products prior to pasteurisation and, although the bacterial cells will be destroyed by this process, the enterotoxins can retain their toxic activity (Bartolomeoli *et al.* 2009). SEs are regarded as heat resistant, with the temperature required for the thermal destruction curve to traverse one log cycle (z value) ranging from 25 to 33°C and the $D_{121^{\circ}\text{C}}$ value (time in minutes at 121°C for 90% destruction) ranging from 8.3 to 34 min (Erickson 2003). However, differences in heat resistance and stability do exist among enterotoxins, i.e. SEC > SEB > SEA and SEE > SEA > SEI, respectively (Erickson 2003). Therefore, heat stability depends on the medium in which the toxin

is present, the pH, the salt concentration and other environmental factors related to the level of toxin denaturation (Balaban & Rasooly 2000). In fact, SEs can show biphasic (faster first initial phase and slower second phase) inactivation at different pHs, and several constituents of foods (e.g. proteins, carbohydrates and chitin) can increase the thermal stability of SEs by up to fivefold, e.g. the time required to inactivate SEB in milk (30 µg/mL) at 100°C is 1.3 times greater than that in Veronal buffer (Erickson 2003).

Staphylococcal food poisoning is the consumption of food containing sufficient amounts of one or more enterotoxins to result in intoxication (Wieneke *et al.* 1993; Argudín *et al.* 2010). The symptoms have a rapid onset (2–4 h) and include nausea, vomiting and abdominal cramping with or without diarrhoea (Atanassova *et al.* 2001; Le Loir *et al.* 2003; Argudín *et al.* 2010). This disease is usually self-limiting and the symptoms typically resolve within 24–48 h after onset (Le Loir *et al.* 2003; Argudín *et al.* 2010). Incidences that require hospitalisation are normally associated with infants or the elderly (Argudín *et al.* 2010).

Foods often associated with staphylococcal contamination and intoxication include meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products (particularly cream-filled pastries and cakes) and sandwich fillings (Veras *et al.* 2008; Argudín *et al.* 2010). Therefore, the foods at greatest risk are those in which the normal bacterial flora has been destroyed, e.g. cooked meats, or inhibited, e.g. cured, salted meats (Institute of Environmental Science and Research Limited 2001). Staphylococci therefore grow well in cooked foods that are high in protein, sugar or salt, foods that are low in acid or foods with moist fillings (Institute of Environmental Science and Research Limited 2001). However, the origins of staphylococcal food poisoning differ widely among countries. Le Loir *et al.* (2003) suggest that this is due to the differences in consumption and food habits in different countries.

SEA alone or together with other SEs/SELs is the most commonly reported enterotoxin in food and is considered to be the main cause of *Staphylococcus aureus*

food poisoning (Vernozy-Rozand *et al.* 2004; Veras *et al.* 2008; Argudín *et al.* 2010). For example, in Japan in 2000, SEA was implicated in a food poisoning outbreak in low fat milk and drinking yoghurt produced from contaminated skim milk powder (Asao *et al.* 2003). When SEA, presumably produced before pasteurisation, was heated three times at 130°C for 2 or 4 s, it still retained immunological and biological activities (Asao *et al.* 2003). SEB, SEC and SED alone have also been implicated in staphylococcal food poisoning throughout the world (Argudín *et al.* 2010).

1.5 Enterotoxigenic Potential of CNS Species in Food

CNS are often associated with food. For example, all *Staphylococcus* species isolated from dried cured Iberian ham and from slightly fermented sausages (fuet, chorizo and salchichón) were CNS (Rodríguez *et al.* 1996; Martin *et al.* 2006), with *S. xylosus* being the main species in both these studies.

Several recent studies have shown that some CNS strains do have the ability to produce enterotoxins. For example, a human isolate of *Staphylococcus warneri* (CCRC 12929) was shown to produce both SEA and SED (Chou & Chen 1997). Mhlambi *et al.* (2010) and Naidoo & Lindsay (2010) isolated CNS species from the dried meat *biltong* and showed that they produced enterotoxins. Furthermore, CNS isolated from various foods, including milk and cheese, were shown to possess not only the SEA gene but also the SEC₁ gene (Cunha *et al.* 2006). Although CNS may carry SE genes, often enterotoxins either are not produced *in vitro* or their production is not detected using tests such as enzyme-linked immunosorbent assay and reversed passive latex agglutination (Rodríguez *et al.* 1996; Cunha *et al.* 2006; Veras *et al.* 2010). This could be due to the toxin being produced by CNS below the detection limit of these methods or the non-expression of these SE genes (Cunha *et al.* 2006).

CNS strains that produce enterotoxins have also been found in dairy and related products. For example, CNS were isolated from goats' milk, whey and cheese (lactic and Camembert) (Vernozy-Rozand *et al.* 1996). *S. xylosus* was predominant in

goats' milk, *Staphylococcus saprophyticus* was predominant in whey, *Staphylococcus simulans* was predominant in lactic cheese and *S. xylosus/equorum* was predominant in Camembert-type cheese (Vernozy-Rozand *et al.* 1996). Eleven out of a total of 187 CNS isolated from goats' milk, whey and cheese products were enterotoxigenic, producing SEE (Vernozy-Rozand *et al.* 1996). SEA, SED, SEE and SEH were produced by 35 CNS strains isolated from fermented fish, meat starter culture, sausage, soy, cured ham, cheese (hard and soft) and water buffalo raw milk (Zell *et al.* 2008). Amongst these CNS strains, SED and SEH were identified as the most common toxins and, of the 35 isolates tested, these enterotoxins were produced by five and eight isolates respectively (Zell *et al.* 2008). CNS strains isolated from Minas soft cheese included *S. saprophyticus*, *S. warneri*, *Staphylococcus epidermidis*, *S. xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus schleriferi* subsp. *schleiferi* (Rall *et al.* 2010). The gene coding for SEA was the most frequently found (18.5%), with genes for SEB and SEC also being present (Rall *et al.* 2010). However, none of the CNS isolates isolated from Minas soft cheese produced any of the enterotoxins (SEA, SEB, SEC or SED) *in vitro* (Rall *et al.* 2010). CNS isolated from sheep milk have been shown to produce SEA, SEC, SED and combinations of SEs, i.e. SECD and SEBCD (Bautista *et al.* 1988). The predominant SE produced by these CNS was SED (Bautista *et al.* 1988).

In addition, CNS have been isolated from foods typically associated with CPS food poisoning, e.g. bakery goods, milk, cheese, sandwiches and pork (Cunha *et al.* 2006). In some instances, they have also been implicated as being responsible for food poisoning outbreaks. For example, CNS isolates were responsible for a food poisoning outbreak associated with unpasteurised milk in Brazil and involved SEC and SED (Do Carmo *et al.* 2004). CNS strains isolated from Minas soft cheese included *S. saprophyticus*, *S. warneri*, *S. epidermidis*, *S. xylosus*, *S. haemolyticus* and *S. schleriferi* subsp. *schleiferi* (Veras *et al.* 2008). The most frequently identified SE gene produced by these CNS was *sea* (18.5%) (Veras *et al.* 2008). Therefore, CNS could play a greater role in SE food poisoning outbreaks than presently thought.

1.6 New Zealand

SE outbreaks have been reported in New Zealand, with yoghurt, hot ham sandwiches, Christmas hams and freeze-dried trampers' meals being implicated (Institute of Environmental Science and Research Limited 2001). However, research on the presence of *S. aureus* in New Zealand has largely centred on strains from clinical and bovine origin (Jarvis & Lawrence 1970, 1971; Grinberg *et al.* 2004) and their enterotoxin production (Jarvis & Lawrence 1970, 1971). Jarvis & Lawrence found that 11 of 35 clinical *Staphylococcus* strains were enterotoxigenic and noted that this was similar to that reported in the United States. However, the predominant enterotoxin was SEB (seven of the 35 clinical strains (20%)), compared with SEA being reported as the leading cause of food poisoning in the United States at the time (Jarvis & Lawrence 1970). Although this same study found that no bovine strains produced SEA, SEB or SEC, it was limited in terms of the number of enterotoxins tested for, with only SEA, SEB and SEC being investigated. This may have been because these were the only SEs identifiable at the time. SED was mentioned but not tested for. As it also appears that strains were not identified to the species level, CNS species may have been included.

Howard (2006) identified and enumerated mastitis pathogens in bulk tank milk from New Zealand dairy cows. Those identified included aesculin-positive streptococci, coliforms, *S. aureus* and CNS species. The CNS group was present in 51% of bulk tank milk samples at greater than 1000 CFU/mL compared with 49, 11 and 0% for streptococci, coliforms and *S. aureus* respectively. These results indicate that CNS species are found associated with dairy cows and these results suggest that the use of this raw milk is a potential source of CNS contamination and therefore that enterotoxin production could occur in the manufacturing plant or in the final product if good manufacturing practices are not adhered to. However, this study identified and differentiated *S. aureus* isolates from CNS only by their ability to produce coagulase, catalase and haemolytic colonies. As no biochemical tests or proteomic or molecular methods were used, it is possible that *S. aureus* isolates were misidentified as other coagulase-positive species or CNS and vice versa.

In 2010 Hudson & On reviewed the methods that are used to evaluate CPS and their production of enterotoxin in dairy products and that could be applied by small businesses producing raw milk cheeses in New Zealand. However, to date, no research investigating CNS species in New Zealand and their presence in food has been reported. Therefore, the question remains as to the presence of CNS in milk and dairy products in New Zealand and their potential to produce enterotoxins. Numerous phenotypic and genotypic methods are available to identify and characterise staphylococci and their enterotoxins. However, the rapid method, matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI–TOF MS), has been suggested as a novel bacterial identification methodology that could contribute greatly to improving the study of CNS and their enterotoxin ability.

1.7 Protein Profiling – A Novel and Rapid Method for Identifying CNS and their Enterotoxins

MALDI–TOF MS detects and examines the pattern of proteins, predominantly ribosomal proteins, directly from samples such as intact bacteria (Dupont *et al.* 2010) and, based on their mass-to-charge ratio (m/z), a reproducible spectrum consisting of a series of peaks is produced within minutes (Carbonnelle *et al.* 2007; Dupont *et al.* 2010).

Traditionally, bacterial identification has been based on phenotypic methods, i.e. Gram-staining and biochemical test patterns, and genotypic methods, i.e. *rpoB* and *sodA* gene sequencing. Although some of these methods are rapid, complete identification by many traditional methods can take from hours to days (Seng *et al.* 2009). MALDI–TOF MS was first proposed as a method for identifying bacteria over 30 years ago (Anhalt & Fenselau 1975; Seng *et al.* 2009). However, it is only now gaining ground as a rapid, inexpensive, reliable and accurate method for bacterial identification (Rajakaruna *et al.* 2009; Seng *et al.* 2009; Dubois *et al.* 2010). This re-emergence is due to the advances in the technology, improvements in the sample preparation and analysis of larger biomolecules (Croxatto *et al.* 2012; Wieser *et al.*

2012). MALDI–TOF MS could therefore replace these phenotypic methods and could rival genotypic methods as the gold standard for the identification of bacteria (Carbonnelle *et al.* 2007).

The incidence of hospital-acquired infection linked to CNS is increasing and, with this, there is an increase in the need for an accurate identification of staphylococci at the species level (Dupont *et al.* 2010). Most commercial identification kits remain unable to differentiate between the CNS species (Dupont *et al.* 2010). Therefore, the identification of *Staphylococcus* species is a prime example of where MALDI–TOF MS is applicable.

In the last decade, there has been an increase in research into the use of MALDI–TOF MS systems in the identification of bacteria, e.g. Keys *et al.* (2004), Ferreira *et al.* (2011) and Carbonnelle *et al.* (2012). A large number of studies have also used this method to identify *Staphylococcus* species (Bernardo *et al.* 2002; Carbonnelle *et al.* 2007; Rajakaruna *et al.* 2009; Dubois *et al.* 2010; Dupont *et al.* 2010; Fox *et al.* 2010, 2011). These studies compared the ability of MALDI–TOF MS to identify staphylococci with the abilities of more traditional phenotypic methods (manual and automated) and/or genotypic methods (*sodA* gene and 16S ribosomal ribonucleic acid (rRNA) sequencing). The MALDI–TOF MS method achieved results that were comparable with those from more traditional methods (Rajakaruna *et al.* 2009; Fox *et al.* 2010; Dubois *et al.* 2010; Carbonnelle *et al.* 2012). Dupont *et al.* (2010) suggested that the MALDI–TOF MS method out-performed the more traditional methods, and it was shown to be more sensitive in the identification of staphylococcal isolates to the species level (Carbonnelle *et al.* 2007; Fox *et al.* 2010; Dubois *et al.* 2010; Dupont *et al.* 2010; Carbonnelle *et al.* 2012). In one study by Dupont *et al.* (2010), MALDI–TOF MS was compared with Phoenix Microbiology, an automated nephelometry system, and Vitex-2, an automatic colorimetric system. The correct identification was obtained for 97.4% of clinical CNS isolates identified by MALDI–TOF MS compared with 79 and 78.6% for Phoenix Microbiology and Vitek-2 respectively. In the study by Dubois *et al.* (2010), 99.3% of clinical and environmental (food and plant) staphylococcal isolates were correctly identified to the species level.

These studies suggest that MALDI–TOF MS is a highly sensitive method that could be used routinely to identify CNS to the species level.

Fox *et al.* (2011) noted that the identification of CNS from environmental samples to species level is not adequately achievable with either traditional methods or MALDI–TOF MS. However, these authors described a method using MALDI–TOF MS and one-dimensional gel electrophoresis that improved the identification of environmental isolates, including *Staphylococcus* species.

MALDI–TOF MS has already been shown to identify bacteria at the genus, species and subspecies level as part of protein profiling and bacterial proteomics. It could also be used to detect recombinant proteins, characterise targeted and unknown proteins, analyse bacterial RNA and DNA and detect virulence factors, including enterotoxins. Methods for use with MALDI–TOF MS to detect enterotoxins have already been studied (Brun *et al.* 2007; Dupuis *et al.* 2008) and could replace the immunological methods that are currently used to detect enterotoxins. This could improve investigations into staphylococcal food-borne poisoning outbreaks (Dupuis *et al.* 2008). This could also mean that MALDI–TOF MS could be used to identify as yet unidentified or previously unidentifiable SEs.

1.8 Aims of this Investigation

The purpose of this study was to isolate, identify and characterise CNS from New Zealand milk and dairy products, and to evaluate their toxin-producing potential.

1.8.1 Isolation of CNS from New Zealand milk and dairy products

Objectives

- a) To isolate CNS bacteria from a variety of processed dairy products.
- b) To select and optimise a suitable method(s) for enumerating CNS from dairy products.

1.8.2 Identification and characterisation of CNS from New Zealand milk and dairy products

Objective

- a) To select and optimise suitable phenotypic and genotypic methods for identifying CNS at the species level: for example, biochemical tests, i.e. BBL™ Crystal™ Gram Positive identification system (BD Diagnostic Systems, Becton Dickinson, Franklin Lakes, NJ, USA) and Remel RapID™ Staph Plus (Oxoid, Basingstoke, UK), protein profiling, i.e. MALDI–TOF MS (Bruker Daltonik GmbH), and molecular methods, i.e. 16S rDNA sequencing.

1.8.3 Evaluation of the toxin-producing potential of CNS isolated from dairy products

Objectives

- a) To select and optimise a suitable method(s) for determining if CNS have the genes present to produce enterotoxins.
- b) To test the enterotoxin-producing ability of selected CNS strains in dairy products.

Chapter Two: Materials and Methods

2.1 General Requirements

The following materials and reagents were obtained from Oxoid, Basingstoke, UK: tryptone soy broth (TSB), tryptone soy agar (TSA) and Remel RapID™ Staph Plus biochemical test kits.

Baird–Parker agar (BP) plates and peptone diluents were obtained from Fort Richard Laboratories, Auckland, New Zealand.

BBL™ Crystal™ Gram Positive identification systems were obtained from BD Diagnostic Systems, Becton Dickinson, Franklin Lakes, NJ, USA.

α -Cyano-4-hydroxy-cinnamic acid was obtained from Bruker Daltonik GmbH, Bremen, Germany.

The standard solution (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) for use in the MALDI–TOF method was obtained from Fluka Analytical, Buch, Switzerland.

The following were obtained from Merck KGaA, Darmstadt, Germany: acetonitrile and ethanol.

Formic acid was obtained from Fisher Scientific, Loughborough, Leicestershire, England.

PCR grade water was obtained from Roche Diagnostics, Mannheim, Germany.

The following were obtained from Zymo Research, Irvine, CA, USA: Quick-gDNA™ MiniPrep method and DNA Clean & Concentrator™-5 kit.

The following materials and methods were obtained from Invitrogen, Carlsbad, CA, USA: 1 Kb Plus DNA Ladder, Platinum® Multiplex PCR Master Mix and E-Gel® EX 2% gels.

The primer sets for the 16S rDNA sequencing (U1392R and Bac27F), SE PCR (SEAfor, SEArev, SEBfor, SEBrev, SECfor, SECrev, SEDfor, SEDrev, SEHfor, SEHrev) and the multiplex SE PCR (SEA-F, SEA-R, SEB-F, SEB-R, SEC-F, SEC-R, SED-F, SED-R, SEE-F and SEE-R) were obtained from Life Technologies, Penrose, Auckland, NZ.

The DNA loading buffer (EZ-Vision™ Two DNA Dye and Buffer 6x) was obtained from AMRESCO Incorporated, Solon, OH, USA.

The Tris-Borate-EDTA (TBE) 10X Solution was obtained from Fischer Scientific, Fair Lawn, New Jersey, USA.

Bio-Rad certified molecular biology agarose was obtained from Bio-Rad Laboratories, Hercules, CA, USA.

The Ridascreen® Set A, B, C, D, E immunoassay kits were obtained from R-Biopharm, Darmstadt, Germany.

2.2 General Methods

2.2.1 Collection of samples and bacterial isolation

As *Staphylococcus* species are naturally associated with milk, BP plates from a routine dairy testing laboratory and dairy products (milk powder, cheese and butter) from various dairy manufacturing plants from around New Zealand were collected. For the dairy products, samples were processed according to ISO 8261 / IDF 122: 2001 (E) (International Organization for Standardization 2001) and then pre-incubated at 37°C for 18 h according to the method described by Silva *et al.* (2005). Single colonies were then isolated by spread plating each sample on to BP plates (incubated at 37°C overnight). A proportion of colonies (at least 10% of the colonies present on each plate) were selected and grown in TSB overnight at 37°C and stock cultures were prepared (stored at -80°C). For colonies originating from BP plates, typical (Fig. 1) and non-typical (Fig. 2) *Staphylococcus* (black, with or without halos) were selected and cultured in TSB overnight at 37°C and then frozen stock cultures were prepared (stored at -80°C). In this way, 460 bacterial isolates were obtained for further study.

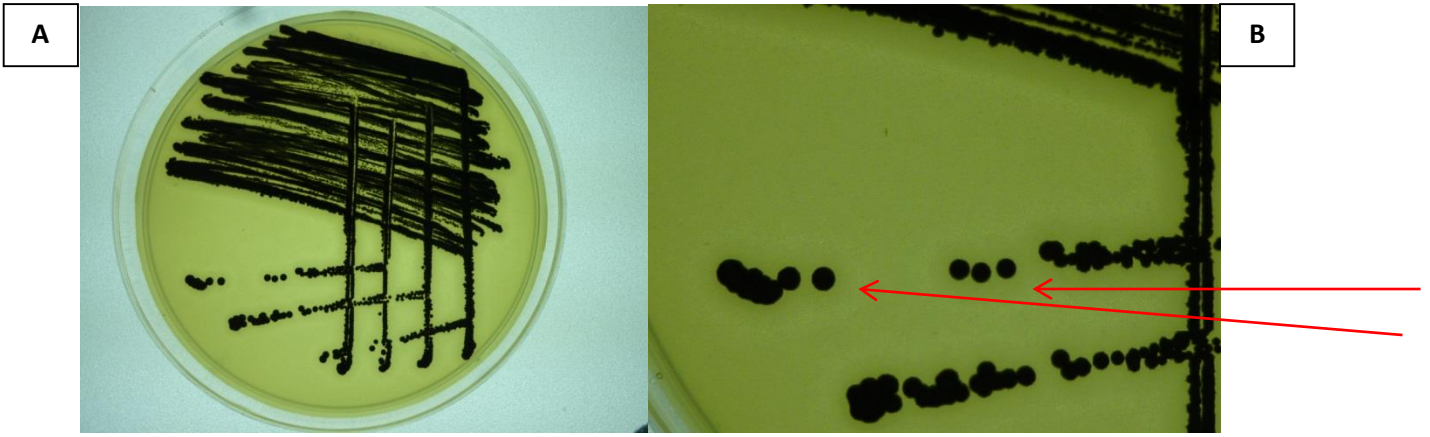


Figure 1 Baird-Parker agar plate showing typical *Staphylococcus aureus* colonies (1A) with a visible clearing or halo around the black colony (1B, indicated with arrows).

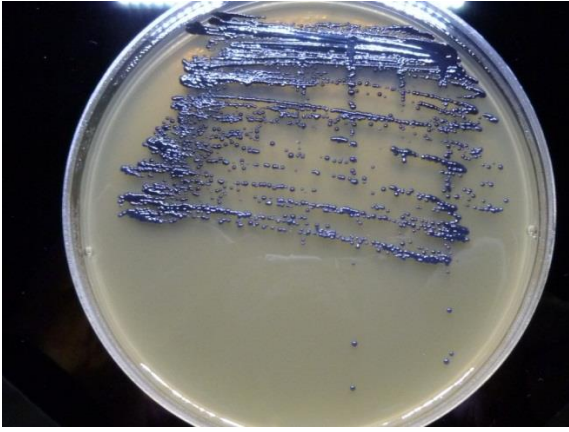


Figure 2. Baird-Parker agar plate showing non-typical black colonies (*Staphylococcus epidermidis*) with no visible clearing or halo.

2.2.2 Positive and negative controls

The reference strain *Staphylococcus aureus* subsp. *aureus* NZRM 917 (New Zealand Reference Culture Collection Medical Section, ESR Kenepuru Science Centre, Porirua, New Zealand) was used as a known and/or negative control for the identification methods.

The positive control isolates, *S. aureus* S27 (SEA producer), *S. aureus* S12 (SEB producer) and *S. aureus* S34 (SED producer) were obtained from the Food Assurance culture collection (Fonterra Research and Development Centre, Palmerston North, New Zealand) and were used as positive controls for the toxin tests and the multiplex toxin PCR method. Their toxin production status was determined by the Institute of Environmental Science and Research.

The positive control isolates 3376 (SEC producer) and 3377 (SEE producer) were obtained from the Institute of Environmental Science and Research and were used as positive controls for the multiplex toxin PCR method.

2.2.3 Phenotypic identification

Bacterial isolates were identified using the BBL™ Crystal™ Gram Positive identification system (Fig. 3A & Fig. 4) and Remel RapID™ Staph Plus biochemical test kits (Fig. 3C & Fig. 5), according to the manufacturers' instructions.

A**C****B****D**

Figure 3. Two Biochemical identification test kits used to identify the 42 Staphylococcus species. The BBL™ Crystal™ Gram Positive identification system (A, B), and the Remel Rapid™ Staph Plus identification system (C, D).

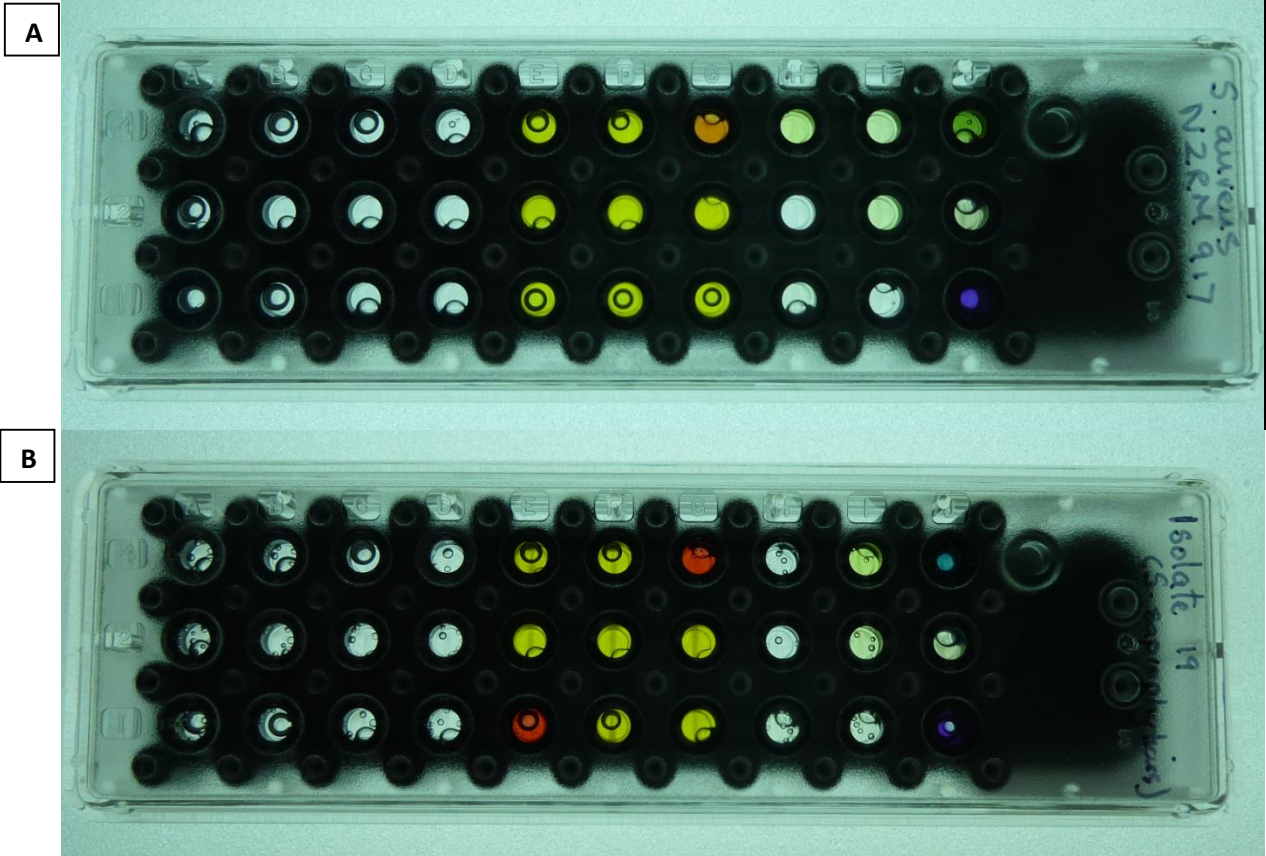


Figure 4. The BBL™ Crystal™ Gram Positive identification system showing a typical result for a *S. aureus* species (A) and a coagulase negative species (*S. saprophyticus*, B).



Figure 5. Remel RapID™ Staph Plus identification system showing a typical result for a *S. aureus* species (A) and a coagulase negative species (*S. saprophyticus*, B).

2.2.4 MALDI-TOF MS analysis

The basic principles of MALDI-TOF MS analysis are shown in Fig. 6. In general MALDI-TOF MS analysis involves a soft ionisation mechanism of proteins (Clark *et al.* 2013). Soft ionisation allows analysis of large biomolecules, including ribosomal proteins of up to 100kDa (Clark *et al.* 2013). In order for this mechanism to work the direct smear or purified protein of a sample is saturated with the matrix, a low-mass organic compound (Clark *et al.* 2013). The matrix is essential for this mechanism as it acts as a scaffold, allowing ionisation to occur and supplies protons for the ionisation of the sample (Clark *et al.* 2013). The drying of the sample and matrix enables them to co-crystallise so that the sample is embedded into the matrix (Clark *et al.* 2013).

The crystallised sample-matrix is irradiated using a UV laser beam, with the intensity determined by a beam attenuator that is standardised according to the manufacturer's settings (Clark *et al.* 2013). The photons from the laser and matrix molecules take up the energy from the beam, and this interaction triggers a sublimation of the matrix into a gas phase (Clark *et al.* 2013). This forms a plume which then results in the ionisation of the sample (Clark *et al.* 2013).

Following ionisation the proteins within the sample are analysed by the mass analyser. This characterises the composition of the sample as a spectrum of mass-to-charge (m/z) ratios (Clark *et al.* 2013). These ratios are electrodynamic measurements of how quickly ions from the sample move through the time of flight (TOF) tube and reach the detector (Clark *et al.* 2013). These spectra are then compared to a database of defined reference spectra, allowing the identification of the micro-organism (Clark *et al.* 2013).

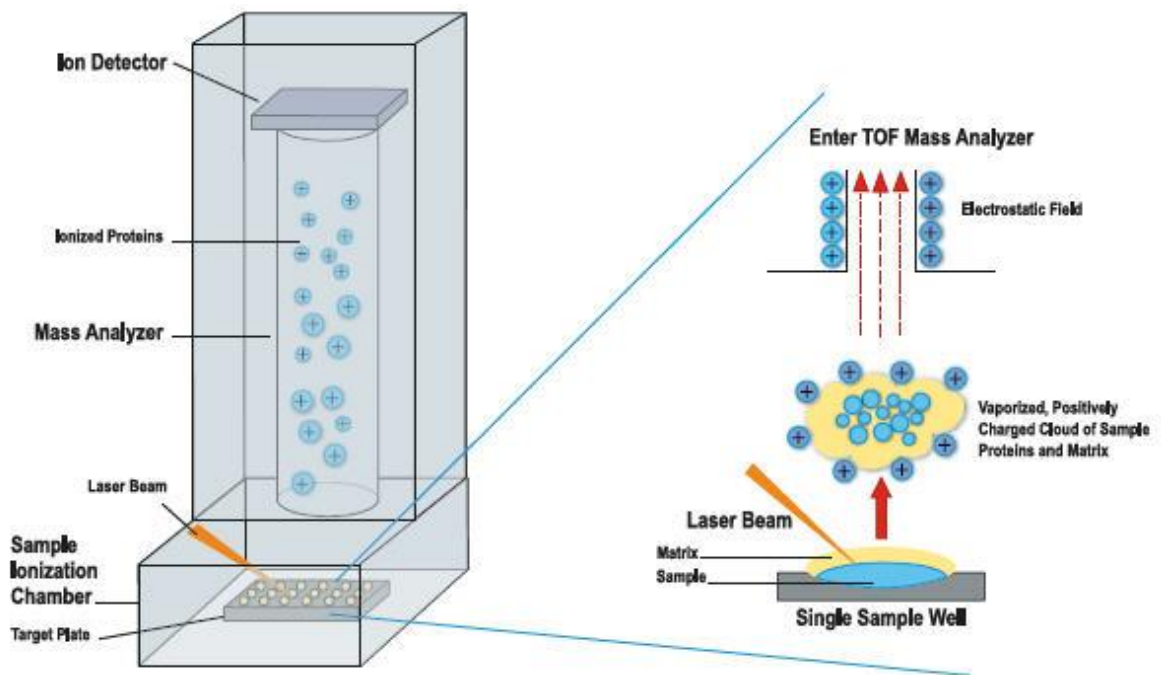


Figure 6. The basic principle of Matrix assisted Laser Desorption Ionisation –time of flight mass spectrometry (MALDI-TOF MS).
 (<http://www.mayomedicallaboratories.com/articles/communique/2013/01-maldi-tof-mass-spectrometry/index.html>)

For the MALDI-TOF MS analysis using the Bruker system, cells from a single colony from an overnight streak plate on TSA were used for each isolate, according to either the direct method or the extraction method, as outlined in the manufacturer's instructions. The direct method was used as an initial screen for each colony. Briefly, the colony was added to the MALDI target plate (Fig. 7B), air dried, overlaid with 1 μ L of matrix, air dried again and then processed in the Microflex LT (Bruker Daltonik GmbH, Bremen, Germany) mass spectrometer (Fig. 7A) using the Flex Control program.

For the extraction method, a loopful of colony was suspended in sterile demineralised water and 100% ethanol. The proteins were extracted using an equal volume of 70% formic acid and 100% acetonitrile, and the resulting supernatant was added to the MALDI target plate, air dried, overlaid with 1 μ L of matrix, air dried again and then processed as described above.

The resulting spectra were analysed using the Bruker Daltonics MALDI Biotyper 3.0 Real Time Classification program and a main spectrum (MSP) dendrogram was generated using the Bruker Daltonics MALDI Biotyper 3.0 Offline Classification software.

A



B



Figure 7. The laboratory bench Microflex LT MALDI-TOF MS set-up (A) and the target plate (B) used for placing samples for analysis.

2.2.5 Identification by 16S rDNA sequencing

Isolates were streak plated on to TSA and incubated overnight at 37°C. A heavy inoculum of each isolate was suspended in 200 µL of peptone diluent and then processed according to the Quick-gDNA™ MiniPrep method (Zymo Research). The universal bacterial primer sets, U1392R (5'-ACG GGCGGT GTG TRC-3') and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3'), were used for the amplification of 16S rRNA in combination with PCR Master Mix (Fermentas Life Sciences, Burlington, Ontario, Canada; www.fermentas.com), according to the manufacturer's instructions, and yielded a product of approximately 1300 bp. Polymerase chain reaction (PCR) amplifications were performed under the following conditions: an initial denaturation of template DNA at 94°C for 3 min; a series of 35 cycles consisting of denaturation (94°C, 30 s), annealing (60°C, 45 s) and extension (1 min 30 s, 72°C); a final extension at 72°C for 7 min. The generated PCR products were further purified using the DNA Clean & Concentrator™-5 kit (Zymo Research) and the resulting DNA concentration was quantified using the NanoPhotometer® P300 (Implen GmbH, Munchen, Germany) (Fig 8). The purified PCR product was sequenced, and the resulting sequences were analysed by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) against 16S rRNA sequences from GenBank (GenBank database of the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/GenBank/>). A phylogenetic tree highlighting the genetic similarities of the isolates was constructed using MegAlign (DNASTAR, www.dnastar.com) after alignment with Clustal V. Accession numbers for the isolates were: JX519584, JX519585, JX519586, JX519587, JX519588, JX519590 and JX519591.



Figure 8. The Implen NanoPhotometer® P300 used to quantify DNA of the 7 isolates sequenced using 16S rDNA.

2.2.6 Immunoassay test

Isolates were grown in TSB overnight at 37°C. At least 10 mL of culture was centrifuged for 5 min at 3500 x *g*. The supernatant was then sterile filtered using a 0.22 µm syringe filter (Merck Millipore, Darmstadt, Germany) and 100 µL of this filtrate was used per well in the assay. The immunoassay test kit, Ridascreen® SET A, B, C, D, E (R-Biopharm, Darmstadt, Germany), and associated reagents were used as outlined in the manufacturer's instructions (Fig. 9).

The absorbance was measured at 450 nm, using a Multiskan GO spectrophotometer (Fisher Scientific, Loughborough, Leicestershire, England). The threshold value was calculated by adding 0.15 to the negative control value. The sample was deemed to contain no SEs or SEs at a concentration below the detection limit if the absorbance test value was less than the threshold value. The isolate was considered to produce SEs if the absorbance test value was greater than or equal to the threshold value.



Figure 9. The complete Ridascreen® SET A, B, C, D, E immunoassay test kit. (<http://www.r-biopharm.com/wp-content/uploads/de/news/lebens-futtermittelanalytik/neues-produkt-ridascreen-set-total-10885/ridascreen-SET-total-de.jpg>)

2.2.7 SE PCR

Enterotoxin SEA, SEB and SED are the most frequently implicated enterotoxins in food poisoning outbreaks (Balaban & Rasooly 2000; Bartolomeoli *et al.* 2009). Therefore isolates were screened for enterotoxin genes SEA, SEB, SED and SEH. Isolates were streak plated on to TSA and incubated overnight at 37°C. A heavy inoculum of each isolate was suspended in 200 µL of peptone diluent and then processed according to the Quick-gDNA™ MiniPrep method. The bacterial primer sets SEAfor (5'-CCTTTGGAAACGGTTAAAAC-3'), SEArev (5'-CTCTGMACCTTYCCATCAA-3'), SEBfor (5'-GGG TATTTGAAGA TGGTAAAATT-3'), SEBrev (5'-AGGCGAGTTGTTAAATTCATAGAGTT-3') and SEDfor (5'-TCAATTTGTGGATAAATGGTGTAC-3'), SEDrev (5'-TTTCC TCCGAGAGTATCA TTAT-3') were used for the amplification of 16S rRNA in combination with PCR Master Mix, according to the manufacturer's instructions, and yielded products of approximately 128, 140 and 154 bp respectively (Derzelle *et al.* 2009). PCR amplifications were performed using the Techne TC-512 thermal cycler (Bio-Techne, Minneapolis, USA) (Fig. 10), under the following conditions: an initial denaturation of template DNA at 95°C for 8 min; 45 amplification cycles (95°C for 10 s, 58°C for 10 s, 72°C for 10 s); a cooling step to 4°C. The E-Gel® system (Invitrogen, Fig. 11) using the 2% E-Gel was set up according to the manufacturer's instructions, using program 7 for 12 min. The seven isolates, *S. aureus* NZRM 917, positive controls (S27, S12 and S34) and a negative control (water) were run on the gel and an image was generated using the Essential V2 (UVitec Limited, Cambridge, UK) gel documentation system (Fig. 12).



Figure 10. The Techne TC-512 thermal cycler used to generate PCR products for SE PCR, multiplex PCR and 16S rDNA sequencing.



Figure 11. The Invitrogen E-Ge® system used for SE PCR analysis.



Figure 12. The UVitec Essential V2 used to photograph the gels generated using both the E-Gel system for the SE PCR results and the traditional method for the multiplex PCR results.

2.2.8 SE Multiplex PCR

As the primers used in the PCR method described in 2.2.8 were specific for *S. aureus* sp, the method and primers employed by Park *et al.* (2011) was used. The 42 isolates were streak plated on to TSA and incubated overnight at 37°C. A heavy inoculum of each isolate was suspended in 200 µL of peptone diluent and then processed according to the Quick-gDNA™ MiniPrep method. The bacterial primer sets SEA-F (5'-CAGCATACTATATTGTTTAAAGGC-3'), SEA-R (5'-CCTCTGAACCTTCCCATC-3'), SEB-F (5'-GTATGGTGGTGTAACTGAGCA-3'), SEB-R (5'-TCAATCTTCACATCTTTAGAATCA-3'), SEC-F (5'-CTCAAGAACTAGACATAAAAGCTAGG-3'), SEC-R (5'-TCAAAATCGGATTAACATTATCC-3'), SED-F (5'-CTAGTTTGGTAATATCTCCTTTAAACG-3'), SED-R (5'-TTAATGCTATATCTTATAGGGTAAACATC-3') SEE-F (5'-CAGTACCTATAGATAAAGTTAAAACAAGC-3') and SEE-R (5'-TAACTTACCGTGGACCCTTC-3') were used. These primer sets were used in combination with Platinum® Multiplex PCR Master Mix (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and yielded products of approximately 400, 351, 271, 319 and 178 bp respectively. PCR amplifications were performed under the following conditions: an initial denaturation of DNA at 95 °C for 10 min was followed by 35 cycles of amplification (95 °C for 30 s, 53 °C for 45 s and 72 °C for 90 s), ending with a final extension at 72 °C for 10 min. A 4% gel was prepared using 4g Bio-Rad agarose and 100mL 1X TBE buffer, heated in a microwave until agarose melted, cooled and poured into a 100mL Bio-Rad casting tray. EZ-Vision loading buffer was prepared according to the manufacturer's instructions and 1µL aliquots were pre-mixed with 5µL each of the 42 isolates, *S. aureus* NZRM 917, positive controls (S27, S12, S34, 3376 and 3377) and a negative control (water). The Bio-Rad gel electrophoresis system was set up according to the manufacturer's instructions and an image was generated using the Essential V2 (UVitec Limited, Cambridge, UK) gel documentation system (Fig. 12).

2.2.9 Interpretation of resulting identities

For this study, we defined a “correct identity” as one for which two or more of the methods (MALDI-TOF and the two biochemical kits) tested gave the same result. When the identities of the isolates were different for all three methods, 16S rDNA sequence analysis was then considered to determine the “correct identity”.

Chapter Three: Results and Discussion

3.1 Product Analysis

Dairy product samples from around New Zealand were provided for this study and included cheese, butter, butter milk powder, skim milk powder, cream, food additives, food bars, curd, yoghurt, dairy spread and UHT milk. Of these product types, cheese produced the greatest number of CNS isolates for further analysis (48 out of 126 CNS isolates, 38%). This was followed by butter and butter milk powder with 33.3% and 11.1% of the total number of CNS isolates, respectively.

Of the product types included in this study cheese also provided the greatest diversity of CNS species, with *S. saprophyticus*, *S. epidermidis*, *S. warneri*, *S. pasteurii* and *S. capitis* all being isolated from this dairy product. While yoghurt only produced 7 CNS isolates, 4 different CNS species were isolated from it and included *S. epidermidis*, *S. warneri*, *S. hominis* and *S. capitis*. For all other product types either one or two species predominated.

3.2 Identification of Coagulase-negative Species

3.2.1 Identification of CNS isolates using the BBL™ Crystal™ Gram Positive identification system, Remel Rapid™ Staph Plus and MALDI-TOF MS

Of the 427 isolates screened using MALDI-TOF MS, 301 were identified as *Enterococcus* species including *E. casseliflavus*, *E. faecium*, *E. faecalis*, *E. faecalis/gallinarum* and *E. gallinarum* (136), *Macrococcus caseolyticus* (70), *Bacillus* species including *B. cereus*, *B. cereus/mycoides*, *B. lichenformis*, *B. mycoides*, *B. oleronius*, *B. pumilus*, *B. sonorensis*, *B. subtilis* and *B. weihenstephanensis/mycoides* (44), *Lysinibacillus fusiformis* (29), *Staphylococcus aureus* (17), *Paenibacillus thiaminolyticus* (2), *Lactococcus lactis* (2) and *Bifidobacterium animalis* (1). The remaining 126 were identified as CNS species. BP agar was originally developed as a selective medium for *S. aureus* (Baird-Parker, 1962) and is the gold standard

medium that is used in food microbiology for the enumeration of staphylococcal species. It was interesting to note, in this current study, that colonies that are traditionally thought of as *Staphylococcus* (a typical black, shiny convex colony surrounded by a clear zone) were isolated 4% of the time on this medium. This emphasised the importance of a confirmation step as routine practice for these bacteria.

Of the CNS isolates identified in this study, 42 were selected, representing isolates from a variety of products, and were further analysed using the BBL™ Crystal™ Gram Positive identification system, the Remel RapID™ Staph Plus method and MALDI-TOF MS applying the full extraction protocol (Table 1). A reference culture, *S. aureus* NZRM 917, was used as a positive control.

Table 1. Identification of 42 CNS isolates using 3 methods including, the BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus, and MALDI-TOF MS.

	Isolate Number	MALDI-TOF Identity	BBL™ Crystal™ Gram Positive identification system	Remel RapID™ Staph Plus method
Agreement between 3 methods	1	<i>S. saprophyticus</i> (2.206)*	<i>S. saprophyticus</i> (97%)**	<i>S. saprophyticus</i> (58%)
	3	<i>S. saprophyticus</i> (2.048)	<i>S. saprophyticus</i> (99.6%)	<i>S. saprophyticus</i> (58%)
	5	<i>S. epidermidis</i> (2.094)	<i>S. epidermidis</i> (>99.99%)	<i>S. epidermidis</i> (>99.9%)
	6	<i>S. warneri</i> (1.998)	<i>S. warneri</i> (99%)	<i>S. warneri</i> (>99.9%)
	7	<i>S. epidermidis</i> (2.181)	<i>S. epidermidis</i> (99.98%)	<i>S. epidermidis</i> (99.8%)
	9	<i>S. capitis</i> (2.049)	<i>S. capitis</i> (99.7%)	<i>S. capitis ss capitis</i> (>99.9%)
	11	<i>S. warneri</i> (1.967)	<i>S. warneri</i> (99.99%)	<i>S. warneri</i> (>99.9%)
	12	<i>S. warneri</i> (2.099)	<i>S. warneri</i> (96%)	<i>S. warneri</i> (>99.9%)
	16	<i>S. saprophyticus</i> (1.996)	<i>S. saprophyticus</i> (99.7%)	<i>S. saprophyticus</i> (68%)
	17	<i>S. saprophyticus</i> (1.888)	<i>S. saprophyticus</i> (99.9%)	<i>S. saprophyticus</i> (68%)
	18	<i>S. saprophyticus</i> (1.8)	<i>S. saprophyticus</i> (99.5%)	<i>S. saprophyticus</i> (>99.9%)
	19	<i>S. saprophyticus</i> (2.11)	<i>S. saprophyticus</i> (99.99%)	<i>S. saprophyticus</i> (>99.9%)
	20	<i>S. epidermidis</i> (2.243)	<i>S. epidermidis</i> (99.7%)	<i>S. epidermidis</i> (>99.9%)
	22	<i>S. saprophyticus</i> (2.018)	<i>S. saprophyticus</i> (99.9%)	<i>S. saprophyticus</i> (>99.9%)
	23	<i>S. saprophyticus</i> (1.891)	<i>S. saprophyticus</i> (>99.99%)	<i>S. saprophyticus</i> (>99.9%)
	24	<i>S. saprophyticus</i> (1.947)	<i>S. saprophyticus</i> (99.9%)	<i>S. saprophyticus</i> (>99.9%)
	25	<i>S. saprophyticus</i> (2.028)	<i>S. saprophyticus</i> (65%)	<i>S. saprophyticus</i> (>99.9%)
	26	<i>S. warneri</i> (1.796)	<i>S. warneri</i> (99%)	<i>S. warneri</i> (>99.9%)
	29	<i>S. capitis</i> (1.812)	<i>S. capitis</i> (99.95%)	<i>S. capitis ss capitis</i> (>99.9%)
	31	<i>S. epidermidis</i> (2.060)	<i>S. epidermidis</i> (99.7%)	<i>S. epidermidis</i> (99.9%)
	32	<i>S. warneri</i> (1.915)	<i>S. warneri</i> (99.9%)	<i>S. warneri</i> (>99.9%)
	33	<i>S. epidermidis</i> (2.17)	<i>S. epidermidis</i> (97%)	<i>S. epidermidis</i> (>99.9%)
	36	<i>S. capitis</i> (2.31)	<i>S. capitis</i> (95%)	<i>S. capitis ss capitis</i> (99.8%)
	38	<i>S. capitis</i> (1.723)	<i>S. capitis</i> (99.7)	<i>S. capitis ss capitis</i> (51%)
40	<i>S. warneri</i> (2.009)	<i>S. warneri</i> (65%)	<i>S. warneri</i> (>99.9%)	
41	<i>S. epidermidis</i> (2.122)	<i>S. epidermidis</i> (99%)	<i>S. epidermidis</i> (>99.9%)	
42	<i>S. capitis</i> (2.203)	<i>S. capitis</i> (98%)	<i>S. capitis ss capitis</i> (99.8%)	
	Reference culture	<i>S. aureus</i> (2.331)	<i>S. aureus</i> (99.7%)	<i>S. aureus</i> (>99.9%)
Agreement between 2 methods	2	<i>S. saprophyticus</i> (2.013)	<i>S. saprophyticus</i> (52%)	<i>S. warneri</i> (99.8%)
	4	<i>S. epidermidis</i> (2.151)	<i>S. epidermidis</i> (>99.99%)	<i>S. aureus</i> (90%)
	10	<i>S. epidermidis</i> (2.03)	<i>S. epidermidis</i> (98.7%)	<i>S. saprophyticus</i> (99%)
	21	<i>S. sciuri</i> (1.884)	<i>S. sciuri</i> (>99.99%)	<i>S. aureus</i> (59%)
	27	<i>S. capitis</i> (2.153)	<i>S. capitis</i> (99.7%)	<i>S. caprae</i> (>99.9%)
	34	<i>S. capitis</i> (2.009)	<i>S. capitis</i> (74%)	<i>S. hominis ss hominis</i> (58%)
	35	<i>S. capitis</i> (2.118)	<i>S. capitis</i> (95%)	<i>S. hominis ss hominis</i> (89%)
	8	<i>S. epidermidis</i> (2.166)	<i>S. intermedius</i> (99.91%)	<i>S. epidermidis</i> (>99.9%)

	30	<i>S. hominis</i> (2.211)	<i>S. saprophyticus</i> (99.6%)	<i>S. hominis ss hominis</i> (>99.9%)
	39	<i>S. hominis</i> (2.246)	<i>S. saprophyticus</i> (>99.99%)	<i>S. hominis ss hominis</i> (99%)
No agreement	13	<i>S. epidermidis</i> (2.107)	<i>S. intermedius</i> (77%)	<i>S. saprophyticus</i> (92%)
	14	<i>S. pasteurii</i> (2.244)	<i>S. haemolyticus</i> (67%)	<i>S. xylosus</i> (96%)
	15	<i>S. pasteurii</i> (2.151)	<i>S. warneri</i> (71%)	<i>S. warneri</i> (>99.9%)
	28	<i>S. warneri</i> (2.27)	<i>Micrococcus sp.</i> (58%)	<i>S. haemolyticus</i> (>99.9%)
	37	<i>S. warneri</i> (2.45)	<i>S. aureus</i> (99%)	<i>Kocuria rosea</i> (>99.9%)

*A score value of >1.7 = genus level identification, >2 = species level identification (as per manufacturer's instruction – Bruker). **Grading based on the API Identification system

The 3 methods used to identify the CNS isolates in this study agreed on 27 occasions (64%). On 10 occasions (24%), corresponding identities between MALDI–TOF analysis and either of the 2 biochemical test kits was obtained. For these isolates, the BBL™ Crystal™ Gram Positive identification system corresponded on more occasions with MALDI–TOF analysis (7 out of 10 times) than the Remel RapID™ Staph Plus method (3 out of 10 times). For 1 isolate, the 2 biochemical kits agreed, but the identity was different when based on MALDI–TOF analysis. For 4 of the isolates, no agreement between the identities was obtained using the 3 methods (Table 1).

Biochemical test methods have been widely used to identify staphylococcal species in both clinical and food-related studies. The results of this study suggest that Remel RapID™ Staph Plus provides an identification of CNS species that is comparable with that of the BBL™ Crystal™ Gram Positive identification system. A previous study compared the BBL™ Crystal™ Gram Positive identification system with the API Staph (bioMérieux Diagnostic, Germany) and API Strep (bioMérieux Diagnostic) methods in identifying Gram-positive cocci, including 77 staphylococcal strains (von Baum et al., 1998). The BBL™ Crystal™ Gram Positive identification system overall gave a 90% rate of correct species identification, similar to that of the established API systems (von Baum et al., 1998). Another study compared API 32 Staph ID (bioMérieux Diagnostic), the BBL™ Crystal™ Gram Positive identification system and the molecular method, restriction fragment length polymorphism of the amplified *tuf* gene (Alexopoulou *et al.*, 2006). However, unlike the previous study, this study found that the API system had a higher overall agreement with the molecular method than the BBL™ Crystal™ Gram Positive identification system (Alexopoulou et al., 2006).

In comparing the ease of use of the 2 biochemical methods, Remel RapID™ Staph Plus provides results more rapidly (4–6 h) than the BBL™ Crystal™ Gram Positive identification system (18–24 h). Whereas the BBL™ Crystal™ Gram Positive identification system requires additional equipment (BBL™ Crystal™ Panel Viewer), which is simple to use, Remel RapID™ Staph Plus is more labour intense because it

requires the addition of reagents to some of the wells. In contrast, MALDI–TOF MS provides results within minutes and, although the principle behind the machine is complex, the laboratory method itself is relatively simple. This method also includes variations that can be applied to the method to aid and improve identification.

Of these 42 isolates, 5 (12%) had an identification that did not agree between the 3 methods or for which the MALDI–TOF identity did not agree. For example, isolate 28 was identified as a *Micrococcus* species by the BBL™ Crystal™ Gram Positive identification system, and isolate 37 was identified as *Kocuria rosea* by Remel RapID™ Staph Plus. To obtain a definitive identification, these 5 isolates were further analysed using 16S rDNA sequencing. Along with these 5 isolates, 2 additional isolates were selected. One in which all 3 methods agreed on the identification (isolate 25) and one where only 2 methods agreed (isolate 4).

3.2.2 Definitive identification of seven CNS isolates using 16S rDNA sequencing

In comparison with 16S rDNA sequencing, the BBL™ Crystal™ Gram Positive identification system correctly identified two out of the seven isolates to the species level and four out of the seven isolates to the genus level. However, it was unable to identify one of the seven isolates that were confirmed as staphylococcal species by 16S rDNA sequencing.

The Remel RapID™ Staph Plus system correctly identified one out of the seven isolates to the species level and five out of the seven isolates to the genus level. Only one out of the seven isolates, confirmed as a staphylococcal species by 16S rDNA sequencing, was not identified by this method.

For the MALDI–TOF MS method, six out of the seven isolates were correctly identified to the species level and one out of the seven isolates was correctly identified to the genus level. This method was therefore able to correctly identify all staphylococcal isolates to at least the genus level when compared with 16S rDNA sequencing.

3.2.3 Comparison of MALDI–TOF MS identification and 16S rDNA sequencing

To further compare the identification for the isolates obtained using MALDI–TOF MS with that obtained using 16S rDNA sequencing, both an MSP dendrogram (Fig. 13) and a phylogenetic tree (Fig. 14) were generated. Identities and clustering of isolates 1 (*S. epidermidis*), 3 (*S. pasteurii*), 4 (*S. pasteurii*), 5 (*S. saprophyticus*) and 7 (*S. pasteurii*) (99–100% similarities using 16S rDNA sequencing) were similar for both the MSP dendrogram (Fig. 13) and the phylogenetic tree (Fig. 14). The only exceptions were isolates 2 and 6. Isolate 2 was identified by MALDI–TOF analysis and 16S rDNA sequencing as *S. epidermidis*. However, it clustered with strains of *S. capitis* on the phylogenetic tree (Fig. 14). *S. epidermidis* and *S. capitis* do form part of the same phylogenetic cluster group based on 16S rDNA sequencing, which may account for this anomaly (Takahashi et al., 1999). Isolate 6 was identified as *Staphylococcus sciuri* (99% sequence similarity) using 16S rDNA sequencing and as *S. warneri* by MALDI–TOF analysis. This isolate clustered with an *S. sciuri* reference strain on the phylogenetic tree (Fig. 14), but with other CNS reference strains on the MSP dendrogram (Fig. 13).

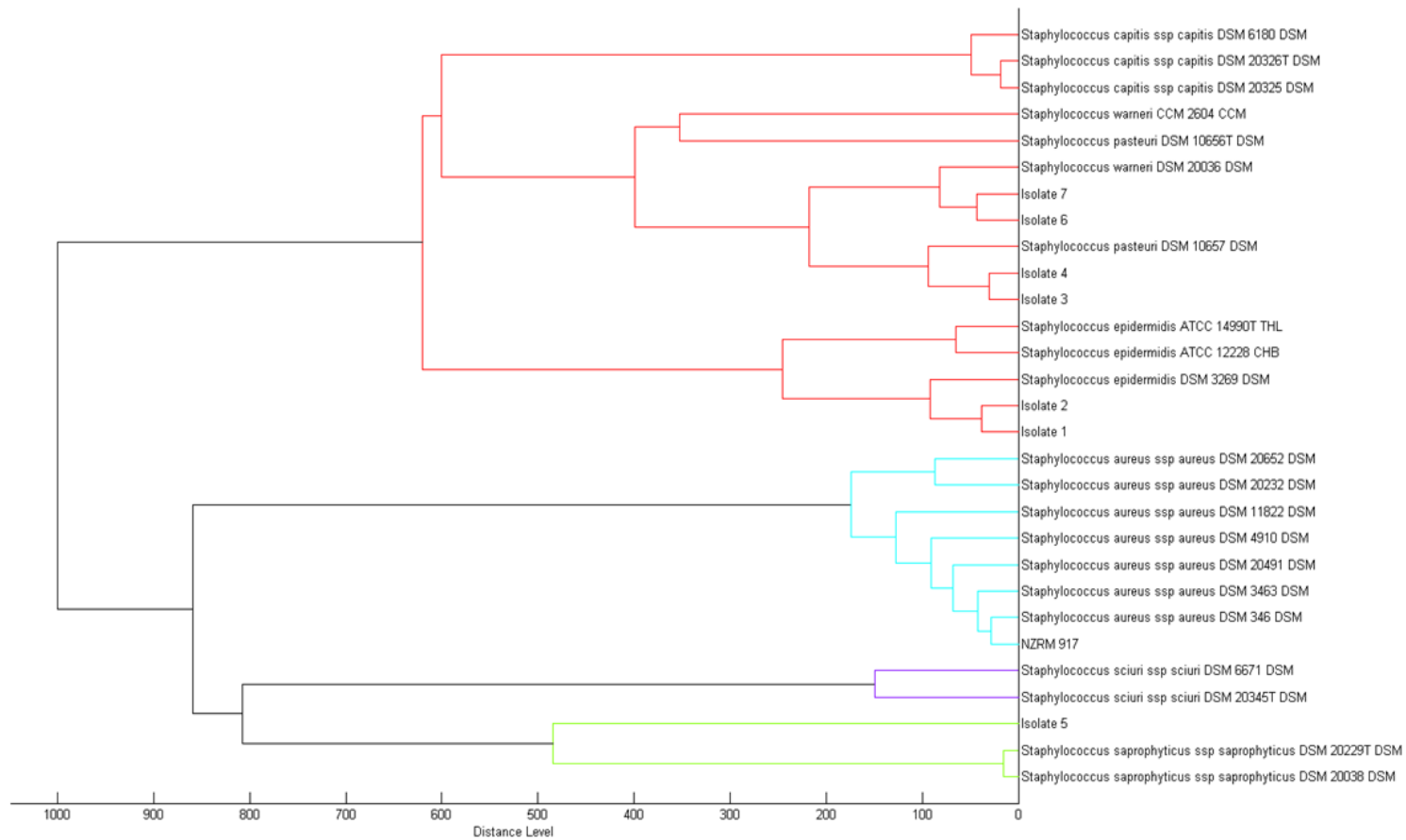


Figure 13. An MSP dendrogram generated by the Bruker Daltonics MALDI Biotyper 3.0 Offline Classification software for seven CNS isolates, for which their identification did not agree among the three methods BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI-TOF MS. Isolate identifications based on 16S rDNA sequencing are as follows: isolates 1 and 2 – *S. epidermidis*, isolates 3, 4 and 7 – *S. pasteuri*, isolate 5 – *S. saprophyticus*, isolate 6 – *S. sciuri* subsp. *sciuri*.

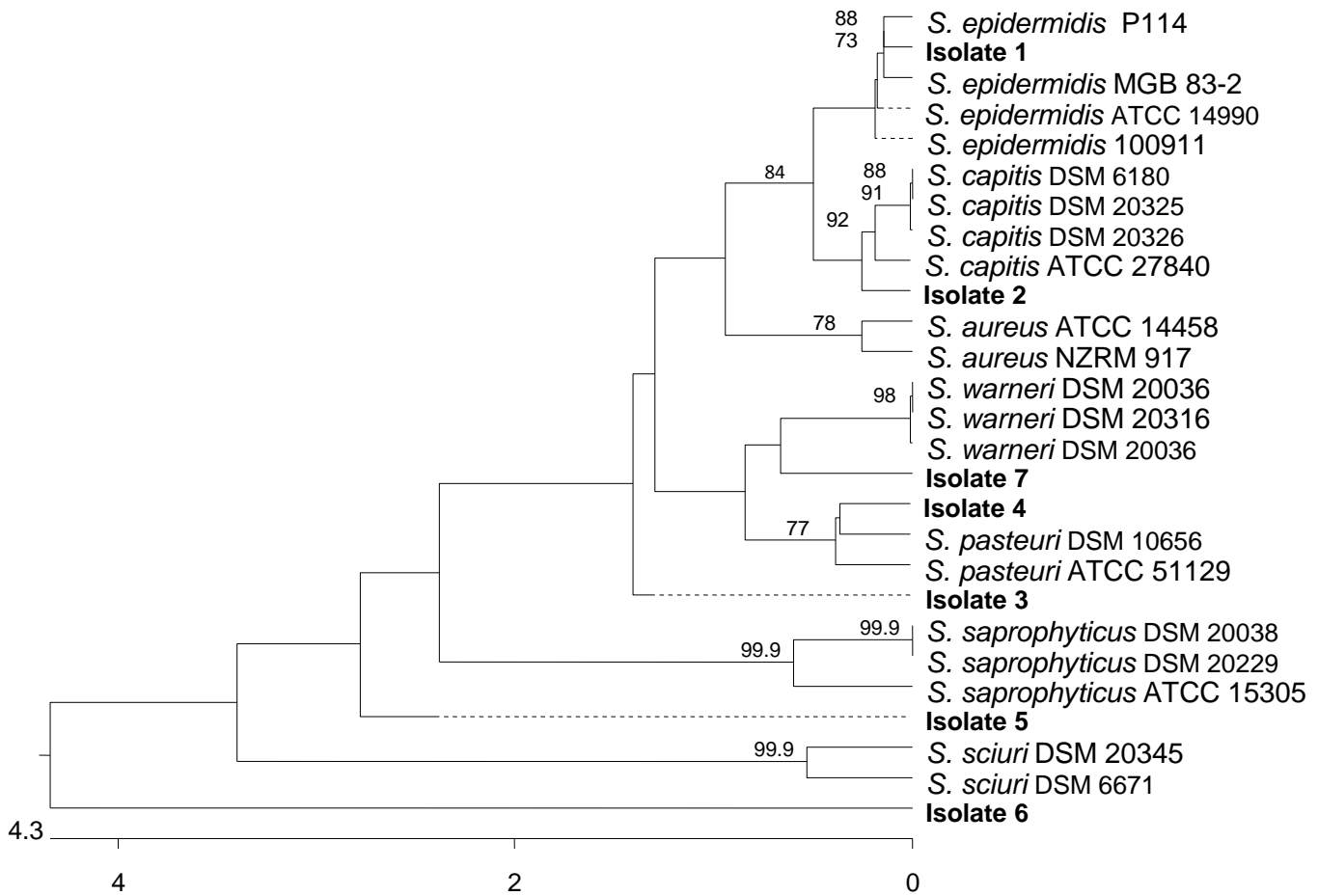


Figure 14. A phylogenetic tree generated by MegAlign using Clustal V alignment of the seven CNS isolates for which the identification did not agree among the three methods (BBL™ Crystal™ Gram Positive identification system, Remel RapID™ Staph Plus and MALDI-TOF MS). Bootstrap values > 70% are shown. Isolate identifications based on 16S rDNA sequencing are as follows: isolates 1 and 2 – *S. epidermidis*, isolates 3, 4 and 7 – *S. pasteurii*, isolate 5 – *S. saprophyticus*, isolate 6 – *S. sciuri* subsp. *sciuri*.

3.2.4 Overall Examination of Identification Results

Biochemical test methods have been used widely to identify staphylococcal species in both clinical and food-related studies. The results from this study suggest that the Remel RapID™ Staph Plus identification system provides an identification of CNS species that is comparable with that of the BBL™ Crystal™ Gram Positive identification system. A previous study compared the BBL™ Crystal™ Gram Positive identification system with the API Staph and API Strep methods in identifying Gram-positive cocci, including 77 staphylococcal strains (von Baum *et al.* 1998). The BBL™ Crystal™ Gram Positive identification system overall gave a 90% rate of correct species identification, similar to that of the established API systems (von Baum *et al.* 1998). Another study compared API 32 Staph ID, BBL™ Crystal™ Gram Positive identification system and the molecular method, restriction fragment length polymorphism of the amplified *tuf* gene (Alexopoulou *et al.* 2006). However, unlike the previous study, this study found that the API system had a higher overall agreement with the molecular method than the BBL™ Crystal™ Gram Positive identification system (Alexopoulou *et al.* 2006).

16S rDNA sequencing was the reference standard for the identification of microorganisms in this study. Overall, the identification of CNS by MALDI–TOF MS did compare well with 16S rDNA sequencing. Although MALDI–TOF MS results, like those of the biochemical methods, are limited by the number and types of microorganisms in the database, reference strains and new strains can be added to a separate database linked to the Bruker database. This is important for the food industry, where many of the strains seen are from the environment and are not likely to be in the existing Bruker database (which is largely based on isolates of clinical origin).

The findings in this study are comparable with those from the study by Loonen *et al.* (2012), in which the phenotypic methods, Vitek-2 and ID 32 Staph, the genotypic methods, partial 16S and *tuf* gene sequencing, and the proteomic method MALDI–TOF MS were compared in differentiating 117 clinical CNS isolates and 25 reference strains. MALDI–TOF MS correctly identified 99.3% of the CNS isolates

compared with 93, 70.4, 85.9 and 92.3% for *tuf* gene sequencing, 16S sequencing, ID 32 Staph and Vitek-2 respectively (Loonen *et al.* 2012). The 16S rDNA sequencing was the least successful method for identifying CNS species in the study by Loonen *et al.* (2012), with 29.6% of isolates incorrectly identified and nine out of the 25 reference strains (36%) misidentified to the species level. Using this method, both *S. lugdunensis* and *S. saprophyticus* were misidentified. This differs from this present study, in which the similarities for the seven isolates sequenced using the 16S gene were between 99 and 100% and this method was able to identify one *S. saprophyticus* isolate. Loonen *et al.* (2012) do note that whole 16S sequencing would improve the differentiation of *Staphylococcus* species using this specific gene.

Matsuda *et al.* (2012) developed a variation on the full extraction method used with MALDI-TOF MS, referred to as the on-plate extraction method, and compared this new method with both the direct method and the full extraction method for identifying 273 clinical staphylococcal isolates and 14 type and reference strains. This new method showed no statistically significant differences from the full extraction method at the genus level ($P = 0.1545$) but significantly higher identification rates than the full extraction method at the species level ($P < 0.0001$), particularly in relation to the species *S. caprae* and *S. saprophyticus* (Matsuda *et al.* 2012). This study found slightly lower identification rates than that of Loonen *et al.* (2012) and this present study, with only 89.5, 80.8 and 60.2% of isolates correctly identified to the species level for the on-plate extraction, full extraction and direct methods respectively (Matsuda *et al.* 2012). Similarly Romero-Gómez *et al.* (2012) reported that MALDI-TOF MS correctly identified only 63.3% of CNS isolates in their study using the full extraction method. However, whereas the sample was spotted on to the target and dried at room temperature in the on-plate extraction method of Matsuda *et al.* (2012), the target was dried at 55°C for the full extraction method. These are not the recommended drying conditions of the manufacturers and could have had an effect on protein quality and the spectrum produced (Matsuda *et al.* 2012). Therefore, this could account for the low score and the incorrect identification obtained by these authors.

MALDI–TOF scores below 2.0 affected the correct identification of staphylococci in the studies by Matsuda *et al.* (2012) and Romero-Gómez *et al.* (2012). However, a clinical study by El Bouri *et al.* (2012) assessed the agreement between MALDI–TOF MS and the phenotypic methods BD Phoenix and API for 928 isolates and found that including scores between 1.7 and 2.0 for 45 staphylococcal isolates aided the MALDI–TOF MS method in achieving a 100% agreement in the identification and differentiation between *S. aureus* and CNS species. This was also observed in the study by Carpaij *et al.* (2011). This study evaluated the use of MALDI–TOF MS for identifying 62 clinical CNS isolates in comparison with *tuf* gene sequencing. Along with using scores above 2.0, these authors also considered scores between 1.8 and 2.0 for duplicate samples as reliable results (Carpaij *et al.* 2011). The species assignment by MALDI–TOF MS was in agreement with *tuf* gene sequencing for all 62 isolates (Carpaij *et al.* 2011).

An emerging view of CNS is that they are essentially opportunistic microorganisms that are important nosocomial pathogens and pose a serious risk for the immunocompromised, the elderly, neonates and patients with an orthopaedic or cardiac prosthesis or prolonged catheter use (d’Azevedo *et al.* 2010). Therefore, because of the increase in the number of nosocomial infections associated with CNS (Alexopoulou *et al.* 2006) and the identification of CNS strains that produce enterotoxins in food, the application of MALDI–TOF MS could be as well suited to the clinical laboratory as to the food microbiology laboratory.

The results from this study showed that MALDI–TOF MS is a rapid, reliable and accurate method for identifying CNS definitively to the genus level and, on most occasions, to the species level in dairy products and is therefore a potential alternative to the traditional phenotypic, such as commercial identification kits, and genotypic, such as sequencing, methods that are currently used (Dupuis *et al.* 2008).

3.3 Staphylococcal Enterotoxin

3.3.1 Immunoassay test results

To determine whether enterotoxins were produced by CNS species, 42 isolates were tested for SEA, SEB, SEC, SED and SEE production using the Ridascreen® SET A, B, C, D, E (R-Biopharm) immunoassay test kit. The three *S. aureus* positive controls, S27, S12 and S34, produced their respective enterotoxins, SEA, SEB and SED. However, none of the 42 CNS isolates produced SEA, SEB, SEC, SED or SEE *in vitro* broth culture.

3.3.2 SE gene identification of seven isolates

As no detectable enterotoxin was produced by any of the 42 CNS isolates, seven of these isolated from different products and of different staphylococcal species were selected to determine whether they possessed the genes for SEA, SEB, SED and SEH using the method of Derzelle *et al.* (2009). None of the seven selected CNS isolates possessed *sea*, *seb*, *sed* or *seh* genes using this method.

3.3.3 SE gene identification using multiplex PCR

As no CNS isolates were identified to possess any of the SE genes (*sea*, *seb*, *sed* or *seh*) tested for the 42 CNS isolates were rescreened using a multiplex PCR method developed by Park *et al.* (2011). In this current study set one from the Park *et al.* (2011) method was used to determine the presence of *sea-see* genes. The *S. aureus* positive control species (S27, S12 and S34) and the *S. aureus* reference strain NZRM 917 were also included.

This method identified that 2 of the 42 CNS isolates possessed a SE gene (Figure 15 and Figure 16). Isolate 12, a *Staphylococcus warneri* (Figure 15, lane 13) gave two distinct bands of equivalent size to those observed for SEB producing control strain (Figure 15, lane 17) and SED (Figure 15, lane 19). Isolate 12 also gave a faint band

of approximately 400bp, equivalent to that observed for the SEA producing control strain (Figure 15, lane 16). Isolate 17, a *Staphylococcus saprophyticus*, gave a band of approximately 319bp (Figure 16, lane 4) that is equivalent to the band observed for the SED producing control strain (Figure 16, lane 19).

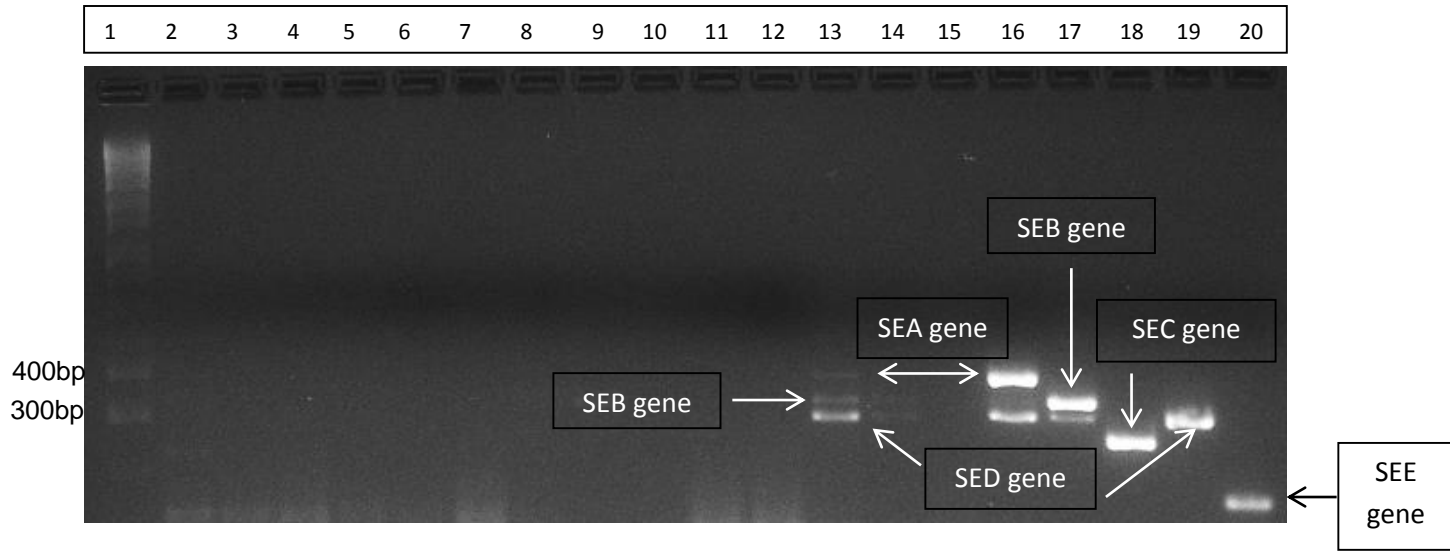


Figure 15. Gel electrophoresis of CNS isolates 1-14 screened for SEA-SEE genes using multiplex PCR. (Lane 1 1Kb ladder, Lane 2 Isolate 1, Lane 3 Isolate 2, Lane 4 Isolate 3, Lane 5 Isolate 4, Lane 6 Isolate 5, Lane 7 Isolate 6, Lane 8 Isolate 7, Lane 9 Isolate 8, Lane 10 Isolate 9, Lane 11 Isolate 10, Lane 12 Isolate 11, Lane 13 Isolate 12, Lane 14 Isolate 13, Lane 15 Isolate 14. Lane 16 – 20 SE producing controls; Lane 16 SEA producing *S. aureus*, Lane 17 SEB producing *S. aureus*, Lane 18 SEC producing *S. aureus*, Lane 19 SED producing *S. aureus*, Lane 20 SEE producing *S. aureus*.)

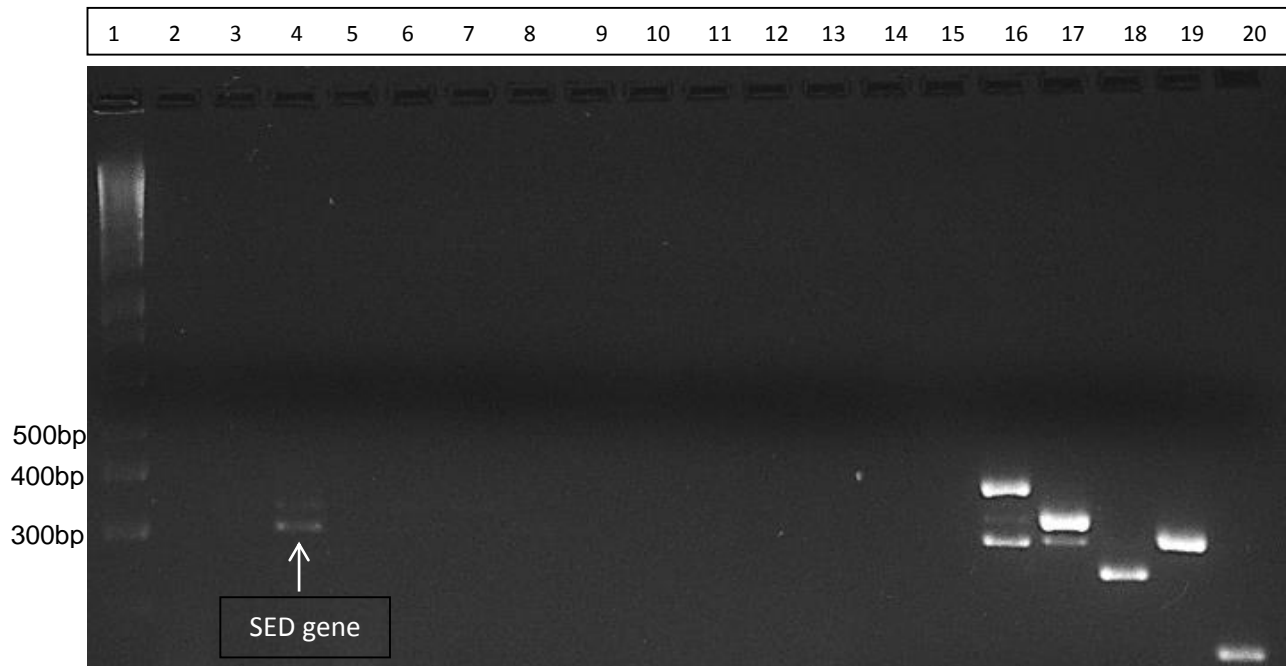


Figure 16. Gel electrophoresis of CNS isolates 15-28 screened for SEA-SEE genes using multiplex PCR. (Lane 1 1Kb ladder, Lane 2 Isolate 15, Lane 3 Isolate 16, Lane 4 Isolate 17, Lane 5 Isolate 18, Lane 6 Isolate 19, Lane 7 Isolate 20, Lane 8 Isolate 21, Lane 9 Isolate 22, Lane 10 Isolate 23, Lane 11 Isolate 24, Lane 12 Isolate 25, Lane 13 Isolate 26, Lane 14 Isolate 27, Lane 15 Isolate 28. Lane 16 – 20 SE producing controls; Lane 16 SEA producing *S. aureus*, Lane 17 SEB producing *S. aureus*, Lane 18 SEC producing *S. aureus*, Lane 19 SED producing *S. aureus*, Lane 20 SEE producing *S. aureus*).

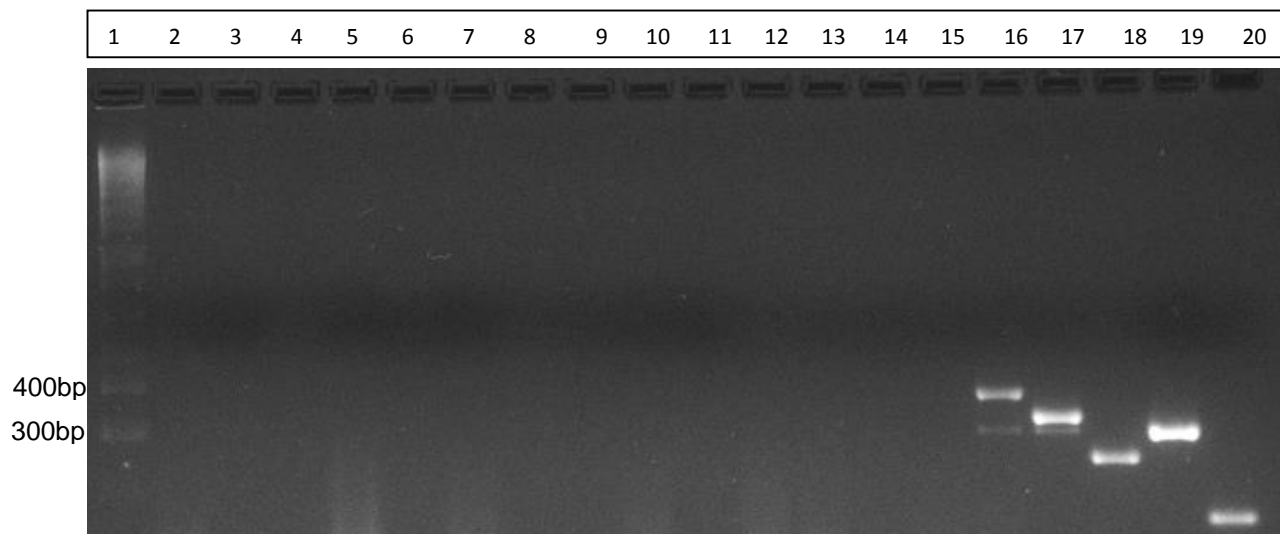


Figure 17. Gel electrophoresis of CNS isolates 29-42 screened for SEA-SEE genes using multiplex PCR. (Lane 1 1Kb ladder, Lane 2 Isolate 29, Lane 3 Isolate 30, Lane 4 Isolate 31, Lane 5 Isolate 32, Lane 6 Isolate 33, Lane 7 Isolate 34, Lane 8 Isolate 35, Lane 9 Isolate 36, Lane 10 Isolate 37, Lane 11 Isolate 38, Lane 12 Isolate 39, Lane 13 Isolate 40, Lane 14 Isolate 41, Lane 15 Isolate 42. Lane 16 – 20 SE producing controls; Lane 16 SEA producing *S. aureus*, Lane 17 SEB producing *S. aureus*, Lane 18 SEC producing *S. aureus*, Lane 19 SED producing *S. aureus*, Lane 20 SEE producing *S. aureus*).

3.3.4 Overall Examination of Enterotoxin Results

Although the Ridascreen® SET A, B, C, D, E (R-Biopharm) immunoassay test kit method describes the use of brain heart infusion broth (BHI) broth for culturing isolates, TSB was used in this study. To confirm that this did not inhibit the production of enterotoxins by the CNS isolates, the *S. aureus* positive controls were grown in both BHI and TSB and put through the immunoassay. Each of the positive controls, S27, S12 and S34, produced detectable levels of their respective enterotoxins, after growth in both BHI and TSB. This confirms that growth in TSB does not affect enterotoxin production.

The Ridascreen® SET A, B, C, D, E (R-Biopharm) immunoassay test kit is prone to cross-reactivity between SEA and SEE, and between SEB and SEC. In this study, S27, a SEA-producing isolate, gave a value above the threshold for the SEA antibody but also reacted with the SEE antibody, when grown in BHI. S12, a SEB-producing isolate, gave a value above the threshold for the SEB antibody and also the SEC antibody when grown in both BHI and TSB. Although the additional reactivity of S27 and S12 to SEE and SEC respectively can be attributed to cross-reactivity, S12 also produced a value above the threshold for SEE. This method therefore identified an additional enterotoxin produced by S12 that was not previously identified using the 3M™ TECRA™ Staph Enterotoxin immunoassay.

The production of SEs by CNS species was not detected using the immunassay; however, these isolates were tested only in pure culture. The production of SEs by these species may occur when tested for directly from food products or when food is artificially contaminated with a CNS isolate at the enterotoxin-producing level (10^3 CFU/mL). The conditions used to manufacture a food product along with the physicochemical conditions of a food may also play a major role in CNS producing enterotoxins.

Although no SE production was detected by the immunoassay, the multiplex PCR sequencing did show that 2 of the 42 CNS isolates analysed possessed SE genes. Other studies have also shown a small proportion of isolates possess SE genes. For example, in the study by Even *et al.* (2010) only one out of 129 CNS isolates was found to possess an enterotoxin gene and in the study by Ruaro *et al.* (2013) none of the 77 CNS isolates from raw milk and cheese possessed an enterotoxin gene.

It is also worth noting that isolate 17 (*S. saprophyticus*) produced a band equivalent to that of SEA gene, the most commonly reported enterotoxin in food and therefore the main cause of *Staphylococcus aureus* food poisoning (Vernozy-Rozand *et al.* 2004; Veras *et al.* 2008; Argudín *et al.* 2010) and isolate 12 (*S. warneri*) produced bands equivalent to both SEB and SED, both of which have also been implicated in staphylococcal food poisoning throughout the world (Argudín *et al.* 2010). Although neither of these isolates produced enterotoxin(s) in the immunoassay if these staphylococci were present in large enough numbers, or exposed to favourable growth temperatures in food ingredients or products, this could result in them producing enterotoxins and could lead to food poisoning of an individual or individuals.

Chapter Four: Summarising Discussion and Conclusion

The third most frequent cause of food poisoning worldwide is due to *Staphylococcus*, with the coagulase positive species, *S. aureus* being identified as the leading cause (Atanassova *et al.* 2001; Veras *et al.* 2008; Podkowik *et al.* 2013). The ability of *S. aureus* to cause food poisoning is due to the production of enterotoxins under favourable conditions. However there is increasing evidence that the coagulase negative staphylococci (CNS) species also possess and are capable of producing these enterotoxins and therefore have been or could potentially be linked to food poisoning outbreaks. While research has been carried out in various countries there is a lack of research into the potential of CNS to cause food poisoning as generally related to food industry and more specifically as linked to dairy in New Zealand.

4.1.1 Identification of coagulase-negative species

A number of dairy products including cheese, butter, milk powder (butter and skim milk), cream, food additives, food bars, curd, yoghurt, dairy spread and UHT milk, were screened in order to isolate staphylococci species. To determine whether these isolates were *S. aureus*, CNS or other species three methods were used, matrix-assisted laser desorption ionisation – time of flight mass spectrometry (MALDI-TOF MS), BBL™ Crystal™ Gram Positive identification system and the Remel RapID™ Staph Plus identification system.

While various studies have used the BBL™ Crystal™ Gram Positive identification system to identify Gram positive species, including staphylococci, the use of the Remel RapID™ Staph Plus identification system to identify and differentiate staphylococci species has not been extensively investigated. However this newer system provides a result within 28 hours (with an incubation time of 4 hours) and includes 40 species of staphylococci compared to the BBL™ Crystal™ Gram Positive

identification system which requires a minimum incubation time of 18 hours therefore results within 48 hours and only includes 25 staphylococci species. The Remel biochemical kit therefore is a fast biochemical alternative to the BBL method and could be a useful method within a microbiology laboratory, particularly clinical. It can be difficult to differentiate between coagulase positive and negative staphylococci species using traditional methods. The use of MALDI-TOF MS could improve rapidity of identification even further providing a fast turnaround time and accurate identity of staphylococci. This could be of significant benefit to both the clinical and food industries, not only in relation to the identification of staphylococci but all bacterial, yeast and mould species in general. However MALDI-TOF MS is not an approved validated method for the food industry, although it is extensively used in clinical settings. Therefore working towards gaining validation of this method for use with food is an important next step in its use in the food industry. This method could be a valuable tool in locating the source of contamination of a product as part of a trace back and identifying staphylococci species responsible for food poisoning outbreaks.

4.1.2 Staphylococcal enterotoxin potential of CNS species

This study used two different PCR methods to determine whether any of the 42 CNS isolates possess an enterotoxin gene. The SE PCR method by Derzelle *et al.* (2009) did not indicate that any of the 42 CNS species analysed possessed enterotoxins SEA, SEB, SED or SEH. However the multiplex PCR method by Park *et al.* (2011) identified 1 species that possesses SED and a second that possesses SEB and SED. The number of isolates identified as possessing one or more of the enterotoxin genes, SEA-SEE, in this study is low, it is still comparative to other studies for example Even *et al.* (2010) and Ruaro *et al.* (2013).

Although 2 CNS isolates were shown to possess enterotoxin genes this was not associated with the production of the enterotoxin *in vitro* (broth culture). However these CNS may be capable of producing their respective SE under the right conditions *in vivo* and while this was not undertaken in this present study it is well worth investigating.

With the increasing evidence of CNS species possessing the enterotoxin genes (Chou & Chen 1997; Cunha *et al.* 2006; Veras *et al.* 2008; Mhlambi *et al.* 2010; Naidoo & Lindsay 2010) and a CNS species being linked to a food poisoning outbreak (Do Carmo *et al.* 2004) this indicates that staphylococci species should not be excluded when testing for SE production in the manufacture of food products. CNS species could therefore be a potential source of enterotoxin production in dairy products; however the risk is likely to be low.

While the risk of food poisoning linked to CNS species is low these staphylococci species have been shown to be a major source of mastitis in dairy herds globally (Zhang & Maddox 2000; Pyorala & Taponen 2009; Tenhagen *et al.* 2009; Sampimon *et al.* 2010) and in New Zealand (Howard 2006). A delay to identify and separate those within the herd suffering from CNS associated mastitis and or even those with an increased CNS population is a potential source of contamination into the milk supply, which under favourable conditions could result in the production of enterotoxins. This could therefore in the future lead to an increase in the risk of CNS to cause food poisoning.

In addition to the CNS mastitis causing species being a potential source of enterotoxin in dairy the consumption of raw unpasteurised milk is increasingly being advocated for in relation to perceived health benefits and improved taste over pasteurised milk. This has resulted in it becoming an increasing consumer trend globally and in New Zealand. With the consumption of raw milk comes the increased risk of exposure to disease causing bacteria, including staphylococci species and this risk is increased further when there is poor processing, hygiene and storage of the milk on farm and then transport, storage and handling by the consumer. This could also serve as another potential source of enterotoxin and therefore increase the risk of food poisoning associated with unprocessed dairy products.

The main methods to detect enterotoxin production available commercially are immunoassay kits and these kits are currently only able to detect enterotoxins SEA-

SEE. MALDI-TOF MS is a potential alternative to these methods and while the use of this method to detect enterotoxins has been studied (Brun *et al.* 2007; Dupuis *et al.* 2008) further research is still required. Therefore MALDI-TOF MS could replace the immunological methods and improve investigations into staphylococcal food poisoning outbreaks breaks (Dupuis *et al.* 2008) by extending to include all currently known SEs and SELs and also as yet unidentified or previously unidentifiable SEs.

4.2 Conclusions

- The Remel RapID™ Staph Plus identification system provides an identification of CNS species that is comparable with that of the BBL™ Crystal™ Gram Positive identification system.
- MALDI-TOF MS is a rapid, reliable and accurate method for identifying CNS definitively to the genus level and, on most occasions, to the species level in dairy products and is therefore a potential alternative to the traditional phenotypic, such as commercial identification kits, and genotypic, such as sequencing, methods that are currently used.
- SE production *in vitro* and in TSB broth by CNS was not detected using the immunoassay method, Ridascreen® SET A, B, C, D, E.
- None of the CNS isolates were found to possess an enterotoxin gene when using the single SE PCR method. However using the multiplex PCR method one isolate was found to possess both the SEB and SED genes, and another the SEA gene.
- The possession of enterotoxin genes by two CNS isolates indicates that coagulase producing *Staphylococcus aureus* species are not the only potential source of enterotoxin production in dairy products, although the risk is likely low.

4.3 Limitations of the Present Study

- This study only screened dairy products from one dairy season, therefore only gives a snapshot of CNS isolates present within the New Zealand dairy industry.

4.4 Future Work

- The enterotoxin immunoassay could be repeated for all 42 CNS *in vivo* i.e. within various dairy products.
- 16S rDNA sequencing could be completed for all 42 CNS isolates.
- The multiplex PCR screening for SEA-SEE genes could be extended to include the remaining 101 CNS isolated from the dairy products in this study.
- Multiplex PCR analysis could be extended to include SEG-SEI and SEJ-SELU genes using the method by Park *et al.* (2011).

References

- Alexopoulou, K., Foka, A., Petinaki, E., Jelastopulu, E., Dimitracopoulos, G., and Spiliopoulou, I. (2006) Comparison of two commercial methods with PCR restriction fragment length polymorphism of the *tuf* gene in the identification of coagulase-negative staphylococci. *Letters in Applied Microbiology* **43**: 450–454.
- Anhalt, J.P., and Fenselau, C. (1975) Identification of bacteria using mass spectrometry. *Analytical Chemistry* **47**: 219–225.
- Argudín, M.Á., Mendoza, M.C., and Rodicio, M.R. (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* **2**: 1751–1773.
- Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H., and Kozaki, S. (2003) An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiology and Infection* **133**: 33–40.
- Atanassova, V., Meindl, A., and Ring, C. (2001) Prevalence of *Staphylococcus aureus* and staphylococcal enterotoxins in raw pork and uncooked smoked ham – a comparison of classical culturing detection and RFLP-PCR. *International Journal of Food Microbiology* **68**: 105–113.
- Baird-Parker, A.C. (1962) An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *Journal of Applied Bacteriology* **25**: 12-19.
- Balaban, N., and Rasooly, A. (2000) Staphylococcal enterotoxins. *International Journal of Food Microbiology* **61**: 1–10.
- Bartolomeoli, I., Maifreni, M., Frigo, F., Urli, G., and Marino, M. (2009) Occurrence and characterization of *Staphylococcus aureus* isolated from raw milk for cheesemaking. *International Journal of Dairy Technology* **62**: 366–371.
- Bautista, L., Gaya, P., Medina, M., and Nuñez, M. (1988) A quantitative study of enterotoxin production by sheep milk staphylococci. *Applied and Environmental Microbiology* **54**: 566–569.

Bernardo, K., Pakulat, N., Macht, M., Krut, O., Seifert, H., Fler, S., Hüniger, F., and Krönke, M. (2002) Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Proteomics* **2**: 747–753.

Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F., and Garin, J. (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. *Molecular and Cellular Proteomics* **6**: 2139–2149.

Carbonnelle, E., Beretti, J.-L., Cottyn, S., Quesne, G., Berche, P., Nassif, X., and Ferroni, A. (2007) Rapid identification of staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology* **45**: 2156–2161.

Carbonnelle, E., Grohs, P., Jacquier, H., Day, N., Tenza, S., Dewailly, A., Vissouarn, O., Rottman, M., Herrmann, J.L., Podglajen, I., and Raskine, L. (2012) Robustness of two MALDI-TOF mass spectrometry systems for bacterial identification. *Journal of Microbiological Methods* **89**: 133–136.

Carpaij, N., Willems, R.J.L., Bonten, M.J.M., and Fluit, A.C. (2011) Comparison of the identification of coagulase-negative staphylococci by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and *tuf* sequencing. *European Journal of Clinical Microbiology and Infectious Diseases* **30**: 1169–1172.

Chou, C.-C., and Chen, L.-F. (1997) Enterotoxin production by *Staphylococcus warneri* CCRC 12929, a coagulase-negative strain. *Journal of Food Protection* **60**: 923–927.

Çitak, S., Varlik, Ö., and Gündoğan, N. (2003) Slime production and DNase activity of staphylococci isolated from raw milk. *Journal of Food Safety* **23**: 281–288.

Clark, A.E., Kaleta, E.J., Arora, A., and Wolk, D.M. (2013) Matrix-assisted laser desorption ionisation-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clinical Microbiology Reviews* **26**: 547-603.

Colombari, V., Mayer, M.D.B., Laicini, Z.M., Mamizuka, E., Franco, B.D.G.M., Destro, M.T., and Landgraf, M. (2007) Foodborne outbreak caused by *Staphylococcus aureus*: phenotypic and genotypic characterization of strains of food and human sources. *Journal of Food Protection* **70**: 489–493.

Coton, E., Desmonts, M.H., Leroy, S., Coton, M., Jamet, E., Christieans, S., Donnio, P.Y., Lebert, I., and Talon, R. (2010) Biodiversity of coagulase-negative staphylococci in French cheeses, dry fermented sausages, processing environments and clinical samples. *International Journal of Food Microbiology* **137**: 221–229.

Croxatto, A., Prod'hom, G., and Greub, G. (2012) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews* **36**: 380–407.

Cunha, M.L.R.S., Rugolo, L.M.S.S., and Lopes, C.A.M. (2006) Study of virulence factors in coagulase-negative staphylococci isolated from newborns. *Memórias do Instituto Oswaldo Cruz* **101**: 661–668.

d'Azevedo, P.A., Comin, G., and Cantarelli, V. (2010) Characterization of a new coagulase negative *Staphylococcus* species (*Staphylococcus pettekoferi*) isolated from blood cultures from a hospitalized patient in Porto Alegre, Brazil. *Revista da Sociedade Brasileira de Medicina Tropical* **43**: 331–332.

Derzelle, S., Dilasser, F., Duquenne, M., and Deperrois, V. (2009) Differential temporal expression of the staphylococcal enterotoxins genes during cell growth, *Food Microbiology* **26**: 896–904.

Do Carmo, L.S., Cummings, C., Linardi, V.R, Dias, R.S., De Souza, J.M., De Sena, M.J., Dos Santos, D.A., Shupp, J.W., Pereira, R.K.P., and Jett, M. (2004) A case study of a massive staphylococcal food poisoning incident. *Foodborne Pathogens and Diseases* **1**: 241–246.

Dubois, D., Leysse, D., Chacornac, J.P., Kostrzewa, M., Schmit, P.O., Talon, R., Bonnet, R., and Delmas, J. (2010) Identification of a variety of *Staphylococcus* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology* **48**: 941–945.

Dupont, C., Sivadon-Tardy, V., Bille, E., Dauphin, B., Beretti, J.L., Alvarez, A.S., Degand, N., Ferroni, A., Rottman, M., Herrmann, J.L., Nassif, X., Ronco, E., and Carbonnelle, E. (2010) Identification of clinical coagulase-negative staphylococci, isolated in microbiology laboratories, by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and two automated systems. *Clinical Microbiology and Infection* **16**: 998–1004.

Dupuis, A., Hennekinne, J.A., Garin, J., and Brun, V. (2008) Protein standard absolute quantification (PSAQ) for improved investigation of staphylococcal food poisoning outbreaks. *Proteomics* **8**: 4633–4636.

El Bouri, K., Johnston, S., Rees, E., Thomas, I., Bonne-Mannathoko, N., Jones, C., Reid, M., Ben-Ismaeil, B., Davies, A.R., Harris, L.G., and Mack, D. (2012) Comparison of bacterial identification by MALDI-TOF MS mass spectrometry and conventional diagnostic microbiology methods: agreement, speed and cost implications. *British Journal of Biomedical Science* **69**: 47–55.

Erickson, M.C. (2003) *Recognition and prevention of staphylococcal enterotoxins as intentional contaminants of foods*. Center for Food Safety, College of Agricultural and Environmental Studies, University of Georgia, Griffin, Georgia.

Even, S., Leroy, S., Charlier, C., Zakour, N.B., Chacornac, J.P., Lebert, I., Jamet, E., Desmots, M.H., Coton, E., Pochet, S., Donnio, P.Y., Gautier, M., Talon, R., and Le Loir, Y. (2010) Low occurrence of safety hazards in coagulase negative staphylococci isolated from fermented foodstuffs. *International Journal of Food Microbiology* **139**: 87–95.

Ferreira, L., Sánchez-Juanes, F., Porrás-Guerra, I., García-García, M.I., García-Sánchez, J.E., González-Buitrago, J.M., and Muñoz-Bellido, J.L. (2011) Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clinical Microbiology and Infection* **17**: 546–551.

Fox, K., Fox, A., Elssner, T., Feigley, C., and Salzberg, D. (2010) MALDI-TOF mass spectrometry speciation of staphylococci and their discrimination from micrococci isolated from indoor air of schoolrooms. *Journal of Environmental Monitoring* **12**: 917–923.

Fox, K., Fox, A., Rose, J., and Walla, M. (2011) Speciation of coagulase negative staphylococci, isolated from indoor air, using SDS PAGE gel bands of expressed proteins followed by MALDI TOF MS and MALDI TOF-TOF MS-MS analysis of tryptic peptides. *Journal of Microbiological Methods* **84**: 243–250.

Gaillot, O., Wetsch, M., Fortineau, N., and Berche, P. (2000) Evaluation of CHROMagar Staph. aureus, a new chromogenic medium, for isolation and presumptive identification of *Staphylococcus aureus* from human clinical specimens. *Journal of Clinical Microbiology* **38**: 1581–1591.

Grinberg, A., Hittman, A., Leyland, M., Rogers, L., and Le Quesne, B. (2004) Epidemiological and molecular evidence of a monophyletic infection with *Staphylococcus aureus* causing a purulent dermatitis in a dairy farmer and multiple cases of mastitis in his cows. *Epidemiology and Infection* **132**: 507–513.

Hennekinne, J.A., Brun, V., De Buyser, M.L., Dupuis, A., Ostyn, A., and Dragacci, S. (2009) Innovative application of mass spectrometry for the characterization of staphylococcal enterotoxins involved in food poisoning outbreaks. *Applied and Environmental Microbiology* **75**: 882–884.

Howard, P. (2006) Mastitis pathogens present in bulk tank milk from seven dairy herds in the Waikato region, New Zealand. *New Zealand Veterinary Journal* **54**: 41–43.

Hudson J. A., and On, S. (2010) *Evaluation of methods for detection of coagulase positive Staphylococcus and staphylococcal toxin in milk and cheese*. Client Report FW 10013. Institute of Environmental Science and Research Limited, Wellington.

Institute of Environmental Science and Research Limited (2001) *Staphylococcus aureus*. Ministry of Health Report, New Zealand. [WWW document]. URL http://www.foodsafety.govt.nz/elibrary/industry/Staphylococcus_Aureus-Science_Research.pdf. Retrieved 10 January 2011.

International Organization for Standardization (2001) *Milk and milk products – General guidance for the preparation of test samples, initial suspensions and decimal dilutions for*

microbiological examination. ISO 8261:2001 (IDF: 2001). International Organization for Standardization, Geneva.

Irlinger, F. (2008) Safety assessment of dairy microorganisms: coagulase-negative staphylococci. *International Journal of Food Microbiology* **126**: 302–310.

Jarvis, A.W., and Lawrence, R.C. (1970) Enterotoxigenic staphylococci in New Zealand. *New Zealand Medical Journal* **72**: 328–331.

Jarvis, A.W., and Lawrence, R.C. (1971) Production of extracellular enzymes and enterotoxins A, B, and C by *Staphylococcus aureus*. *Infection and Immunity* **4**: 110–115.

Keys, C.J., Dare, D.J., Sutton, H., Wells, G., Lunt, M., McKenna, T., McDowall, M., and Shah H.N. (2004) Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterisation of bacteria implicated in human infectious diseases. *Infection, Genetics and Evolution* **4**: 221–242.

Kloos, W.E., and Bannerman, T.L. (1994) Update on clinical significance of coagulase-negative staphylococci. *Clinical Microbiology Reviews* **7**: 117–140.

Larkin, E.A., Carman, R.J., Krakauer, T., and Stiles, B.G. (2009) *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. *Current Medicinal Chemistry* **16**: 4003–4019.

Le Loir, Y., Baron, F., and Gautier, M. (2003) *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research* **2**: 63–76.

Loonen, A.J.M., Jansz, A.R., Bergland, J.N.B., Valkenburg, M., Wollfs, P.F.G., and van den Brule, A.J.C. (2012) Comparative study using phenotypic, genotypic, and proteomics methods for identification of coagulase-negative staphylococci. *Journal of Clinical Microbiology* **50**: 1437–1439.

Martin, B., Garriga, M., Hugas, M., Bover-Cid, S., Veciana-Nogues, M. T., and Aymerich, T. (2006) Molecular, technological and safety characterization of Gram-positive catalase-positive cocci from slightly fermented sausages. *International Journal of Food Microbiology* **107**: 148–158.

Matsuda, N., Matsuda, M., Notake, S., Yokokawa, H., Kawamura, Y., Hiramatsu, K., and Kikuchi, K. (2012) Evaluation of a simple protein extraction method for species identification of clinically relevant staphylococci by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology* **50**: 3862–3866.

Mhlambi, S.G., Naidoo, K., and Lindsay, D. (2010) Enterotoxin-producing *Staphylococcus* strains associated with South African *biltong* at point of sale. *Journal of Food Safety* **30**: 307–317.

Naidoo, K., and Lindsay, D. (2010) Pathogens associated with *biltong* product, and their *in vitro* survival of hurdles used during production. *Food Protection Trends* **30**: 532–538.

Pinchuk, I.V., Beswick, E.J., and Reyes, V.E. (2010) Staphylococcal enterotoxins. *Toxins* **2**: 2177–2197.

Podkowik, M., Park, J.Y., Seo, K.S., Bystron, J., and Bania, J. (2013) Enterotoxigenic potential of coagulase-negative staphylococci. *International Journal of Food Microbiology* **65**: 34-40.

Pyorala, R., and Taponen, S. (2009) Coagulase-negative staphylococci – emerging mastitis pathogens. *Veterinary Microbiology* **134**:3-8.

Rajakaruna, L., Hallas, G., Molenaar, L., Dare, D., Sutton, H., Encheva, V., Culak, R., Innes, I., Ball, G., Sefton, A.M., Eydmann, M., Kearns, A.M., and Shah, H.N. (2009) High throughput identification of clinical isolates of *Staphylococcus aureus* using MALDI-TOF-MS of intact cells. *Infection, Genetics and Evolution* **9**: 507–513.

Rall, V.L.M., Sforcin, J.M., de Deus, M.F.R., de Sousa, D.C., Camargo, C.H., Godinho, N.C., Galindo, L.A., Soares, T.C.S., and Araújo, J.P. Jr. (2010) Polymerase chain reaction detection of enterotoxins genes in coagulase-negative staphylococci isolated from Brazilian Minas cheese. *Foodborne Pathogens and Disease* **7**: 1121 – 1123.

Rodríguez, M., Núñez, F., Córdoba, J.J., Bermúdez, E., and Asensio, M.A. (1996) Gram-positive, catalase-positive cocci from dry cured Iberian ham and their enterotoxigenic potential. *Applied and Environmental Microbiology* **62**: 1897–1902.

Romero-Gómez, M.P., Gómez-Gil, R., Paño-Pardo, J.R., and Mingorance, J. (2012) Identification and susceptibility testing of microorganism by direct inoculation from positive blood culture bottles combining MALDI-TOF and Vitek-2 Compact is rapid and effective. *Journal of Infection* **65**: 513–520.

Ruaro, A., Andrighetto, C., Torriani, S., and Lombardi, A. (2013) Biodiversity and characterisation of indigenous coagulase-negative staphylococci isolated from raw milk and cheese of North Italy. *Food Microbiology* **34**: 106-111.

Sampimon, O., van den Borne, B.H.P., Santman-Berends, I., Barkema H.W., and Lam, T. (2010) Effects of coagulase-negative staphylococci on somatic cell count in Dutch dairy herds. *Journal of Dairy Research* **77**:318-324.

Seng, P., Drancourt, M., Gouriet, F., La Scola, B., Fournier, P.E., Rolain, J.M., and Raoult, D. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases* **49**: 543–551.

Silva, B.O., Caraviello, D.Z., Rodrigues, A.C., and Ruegg, P.L. (2005) Evaluation of Petrifilm for the isolation of *Staphylococcus aureus* from milk samples. *Journal of Dairy Science* **88**: 3000-3008.

Takahashi, T., Satoh, I., and Kikuchi, N. (1999) Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. *International Journal of Systematic Evolutionary Microbiology* **49**:725-728.

Tenhagen, B.A., Hansen, I., Reinecke, A., and Heuwieser, W. (2009) Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition. *Journal of Dairy Research* **76**:179-187.

Udo, E.E., Al-Bustan, M.A., Jacob, L.E., and Chugh, T.D. (1999) Enterotoxin production by coagulase-negative staphylococci in restaurant workers from Kuwait City may be a potential cause of food poisoning. *Journal of Medical Microbiology* **48**: 819–823.

Vasconcelos, N.G., and Cunha, M.L.R.S. (2010) Staphylococcal enterotoxins: molecular aspects and detection methods. *Journal of Public Health and Epidemiology* **2**: 29–42.

Veras, J.F., do Carmo, L.S., Tong, L.C., Shupp, J.W., Cummings, C., Dos Santos, D.A., Cerqueira, M.M.O.P., Cantini, A., Nicoli, J.R., and Jett, M. (2008) A study of the enterotoxigenicity of coagulase-negative and coagulase-positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil. *International Journal of Infectious Diseases* **12**: 410–415.

Vernozy-Rozand, C., Mazuy, C., Prevost, G., Lapeyre, C., Bes, M., Brun, Y., and Fleurette, J. (1996) Enterotoxin production by coagulase-negative staphylococci isolated from goats' milk and cheese. *International Journal of Food Microbiology* **30**: 271–280.

Vernozy-Rozand, C., Mazuy-Cruchaudet, C., Bavai, C., and Richard, Y. (2004) Comparison of three immunological methods for detecting staphylococcal enterotoxins from food. *Letters in Applied Microbiology* **39**: 490–494.

Von Baum, H., Klenne, F.R., Geiss, H.K., and Sonntag, H.G. (1998) Comparative evaluation of a commercial system for identification of Gram-positive cocci. *European Journal of Clinical Microbiology and Infectious Diseases* **17**: 849-852.

Wieneke, A. A., Roberts, D., and Gilbert, R.J. (1993) Staphylococcal food poisoning in the United Kingdom, 1969-90. *Epidemiology and Infection* **110**: 519–531.

Wieser, A., Schneider, L., Jung, J., and Schubert, S. (2012) MALDI-TOF MS in microbiological diagnostics – identification of microorganisms and beyond (mini review). *Applied Microbiology and Biotechnology* **93**: 965–974.

Zhang, S., and Maddox, C.W. (2000) Cytotoxic activity of coagulase-negative staphylococci in bovine mastitis. *Infection and Immunity* **68**:1102-1108.

Zell, C., Resch, M., Rosenstein, R., Albrecht, T., Hertel, C., and Götz, F. (2008) Characterization of toxin production of coagulase-negative staphylococci isolated from food and starter cultures. *International Journal of Food Microbiology* **127**: 246–251.