Chapter I

Introduction, problem statement and literature review

1. Introduction

Food safety and security presents a continual challenge for the food industry, food and biological scientist, as well as the legislating authority (Holzapfel, Geisen and Schillinger, 1995). Although modern technologies and safety concepts like HACCP have been introduced, cases of food poisoning, intoxication, and spoilage are continually increasing (Holzapfel *et al.*, 1995; Meng and Doyle, 2002).

In the United States of America, USA, food borne illnesses account for 76 million reported cases at the Centre of Disease and Prevention (Cleveland, Montville, Nes, and Chikandas, 2001). Government expenditure on food borne illnesses caused by *Campylobacter jejuni, Clostridium perfringens, Escherichia coli* 0157:H7, *Listeria monoctogenes, Salmonella* species, *Staphylococcus aureus* and *Toxaplsma gondii* was estimated at US$ 6.5-34 million. As a result of these reports and the high government expenditure there is increased pressure on the need to control pathogenic and spoilage bacteria in the USA (Cleveland *et al.*, 2001). In Europe, the same pathogenic microorganisms show a similar pattern. The three major pathogens that cause food borne illnesses are, *E. coli* 0157, *Salmonella* species and *Campylobacter* (Postnote, 1997).

The concept of emerging pathogens, which is described by the survival and growth of pathogens under conditions that had been previously considered to be safe, has prompted the food industry, the public and governments to question the effectiveness of the presently used food preservation methods (Cleveland *et al.*, 2001; Meng and Doyle, 2002). The survival and growth of food pathogens like *L. monocytogenes* and *B. cereus* under refrigeration temperatures of 4 and 8 °C respectively challenges the low temperature food preservation whilst posing a potential microbial hazard amongst consumers (Jay, 2000; Kotiranta, Lounatmaa and Haapasalo, 2000).

Amongst food consumers the increased consumption of food formulated with certain chemical preservatives e.g. sulphites and nitrates has raised concerns over the possible adverse health effects of these substances (Roller 1995). These concerns have been accompanied by the increasing demand for convenient foods with extended shelf life (Caplice and Fitzgerald, 1999). These demands in turn, have increased pressure on food manufactures to remove chemically synthesized additives from processed food

and to provide more natural alternatives for maintaining the safety and extending the shelf life of food (Roller, 1995: Cleveland *et al.*, 2001). As an alternative to chemical preservatives, interest has been generated in the potential of naturally produced antimicrobial agents such as bacteriocins from lactic acid bacteria, LAB (Rodriguez, Martienz, Hom and Dodd, 2003). Bacteriocins are antimicrobial peptides that exert antagonism against closely related LAB, Gram-positive spoilage and pathogenic bacteria (Rodriguez *et al.*, 2003)

Food preservation by LAB can be attributed to the reduction or lowering of pH that extends the shelf life of fermented food through the destruction of putrefactive, pathogenic and toxigenic bacteria thereby improving the microbial quality of food (Holazapfel *et al.*, 1995). In addition, LAB improve the sensory attributes and commercial value of products like cheese (Ross, Morgan and Hill, 2002; Rodriguez *et al.*, 2003).

Two groups of LAB occur in bacterial ripened cheeses. These are the starter culture LAB and the non-starter lactic acid bacteria, NSLAB (Peterson and Marshall, 1990). The latter group is composed of species lactobacilli, pediococci and micrococci that work in synergy to affect the typical cheese flavour in hard and semi-hard cheeses (Franklin and Sharpe, 1963; Jordan and Cogan, 1993; Beresford, 2003).

Pediococci exhibit antimicrobial activity through the production of lactic acid. However, some strains of *Pediococcus* species produce antimicrobial peptides, bacteriocins known as pediocins. The isolation of bacteriocin producing strains of *Pediococcus* species or the use of purified bacteriocin may offer an additional hurdle in food preservation (Roller 1995; Ross *et al.*, 2002). This study was conducted to determine and identify species of pediocin producing pediococci that may occur in South African farm-style cheeses produced under traditional, less commercialised conditions with or without the use of starter culture.

1.2. Literature review

1.2.1. Cheese ripening

Cheese ripening is a complex process that results in the maturation and development of flavour within bacterial ripened cheeses. Ripening begins with the primary changes in the curd that involve the breakdown of carbohydrates, proteins and fat (Franklin and Sharpe, 1963; Bhowmik, Riesterer, Van Boekel and Marth, 1990). These changes are followed up by secondary changes that involve the transformation of the primary products into amines, organic acids, sulphur compounds, ketones, lactone aldehyde and trans fatty acids (Soda, 1993; Bhowmik *et al.*, 1990; Rehman, Fox and McSweeney, 2000a; Beresford, 2003).

As previously defined LAB occur in two groups in cheese. Firstly LAB are deliberately added as starter culture to the cheese milk, alternatively LAB occur as the adventitious contaminant NSLAB (Peterson and Marshall, 1990). During cheese ripening LAB are essential because they provide proteolytic and lypolytic enzyme systems required for cheese ripening (Bhowmik *et al.*, 1990; Soda, 1993; Fox, McSweeney and Lynch, 1998; Rehman *et al.*, 2000a). Apart from the enzyme systems, LAB provide conditions favourable for the cheese ripening through the reduction in pH and the electrode potential (Soda, 1993; Beresford, Fitzsimons, Brennan and Cogan, 2001).

1.2.2. Non-starter lactic acid bacteria, NSLAB

In cheese ripening NSLAB originate from the factory environment, processing equipment and the raw milk as adventitious contaminants (Peterson and Marshall, 1990; Martley and Crow, 1993). The composition of the lactic microflora is heterogeneous. Broadbent, Brotherson, Johnson and Oberg, 2002, attributed the diversity in LAB to the influence of milk heat treatment, plant sanitation and the native microbiota of the plants and soil in the region. In their work Broadbent *et al.*, 2002, further explained that more diversity and complexity exists amongst the lactic microflora in bacterial ripened cheese produced using raw milk. Isolation of NSLAB has identified facultative heterofermentative mesophillic lactobacilli and less frequently pediococci in Cheddar and other bacterial ripened cheeses (Bhowmik and Marth, 1990; Peterson and Marshall, 1990; Rehman, Banks, McSweeney and Fox,

2000b; Beresford, 2003). Early reports on the influence of pediococci among the NSLAB showed increases in the flavour score of Cheddar cheese (Franklin and Sharpe, 1963; Fryer and Sharpe, 1966). In later reports no individual role has been ascribed to pediococci, instead it thought to form part of the complex microflora that acts synergistically to effect flavour development (Law, Castanon and Sharpe, 1976; Tzanetakis and Litopoulou-Tzanetaki 1989a; Beresford, 2003).

1.2.2.1. Characterisation of non-starter lactic acid bacteria, NSLAB

Characterisation of NSLAB involves the isolation and phenotypic characterisation of LAB from cheese on either Rogasa agar (Rogosa, Mitchell and Wiseman, 1951) or MRS agar (de Man, Rogosa and Sharpe, 1960). The colonies are microscopically examined and subsequently identified to species level through physiological and morphological characterisation as well as through carbohydrate fermentation (Crow, Curry and Hayes, 2001). NSLAB initially begin at low numbers of 10^2 to 10^3 cfu/g (Crow *et al.*, 2001). The population of NSLAB increases to cell densities greater than 10^7 cfu/g in Cheddar, Figure 1, and in other cheeses ripened for more than three or four months (Kosikowski and Mistry, 1999a; Rehman *et al*, 2000a). The method of cultivation and phenotypic characterisation of NSLAB provides an overview of the NSLAB selected for the given media (Broadbent *et al.*, 2002). Phenotypic characterisation may be unreliable since the phenotype character depends on the culture and the environmental conditions used in the assay. Another setback of phenotypic characterisation is the failure to identify the species to strain level (Beresford *et al.*, 2001; Crow *et al.*, 2001).

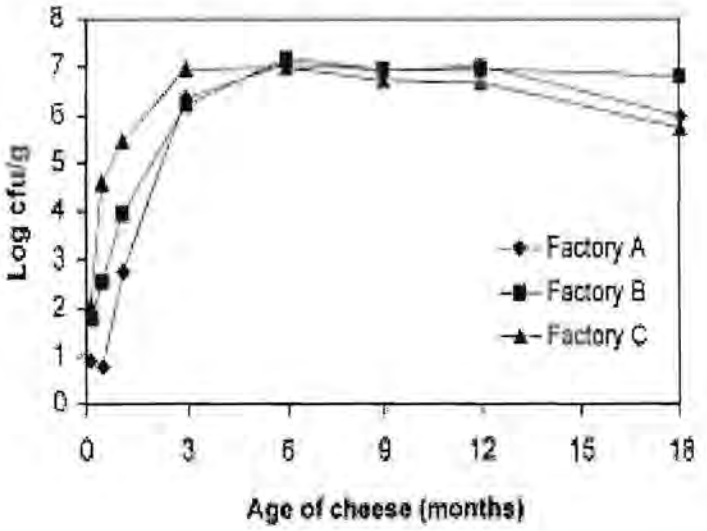

Figure 1. Non-starter lactic acid bacteria cell density in New Zealand Cheddar cheese over a period of 18 months (Crow *et al.*, 2001)

1.2.3. Factors influencing growth of non-starter lactic acid bacteria

Use of either raw or heat treated milk is a major influential factor on the growth of NSLAB in cheese (Franklin and Sharpe, 1963). Severe heat treatment reduces the incidence of NSLAB while raw milk introduces a higher diversity of the LAB in the cheese (Franklin and Sharpe, 1963; Broadbent *et al.*, 2002). The growth of secondary microflora is also influenced by intrinsic properties like pH, water activity (a_w), substrate availability and extrinsic factors like temperature, relative humidity and atmospheric properties of the cheese (Soda, 1993; Fox *et al.*, 1998). In an earlier report by Fox *et al.*, 1998, temperature was noted as the most influential factor on the growth of NSLAB, as higher counts of NSLAB were observed at 8 °C compared to 1 °C. Later Broadbent *et al.*, 2002, showed that amongst both extrinsic and intrinsic factors influencing growth of secondary microflora, NSLAB were mostly influenced by temperature, pH, moisture, salt in moisture, substrate availability, presence of antimicrobials, preservatives and the oxidation reduction potential of the cheese.

1.2.3.1 Moisture

The moisture content of cheese determines the microflora growing on cheese. The water activity across the entire cheese structure is uneven with the centre usually having a higher water activity compared to the periphery (Kosikowski and Mistry, 1999b). The initial a_w, at the manufacture of cheese is approximately, 0.99, this is

sufficient for the growth of LAB (Beresford *et al.*, 2001). Processing of cheese through whey drainage, salting and during ripening reduces the water activity to range between 0.917-0.988 (Rüegg and Blanc, 1981), which results in the decrease in the population of the starter culture. Reducing the water activity beyond the optimum requirement of LAB will influence the metabolic activity and the multiplication of LAB (Beresford *et al.*, 2001).

1.2.3.2. Salt

Sodium chloride reduces the water activity of the cheese thereby contributing towards the inhibition of LAB. However, it is the percentage of salt dissolved in moisture that inhibits growth of bacteria as opposed to actual salt added (Rapposch, 1997). Normally salted cheeses have a salt content ranging from 0.7-7g/100g of cheese (Beresford *et al.*, 2001). In Cheddar cheese the percentage salt normally ranges between 1.5 to 2.0 % (Kosikowski and Mistry, 1999b).

1.2.3.3. Redox potential

Growth of NSLAB like all other microbes is either enhanced or restricted by a positive or negative oxidation-reduction potential E_h (Broadbent *et al.*, 2002). In the development of cheese flavour the changes in the E_h are essential. Kristoffersen, 1967, reported an initial reduction in the E_h followed by a slight increase and a further decline of the E_h. The initial decrease was attributed to oxidative fermentation of residual lactose by LAB while the latter drop in E_h was attributed to the growth and metabolic activity of NSLAB. In bacterial ripened cheese the E_h is normally –250 mV. Such an oxidised interior facilitates the growth of obligatory or facultative anaerobic microorgamisms (Beresford *et al.*, 2001).

1.2.3.4. pH

The growth of bacteria is favoured by neutral pH. Below pH 5 growth is restricted to some acid tolerant bacteria. Production and accumulation of lactic acid and other organic acids within the curd lowers the pH to a range of 4.5-5.3 (Lane, Fox, Walsh, Folkertsma and McSweeney, 1997).

1.2.3.5. Substrate availability

The substrate utilized by NSLAB as energy source has not been fully elucidated (William, Withers and Banks, 2000). In studies with lactobacilli species isolates from cheese, William *et al.*, 2000, reported that the source of energy was derived from lactic acid, citric acid and carbohydrate moieties from glycoproteins, as well as fatty acids, glycerol and amino acids. In other reports, Rapposch, Eliskases-Lechner and Ginzinger, 1999, suggested that autolysis of starter culture was a probable source of ribose and other essential nutrients for the growth of NSLAB.

1.2.3.6. Ripening temperature

The ripening temperature of cheese should be a compromise between the ideal temperature for optimum flavour development and the temperature needed to prevent growth of spoilage microbes (Gilles and Fryer, 1984; Beresford *et al.*, 2001; Crow *et al.*, 2001). Ripening of Cheddar at different temperatures allows the variation in the population of the NSLAB. A ripening temperature of 15 °C was shown to promote accelerated growth in NSLAB from an initial population of 10^2 cfu/g to a 10^7 cfu/g after two months. At a temperature of 10 °C the increase in the population of NSLAB was slower, after two months the population of NSLAB was 10^6 cfu/g and levels of 10^7 cfu/g were attained after the third month (Rehman *et al.*, 2000a). At temperatures above 15 °C, Cromie *et al.*, 1987, reported an increase in the population of NSLAB within the first four weeks; however, the microbial quality of the Cheddar may be compromised by the occurrence of spoilage microbes (Fox *et al.*, 1998).

1.2.4. Characteristics of *Pediococcus* genus

The *Pediococcus* genus is composed of Gram-positive, non-sporeforming facultative anaerobic acid producing bacteria (Garvie, 1986). Several species constitute the *Pediococcus* genus. They differ in morphological, nutritional, physiological and genetic characteristics (Weiss, 1992).

1.2.4.1. Habitats

Among the *Pediococcus* genus, strains of *P. pentosaceus* and *P. acidilactici* commonly occur on plant material at low microbial counts. However, their numbers increase as part of LAB during early fermentation of vegetables (Dellaglio, Vescovo, Morelli and Torriani, 1984). Strains of other species like *P. parvulus, P. inopintus* and

P. dextrinicus also occur as part of the vegetative flora, while strains of *P. dextrinicus* and *P. damanosus* constitute part of LAB responsible for wine or beer spoilage (Strasser de Saad and Manca de Nadra, 1993). In other foods pediococci are closely associated with proteinious food i.e. meat and fish (Johnson and Steele, 1997; Buckenhuskes, 1997). In cheese pediococci occur as part of the secondary lactic flora (Darce, 1958a, b; Peterson and Marshall, 1990). The occurrence of this genus in vegetables and meat as well as its potential as bio-preservative has resulted in the utilisation of the genus in the industrial fermentation of meat and vegetables (Johnson and Steele, 1997; Buckenhuskes, 1997).

1.2.4.2. Morphological properties

The *Pediococcus* genus has been identified as a member of the Streptococcaceae family (Raccach, 1987). Gunther and White, 1961, described cells of pediococci as being spherical, uniform in size and uniquely dividing along two planes at right angle to form tetrads under favourable conditions (Garvie, 1986). In cases where the tetrad structures are not visible, cultures usually occur as single cells, pairs or short chains formed from pairs of cells as well as irregular clusters that have a diameter of 0.36-2.0 μm (Weiss, 1992; Simpson and Taguchi, 1998; Facklam, 2001).

Colonies of pediococci vary in size across a range of 1.0-2.5 mm. The colony morphology is mostly characterised by a smooth, round and greyish white colour (Garvie, 1986). Some species of pediococci are facultatively anaerobic while others are microaerophilic (Raccach, 1987; Weiss, 1992). Amongst the *Pediococcus* species, *P. damanosus* and *P. parvulus* are more anaerobic whilst *P. urinaeequi* is aerobic. However, both *P. pentosaceus* and *P. acidilacitici* can grow under facultatively anaerobic conditions (Garvie, 1986; Raccach, 1987; Weiss, 1992).

1.2.4.3. Physiology

Species of pediococci can be identified through the determination of the range of temperature, pH and salt, NaCl, at which growth occurs (Garvie, 1986). Further identification can be done through measuring physiological characteristics like carbohydrate fermentation, hydrolysis of arginine and the isomer(s) of lactic acid produced (Weiss, 1992)

Table 1. Characteristics differentiating the species of *Pediococcus*[a] (adapted from Garvie, 1986 and Raccach[b], 1987; 1999)

Character	*P. damanosus*	*P. parvulus*	*P. inopinatus*	*P. dextrinicus*	*P. pentosaceus*	*P. acidilactici*	*P. halophilus*	*P. urinaeequi*
Growth at								
35 °C	-	+	+	+	+	+	+	+
40 °C	-	-	-	+	+	+	-	+
50 °C	-	-	-	-	-	+	-	-
Heat resistance[b]	-	-	-	-	***d***	+	-	-
Growth at								
pH 4.2	+	+	-	-	+	+	-	-
pH 7.5	-	+	***d***	+	+	+	+	+
pH 8.5	-	-	-	-	***d***	***d***	+	+
Growth at								
4 % NaCl (w/v)	-	+	+	+	+	+	***d***	+
6.5 % NaCl	-	+	***d***	-	+	+	+	+
18 % NaCl	-	-	-	-	-	-	+	-

[a] Symbols: +: 90 % or more strains are positive

d: 11-89 % of strains are positive

-: 90 % or more of strains are negative

Most species of pediococci grow well at 30 °C, however the optimum range stretches from 25-40 °C (Garvie, 1986). Compared to *P. acidilactici* that has an optimum growth temperature of 40 °C, *P. pentosaceus* grows optimally across a range of 28-32 °C. However strains of *P. acidilactici* are cable of growing at temperatures of 50 °C (Weiss, 1992; Raccach, 1999). The optimum pH for growth of pediococci lies between 6.0-6.5. At pH 4.2 half of the species will grow, whilst at pH 7 *P. damanosus* is the only species that will not grow (Garvie, 1986; Weiss, 1992). In the presence of sodium chloride all species of pediococci with the exception of *P. damanosus* can grow at salt level of 4.5 and 6.5 % (w/v), however at salt concentrations of 10 % (w/v) none of the species can grow with the exception of *P. halophilus* that has maximum tolerance of 18 % (w/v) (Garvie, 1986; Raccach, 1987; Weiss, 1992; Simpson and Taguchi, 1998).

1.2.4.4. Genetics

Genetically analysis of 16S ribosomal RNA, rRNA, of *Pediococcus* species shows that they posses a low G + C ratio that ranges from 32-42 % (Garvie, 1986). Compared to other LAB pediococci exhibits a close phylogenetic relationship to the *Lactobacillus* genus. As a result of this relationship pediococci are commonly found in association with lactobacilli and *Leuconostoc* in plant habitats (Raccach, 1987)

A close association, inter-species relationship, exists among members of the *Pediococcus* species. This is mostly shown between *P. pentosaceus* and *P. acidilactici*. This close relationship can be differentiated by DNA-DNA hybridisation of these two species (Garvie, 1986). However, unlike all other species *P. dextrinicus* shows less phylogenetical relationship compared to other species that have G+C ratio of 32-42 %. On the contrary *P. dextrinicus* has a DNA homology of 4-8 % (Raccach, 1999).

Pediococci harbour plasmids that are variable in size. Dellagalio *et al.*, 1984 and Hoover *et al.*, 1986 reported sizes of 1.3–127 mDa and 1.3–30 mDa respectively (Weiss, 1992; Raccach, 1999). A maximum of three plasmids have been isolated from a single strain of *Pediococcus* species. Generally plasmids encode for a number of traits that include bacteriocin, production, bacteriocin immunity and the fermentation of carbohydrates namely lactose, raffinose, melibiose and sucrose (Daeschel and Klaenhammer 1985; Raccach, 1999).

1.2.4.5. Nutrition requirements

Pediococci are chemoorganotrophs microorganisms that are complex in their nutritional requirement. They require a source of a fermentable carbohydrate in the form of monosaccharides or disaccharides and an array of vitamins, amino acids and ions for their growth (Garvie, 1986).

1.2.4.5.1. Carbohydrate metabolism

According to Ramano *et al.*, 1979, the fermentation pathway of pediococci involves the transportation of a monosaccharide like glucose across the pediococcal cell using phosphoenolpyruvate: phosphotransferase system (PEP: PTS). Inside the cell the glucose enters the glycolysis pathway using the Embden-Meyerhof-Parnas (EMP)

pathway to yield pyruvate (Raccach, 1999). The pyruvate is reduced to lactic acid and this is coupled with re-oxidation of NADH to NAD^+. The overall pathway is homolactic fermentation implying that 90 % or more of the end product is lactic acid (Simpson and Taguchi, 1998; Raccach, 1999).

$$C_6H_{12}O_6 + 2ADP + 2P_i = 2CH_3CHOCOOH + 2ATP$$

Glucose Lactic acid

Amongst other monosacharides pediococci may ferment fructose, ribose, arabinose to produce lactate as the primary product together with small quantities of ethanol and acetate (Raccach, 1999). Xylose may also be fermented to produce equal quantities of lactate and ethanol.

1.2.4.5.2. Other energy sources

P. pentosaceus can metabolise L(+) lactate under anaerobic conditions and convert it to D(-) lactate until a racemic (DL) lactate is formed (Raccach, 1987). Under aerobic conditions, *P. pentosaceus* forms both recemic lactate, L(+) lactate and D(-) lactate. The two forms are utilised in the production of acetate and carbon dioxide (Thomas, Mckay and Morris, 1985; Liu, 2003). The metabolism of lactate ensures that the redox potential of the fermenting food is kept low thus contributing to successive steps during the process i.e. cheese ripening and to the stability of the food (Raccach, 1999; Liu, 2003). Under aerobic conditions, glycerol may be utilised by some *Pediococcus* species more specifically psuedo-catalase positive pediococci to produce lactate, acetic acid and acetone together with carbon dioxide (Simpson and Taguchi, 1998).

1.2.4.5.3. Vitamin and organic base

Amongst other nutritional requirements biotin has been shown to be essential for pediococci, whilst vitamin B_{12} and Tween 80 have been reported to stimulate growth of pediococci (Garvie, 1986). Specific requirements differ within the respective species, with biotin and pyridoxal being essential for *P. acidilactici*, while pyridoxal is essential for *P. pentosaceus*. Folic acid or thymidine is an essential requirement for the growth of strains of *P. dextrinicus*, *P. parvulus* and *P. pentosaceus* (Raccach, 1999).

1.2.4.5.4. Nitrogen metabolism and mineral requirement

Pediococci require a range of amino acids for their growth namely alanine, aspartic acid, glutamic acid, arginine, histidine, isoleucine, phenylalanine, proline, threonine, tyrosine, valine, trytophan, cysteine, glycine and leucine. The requirement for methionine, serine and lysine is strain specific (Garvie, 1986; Raccach, 1987). In the absence of a nitrogen source some strains of *Pediococcus* species fail to grow (Simpson and Taguchi, 1998).

Metals or ions are essential for pediococci growth and their metabolic activity; the required metals include potassium, phosphate, magnesium, calcium, zinc, iron and manganese (Raccach, 1999).

1.2.5. Public health

Generally pediococci is composed of harmless vanomycin resistant bacteria. No cases of food poisoning have been associated with any strains of *Pediococcus* species (Facklam, 2001). However some strains within the genus have been shown to decarboxylate histidine to histamine. Above the level of 100 mg in every 100 g of food, histamine can cause illnesses amongst consumers (Raccach, 1987; 1 Raccach 1999; Facklam, 2001).

1.2.6. Occurrence of Pediococci in dairy products

Despite the inability of pediococci to utilise lactose, strains of *Pediococcus* species have been isolated from a number of dairy products. Some of these products includes goat milk (Tzanetakis and Litopoulou-Tzanetaki, 1989a, b; Stanley, 1998) and cow milk (Perry and Sharpe, 1960; Franklin and Sharpe 1963; Roudrguez *et al.*, 2003). In other reports, pediococci has been isolated from both commercial and artisan or farm-style cheese (Darce, 1958a, b) and yoghurt (Litopoulou-Tzanetaki *et al.*, according to Tzanetakis and Litopoulou-Tzanetaki, 1989a).

Farm-style cheeses refer to cheeses produced from goat, ewe, cow and buffalo milk using traditional or less commercialised techniques (Kupiec and Revell, 2001). Starter cultures may be used, however in the absence of starter LAB cheese producers rely on the LAB naturally present in cheese milk as adventitious contaminates to grow and produce lactic acid (Cogan, Barbosa, Beuvier, Bianchi-Salvadori, Coccnceli,

Fernandess, Gomez, Gomez, Kalantzopoulos, Ledda, Medina, Rea and Rodriguez, 1997; Kupiec and Revell, 2001).

1.2.6.1. Milk

Goat milk is commonly used in the cheese production in Greece. In reports by Tzanetakis and Litopoulou-Tzanetaki, 1989 b, pediococci were isolated as part of the LAB that makes up goat milk. In their report, pediococci comprised 11 % of the total LAB. Among the 13 samples analysed by Tzanetakis and Litopoulou-Tzanetaki, 1989 b, the occurrence of pediococci showed seasonal variation. In winter the occurrence of pediococci among the total LAB was higher, 11 %, compared to, 2.3 % and 0 %, in spring and summer respectively. Identification and characterisation of the pediococci identified *P. pentosaceus* as the single specie occurring in goat milk. Generally pediococci occurred in 24 % of the 54 goat milk samples. Their results were similar to those of Perry and Sharpe, 1960. In their work pediococci isolates were present in 30 % of the milk samples from cows (Tzanetakis and Litopoulou-Tzanetaki, 1989b).

1.2.6.2. Cheese

Strains of *Pediococcus* species have been isolated from a number of cheeses, namely Cheddar, Comté, Machengo, Orinotyri and El-klila (Darce, 1958a, b; Bouton and Ohta, 1996; Bouton, Guyot and Grappin, 1998; Prodromou, Thasitou, Haritonidou, Tzanetakis and Litopoulou-Tzanetaki, 2001; Gerasi, Litopoulou-Tzanetaki and Tzanetakis, 2003). However, most reports have been from Cheddar cheese where pediococci has been isolated as part of the NSLAB.

Reports from Cheddar cheese show that pedicocci occur at different level. In earlier reports, the levels of pedicocci were high, 10^7-10^8 cfu/g (Darce, 1958a; Tzanetakis and Litopoulou-Tzanetaki, 1989a). In more recent reports, Crow *et al.*, 2001, reported lower levels of, 10^2-10^4 cfu/g. The variation in the number and type of NSLAB is greatly influenced by the severity of heat treatment to which the milk is subjected (Franklin and Sharpe, 1963; Elliot and Mulligan, 1968; Grappin and Beuvier, 1997). Severe heat treatment reduces the NSLAB and the species present in cheese allowing the survival of thermal tolerant species of LAB (Tunner, Lawrence and Lelièvre, 1986).

Among the 390 LAB isolates from American Cheddar cheese, four isolates, 1 %, isolates were characterised as pediococci (Litoupoulou-Tzanetakis *et al.*, 1989a). Similar results were obtained among 2000 isolates of LAB where 20 isolates were characterised as pediococci in Canadian Cheddar (Elliot and Mulligan, 1968). In El-klila a traditional cheese produced from raw un-pasteurised cow or goat milk, 33.3 %, of 60 LAB isolates were characterised as pediococci (Boubeki and Ohta, 1996) while 40 % of the isolates were identified as pediococci in Manura cheese (Gerasi *et al.*, 2003). Prodromou *et al.*, 2001, reported similar results from Orinotyri cheese. Orinotyri is a cheese produced from the spontaneous fermentation of ewe milk. Among the 128 LAB isolates from Orinotyri cheese, 25.9 % were characterised as pediococci. Whilst in Comté a hard Swiss type cheese produced from raw milk, 16 pediococci isolates were among the LAB (Bouton *et al.*, 1998).

During the ripening of cheese, *Pediococcus* species have been isolated at different stages of the ripening process. In Cheddar cheese, pediococci isolates were present at levels of 10^2-10^4 cfu/g after the second week (Darce, 1958). In Canadian Cheddar cheese, pediococci was noted after the fourth week (Elliot and Mulligan, 1968) while in American Cheddar the presence of pediococci was shown in the sixth and twelfth month of cheese ripening (Litoloupou-Tzanetaki *et al.*, 1989a). However, in other reports on Irish Cheddar, Jordan and Cogan, 1993, failed to isolate pediococci during the course of Cheddar ripening. The absence of pediococci in Irish Cheddar may have been due to the practise of more stringent hygienic conditions during processing and cheese ripening. In artisan cheese, Comté cheese, pediococci was isolated after the first, third and fifth month during the course of ripening (Bouton *et al.*, 1998). Prodromou *et al.*, 2001, reported the occurrence of pediococci in fresh, 10 d, and after three months of ripening.

Among the *Pediococcus* species, *P. pentosaceus* and *P. acidilactici* have been characterised as the predominant species involved in cheese ripening (Tzanetakis and Litopoulou-Tzanetaki, 1989a; Prodromou *et al.*, 2001). However, no published reports have related the occurrence of either *Pediococcus* species with any stage of cheese ripening.

1.2.7. Food poisoning

Food poisoning can be defined as an acute condition that is usually presented as gastroenteritis. The symptoms of food poisoning are usually manifested after a few hours or days of consuming food containing pathogenic microorganisms or the toxins produced from these microbes (Eley, 1996).

1.2.7.1. *Bacillus cereus*

Bacillus cereus is a Gram-positive facultative anaerobic spore-forming rod that is widely distributed in nature (soil, dust and water) and in raw as well processed food (Eley, 1996; Jay, 2000). The ubiquitous nature of *B. cereus* makes it difficult to eradicate this pathogen from food. Apart from the survival of *B. cereus* under stressful conditions, it has been shown that some strains cause food poisoning at an infective dose as low as 10^3-10^4 cfu/g (Anderssem, Rönner and Granum, 1995). Within the dairy industry *B. cereus* is renowned as one of the most problematic food pathogens for the following reasons (Anderssem *et al.*, 1995).

- Occurrence of *B. cereus* is almost unavoidable in milk
- Attachment of hydrophobic spores of *B. cereus* to the pipelines
- Survival of spores during pasteurisation and the elimination of competing flora
- Survival and growth of pychrotrophic strains of *B. cereus* at temperatures of 4 to 6 °C

1.2.7.1.1. Pathogenesis

Food poisoning due to *B. cereus* occurs through the production of two toxins that cause the emetic and diarrheal syndromes respectively (Eley, 1996). Apart from the enterotoxins, pathogenesis of *B. cereus* is also expressed through the production of extracellular products like lecithinase, proteases, beta-lactamase and cereolysin (Jay, 2000; Kotiranta *et al.*, 2000).

1.2.7.1.2. Emetic syndrome

Low molecular weight heat stable toxins are responsible for food poisoning resulting in the emetic syndrome. These toxins are produced during spore formation (Jay, 2000). The symptoms of the emetic type include nausea and vomiting with occasional

diarrheal. These symptoms appear after five hours of ingesting the emetic toxin and last for 24 h (Kotiranta *et al.*, 2000).

1.2.7.1.3. Diarrheal syndrome

The diarrheal syndrome is caused by the germination of ingested spores within the gastrointestinal tract. Diarrheal enterotoxins cause fluid secretion into the gut, hence watery diarrhea, abdominal cramps and cramps are common symptoms that characterise this syndrome that may lasts for 12-24 h (Kotiranta *et a*l., 2000).

1.2.7.1.4. Incidence and epidemiology

B. cereus is mostly associated with cereals especially rice, other vehicle foods include pasta, milk pudding and pasteurized cream (Eley, 1996). The emetic type of food poisoning is mostly implicated with the consumption of pasta, rice or fried rice, while the diarrhoeal type is mostly transmitted by milk products (Kotiranta *et al.*, 2000). In reports from USA, Canada, England and Wales, Chinese food was mostly implicated as the vehicle food for *B. cereus* poisoning. Wong, Chen and Chen (1988) reported the occurrence of *B. cereus* in ice-cream (52 %), soft ice-cream (35 %), milk powder (29 %), fermented milks (17 %) and pasteurized together with fruit flavored milks (2 %) (Jay, 2000).

Reported cases of food poisoning by *B. cereus* are few because of the relatively mild symptoms (Andersson *et al.*, 1995). Amongst the reported cases between 1950-1985 the diarrheal type was reported in Hungary, Finland, Bulgaria and Norway. Within the same period in Japan and the UK the emetic type was most prevalent (Eley, 1996; Kotiranta *et al.*, 2000). Between the years 1973-1985 food poisoning caused by *B. cereus* accounted for the respective percentages in each of the following countries Finland (17.8 %), Netherlands (11.5 %), Scotland (0.8 %), England (0.7 %) and Wales (2.2 %), while Canada and Japan had 0.7 % (Kotiranta *et al.*, 2000).

1.2.7.2. *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, non-spore forming catalase positive facultative anaerobic rod (Weiss, 1992). It is ubiquitous and has been isolated in decaying vegetation, soil, milk, animal waste sewage and fresh water (Meng and Doyle, 1998; Jay, 2000). Farber and Peterkin (1991) showed the occurrence and

prevalence of *L. monocytogenes* as part of the intestinal microflora in both humans (2 to 6 %) and animals cattle, poultry and swine (10 to 50 %). *L. monocytogens* has been a cause of human illness for more than 60 years. In 1981 food was firstly associated as a vehicle for the transmission of *L. monocytogenes* (Rocourt and Bille, 1997). These reports were further supported by investigations that confirmed food as the primary vehicle for the transmission of listeriosis (Meng and Doyle, 1998).

L. monocytogenes is an opportunistic pathogen; it mostly infects individuals, whose immune system is disturbed, including pregnant women, newborns, immune-comprised persons and the elderly (Rocourt and Bille, 1997). Public health concerns over *L. monocytogenes* stem from the manifestation of listeriosis in non-enteric forms e.g. meningitis, septicaemia and abortion as well as the relatively high case-fatality rate of 20 to 30 % (Rocourt and Bille, 1997; Meng and Doyle, 1998).

L. monocytogenes can survive and grow over a broad temperature range of 1 to 40 °C and within a pH range of 4.1 to 9.6 (Jay, 2000) as well as under conditions with minimal growth nutrients (Meng and Doyle, 1998). As a result of its wide distribution and survival under refrigeration temperature and acidic conditions, *L. monocytogenes* possess as a constant contaminate of food along the food chain (Meng and Doyle, 1998).

1.2.7.2.1. Pathogenesis

Virulence or pathogenesis of *L. monocytogenes* is mostly caused by the production of β-haemolysin designated as listeriolysin O and cytotonic toxins as well as from phosphatase activity (Pearson and Marth, 1990; Eley, 1996). β-haemolysin is a cytolysin that causes the lysis of tissue cells or the erythrocytes (Jay, 2000).

Goulet, Rocourt, Rebiere, Jaquet, Moyse, Dahaumont, Saivatand and Veit, 1998, identified human strains of *L. monocytogenes* associated with listeriosis outbreaks as belonging to serovar 4b (Meng and Doyle, 1998)

1.2.7.2.2. Incidence and epidemiology

Parber and Peterkin, 1991, isolated *L. monocytogenes* from raw milk (3-4 %) and dairy products (3 %) as well as eggs (Meng and Doyle, 1998). In their review Rocourt and Bille, 1997, identified refrigerated, processed, and ready-to-eat foods as being mostly implicated with listeriosis outbreaks. Some of these products include pasteurised milk and soft cheese. These products are mostly implicated with listeriosis because these foods are neither cooked nor reheated before consumption (Meng and Doyle, 1998).

In the USA an estimated number of 1 700 people fall ill annually as a result of listeriosis, where the fatality rate is pegged 20-30 % (CDC, 2002). In Europe dairy products account for more than half of the listeriosis outbreaks (Lunden and Korleala, 2002). Reports from Switzerland and France have implicated the use of un-pasteurised raw milk in the production of soft cheese as the vehicle for *L. monocytogenes*; while in Finland, butter was implicated with listeriosis outbreak in 1998-1999 (Lunden and Korleala, 2002).

1.2.8. Antimicrobial activity of Pediococci

Pediococci like other genera of LAB exert an inhibitory action against other microorganisms primarily through the production of lactic acid and carbon dioxide (Vandenbergh, 1993; Ross *et al.*, 2002). However, some strains of *Pediococcus* species especially from *P. pentosaceus*, *P. acidilactici* (Manca de Nadra, Sandino de Lamelas, Stresser de Saad, 1998) and *P. damanosus* (Skyttä, Hikara and Mattila-Sandholm, 1993; Nel *et al.*, 2001) produce bacteriocins known as pediocins. As defined by Tagg *et al.*, 1976, bacteriocins are heterogeneous antibacterial peptides that are either bactericidal (i.e. destructive) or bacteriostatic (i.e. inhibitory) to LAB closely related to the bacteriocin-producing microorganism as well as to other Gram-positive bacteria (Schillinger *et al.*, 1996; Waite and Hutkins, 1998). However some pediocins have been shown to exhibit antimicrobial activity against both Gram-positive and some Gram-negative bacteria such as *Staphylococcus areus* and *Escherichia coli* (Spelhauger and Harlander, 1989; Skytta *et al.*, 1993; Bennik, Verheul, Abee, Naaktgeboren-Stoffels, Gross and Smid, 1997; Manca de Nadra *et al.*, 1998).

The production of pediocins by pediococci strains is a plasmid related trait (Daeschel and Klaenhammer, 1985). Pediocin production and immunity of *P. pentosaceus* has been reported to be encoded on the 13.6 mega-Dalton plasmid (Litopoulou-Tzanetaki *et al.*, 1989b)

Pediocin and pediocin-like bacteriocins are characterised as being small heat stable non-lanthionine and their molecular mass is approximately less than 80 kDa (Piva and Headon, 1994; Ennahar, Sashihara, Somnomoto, and Ishizaki, 2000a). As a result of these structural characteristics, pediocins and pediocin-like bacteriocins are classified as class II bacteriocins (Caplice and Fitzgerald, 1999). However, amongst the class II bacteriocins pediocins are sub-classified class IIa. Class IIa bacterciocins are unique in their activity against major food pathogens like *L. monocytogenes*. Hence pediocins and pediocin-like bacteriocins are designated as antilisterial bacteriocins (Ennahar, Deschamps and Richard, 2000b).

1.2.8.1. Primary, secondary and tertiary structure of pediocins

1.2.8.1.1. Primary structure

The primary structure of pediocins is made up of 37 to 48 amino acids with the N-terminal being designated as the YGNGVXaaC motif. This designation arises due to the common amino acid sequence identified as Try-Gly-Asn-Gly-Val-Xaa-Cys (Ennahar *et al.*, 2000a). Present in the N-terminal half of the peptide are two or four cysteine amino acids that are responsible for the formation of S-S bridge or disulphide bonds (Cleveland *et al.*, 2001). The motif functions as the site for attachment on the cell membrane of sensitive bacteria (Eijisink *et al.*, 1998). Pediocins have an overall cationic charge and exhibits an isoelectric point, pK*I*, within the range of 8.3 to 10 (Ennhar *et al.*, 2000a, b). A high number of glycine residues and non-polar residues are present in the primary structure; these residues impart a high degree of conformational freedom that is important in the interaction of the bacteriocin-membrane attachment (Ennhar *et al.*, 2000a).

1.2.8.1.2. Secondary structure

The secondary structure of pediocins has not been fully elucidated. However, depending on the surrounding medium pediocins take on various secondary structures. In aqueous medium for example, pediocins take on a random coil

configuration, while in non-aqueous solutions a helical configuration characterises the secondary structures (Ennahar *et al.*, 2000a). Pediocins together with other class II bacteriocins are referred to as cytobiotics as a result of the presence of two or four cysteine amino acid residues located within the N-terminal that accounts for the antimicrobial activity (Bhunia *et al.*, 1991). The presence of another pair of cysteine residues facilitates the formation of another disulphide linkage. The two linkages form a six-member ring that is important for the stabilization of the ß-sheet secondary structure (Bhunia *et al.*, 1991; Chen *et al.*, 1997).

The C-terminal amino acid residues adopt an amphiphilic α-helix structure. However the spanning of the α-helix structure leaves portions of one or two non-helical segments (Bhunia *et al.*, 1991; Ennhar *et al.*, 2000a)

1.2.8.2. Factors influencing pediocin production

Pediocin production amongst pediococci is influenced by growth medium, incubation temperature, time and pH (Carolissen-MacKay, Arendse and Hastings, 1997). Reports on optimising pediocin production have centered on *P. acidilactici*, *P. pentosaceus* and *P. damanosus* (Biswas, Ray, Johnson and Ray 1991; El-Adawy, 2001; Nel *et al.*, 2001).

1.2.8.2.1. Temperature and time

El-Adawy, 2001, reported that the optimum temperature for pediocin production in *P. pentosaceus* coincided with the optimum growth temperature of 30 °C. This finding was similar to those of Biswas *et al.*, 1991 and Nes *et al.*, 2001, both used *P. acidilactici* and *P. damanosus* respectively. Using *P. acidilactici,* Biswas *et al.*, 1991, reported on an optimum time of 16 h, whilst Skyttä *et al.*, 1993, reported that both *P. pentosaceus* and *P. damanosus* favoured an optimum time of 24 to 48 h. In all three reports approximately 80 % of pediocin production occurred in the logarithmic phase of pediococcal growth (Biswas *et al.*, 1991; Skyttä *et al.*, 1993; El Adawy, 2001; Nes *et al.*, 2001).

1.2.8.2.2. pH

Amongst the reports on pediocin production, low pH as been shown to be the most ideal for optimum pediocin production. Biswas *et al.*, 1991 reported a higher activity

from *P. acidilactici* incubated a below pH of 5. Similar reports were observed for *P. damanosus* and *P. pentosaceus* (Skyttä *et al.*, 1993). According to Biswas *et al.*, 1991 the acidic pH may be as important for the post-translation of pediocin peptide as is the case is with nisin. However some authors differ from the idea of post-translation of pediocins arguing that the simple amino acid sequence of pediocins does not show evidence of post-translation modification (Eijsink *et al.*, 1998). Apart from the modification of the peptide, an acidic pH has been reported to be essential for desorption of pediocin from the pediococcal cells thereby increasing the bacteriocin titre (Biswas *et al.*, 1991; Guerra and Pastrana, 2002)

1.2.8.2.3. Growth medium

The most commonly used medium for the growth of pediococci is MRS broth (Raccach, 1987). Using MRS as the base medium, various additives enhance the production of pediocins depending on the strain and species of pediococci. El-Adawy, 2001, showed that supplementing MRS with 0.05 % (w/v) L-cysteine optimised pediocin production and activity in *P. pentosaceus*. Similarly Nel *et al.*, 2001, reported optimum production and activity of *P. damanosus* using 1.7 % (w/v) bacteriological peptone, 3 % (w/v) Tween 80 and 0.014 % (w/v) Manganese Sulphate $MnSO_4$. Contrary to Nel *et al*, 2001, Piva and Header, 1994, had earlier reported that supplementing M17 broth with 0.01 % (w/v) Tween 80 reduced pediocin production and activity of *P. pentosaceus*. In their report Piva and Header, 1994, had reported on the formation of micelles between Tween 80 and media peptides. The formation of micelles would reduce the availability of pediocins and thereby reduce the activity of the pediocins. However, according to Nel *et al.*, 2001, Tween 80 stimulated cell growth and desorption of pediocins from the glass surface.

1.2.8.3. Mode of action of pediocins

Bacteriocins from LAB inclusive of pediocins target the cell membrane as the site of action. Once attached the bacteriocins deplete or dissipate the proton motive force (PMF) of cell membrane (Montville, Winkowski and Luderscher, 1995). Dissipation of the PMF arises from the depletion of either components of the PMF i.e. the membrane potential ($\Delta\psi$) or the pH gradient (ΔpH). In a normal cell the PMF is responsible for ATP synthesis, active transport and bacterial motion. These functions are dependent on the gradients existing within the components of the PMF that drive

all the energy driven processes (Montville *et al.*, 1995; Montville and Chen, 1998). Pediocins reduce the pH gradient at low concentrations whilst higher concentrations are required for the membrane potential on a normal cell membrane (not energized) (Montville *et al.*, 1995). As a result of the dissipation of the PMF, ATP is depleted in an effort to restore the PMF or due to the shift in the equilibrium triggered by the loss of inorganic phosphate (Waite and Hutkins, 1998).

$$ATP = ADP + P_i$$

The loss of intracellular molecules and ions as well as the dissipation of the PMF renders the cell cytoplasm unprotected against the environment. This is accompanied by an arrest of energy dependent cellular processes leading to growth inhibition and finally death of the vegetative cell (Montville *et al.*, 1995; Montville and Chen, 1998).

1.2.8.3.1. Mechanism of pore formation in vegetative cells

The structure of pediocins is well adapted for its role in the binding and pore formation on the cell membranes. Two models have been reported to describe pore formation namely, the barrel-stave and the detergent fashion model (Montville *et al.*, 1995). However, the most acceptable model explaining the action of pediocins is the barrel and stave model (Montville and Chen, 1998). As postulated in the latter model, the action of the pediocin is achieved by binding, insertion and oligomerization of pediocin monomers in the cytoplasmic membrane (Montville *et al.*, 1995; Montville and Chen, 1998).

Initially the pediocin binds to the membrane through electrostatic interaction between the positively charged and polar residues of the pediocin with the anionic phospholipid heads of the membrane (Montville *et al.*, 1995; Ennahar *et al.*, 2000a). Upon attachment to the cell membrane, the pediocin interacts and inserts into the cell membrane. Interaction of the pediocin is supported by hydrophobic and amphiphilic domains of the C-terminal of the pediocin and the lipid acyl chains (Ennahar *et al.*, 2000a). Upon insertion the pediocin oligomerize to form pores in the cytoplasmic membrane. The hydrophilic residues will be facing the interior of the pore whilst the hydrophobic regions face the hydrophobic regions of the phospholipids molecules in the interior of the membrane Figure 3 (Montville *et al.*, 1995; Bennik *et al,* 1997; Ennahar *et al.*, 2000a).

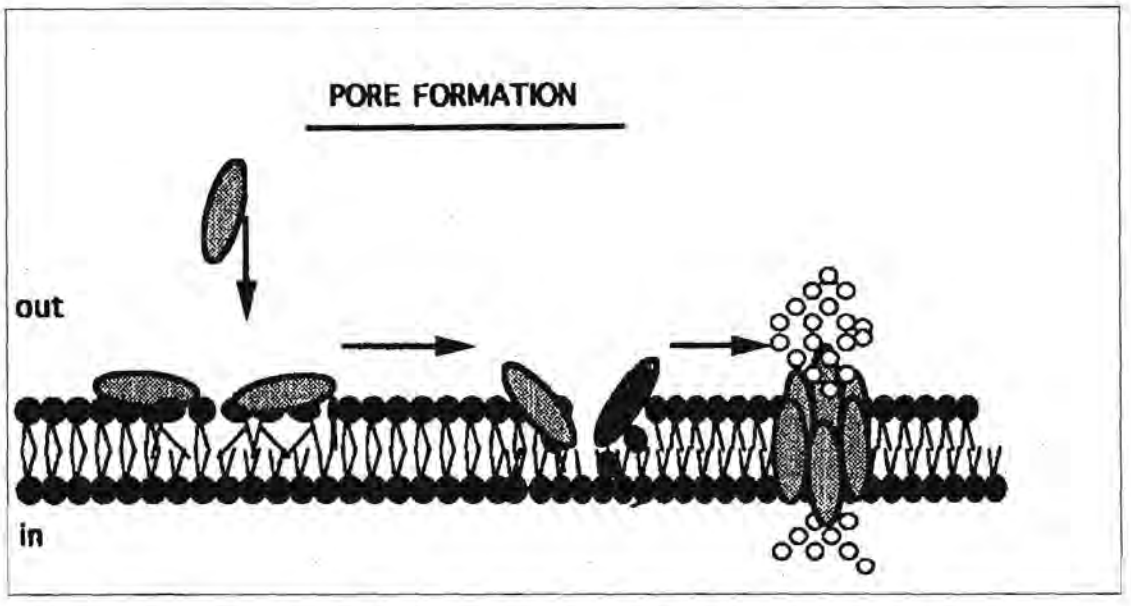

Figure 2 Pore formation of bacteriocins using the Barrel stave model (Montville and Winkowski, 1997).

1.2.8.4. Antimicrobial activity of pediococci against *L. lactis*, *B. cereus* and *L. monocytogenes*

Antimicrobial activity of pediococci has been widely reported against *Listeria* species, with most work being targeted at *L. monocytogenes*. Spelhaug and Harlander, 1989, reported on *B. cereus* and *Lactococcus* species as being part of the inhibitory spectrum of pediococci (El-Adawy, 2001). Primary work on both pathogens has shown the susceptibility of these pathogens on agar and in culture broth. Despite being inhibitory it becomes essential to determine the effectiveness of pediocins in food systems.

Effectiveness of pediococci in food systems has been mostly shown against *L. monocytogenes* (Cleveland *et al.*, 2001). Other reports have been determined against *Clostridium botulinum* (Okerekere and Montville, 1991). Amongst dairy products antimicrobial activity of pediococci against *L. monocytogenes* has been reported in milk, cottage cheese and Cheddar. In their work Raccach and Geshel, 1993, using milk as typical food system reported a 4 Log_{10} reduction in the population of *L. monocytogenes*. In smear-ripened cheese Ennaher, Assobhel and Hassel, 1998, inhibited the growth of *L. monoctogenes*. Similar work by Loessner, Guenther, Steffan and Scherer, 2003 showed the reduction of *L. monocytogenes* growth on the surface of smear ripened cheese where the final count was reported to be 10^2 cfu/g.

Buyong *et al.*, 1998, reported on the antimicrobial activity of pediocin producing recombinant *Lactococcus Lactis* subsp *lactis* starter culture in Cheddar cheese. In their findings, Buyong *et al.*, 1998, observed a single Log_{10} reduction in the population of *L. monocytogenes* in the first week of Cheddar ripening. By the end of the third month, counts of *L. monocytogenes* had been reduced to 1 Log_{10} cfu/g.

1.2.8.5. Factors affecting pediocin activity in food systems

Although pediocins have been shown to be effective by various authors, there are a number of factors influencing the effectiveness of these antimicrobial peptides. Early reports by Choi and Beuchat, 1994, showed the loss in antimicrobial activity of pediocin upon prolonged incubation of food systems. Other influential factors include the chemical composition and the physical condition of the food (Cleveland *et al.*, 2001). Since pediocins are protein in nature the presence of proteolytic enzymes originating from the food or endogenous flora may reduce the antimicrobial activity. (Bennik *et al.*, 1997).

Another influential factor is the development of induced strain resistance amongst bacterial cells previously exposed to bacteriocins. Resistance amongst cells occurs as an adaptive mechanism that requires 2-10 times the minimum inhibitory concentration of the original cells (Bennik *et al.*, 1997). The mechanism for the development of resistance amongst the bacterial cells has been proposed to occur through changes in the composition of cell membrane or through the production of proteolytic enzymes that are active against the bacteriocin (Cleveland *et al.*, 2001; Loessner *et al.*, 2003).

1.2.9. Methods of determining antimicrobial activity of bacteriocins

1.2.9.1. Agar diffusion

The agar diffusion methods are convention methods used in determining the bacteriocin activity of LAB. Other modifications of the agar diffusion method include the agar well diffusion, disc and spot diffusion (Piddock, 1990). The method is based on the fact that bacteriocins diffuse in the solid or semi-solid agar medium that is subsequently inoculated with an indicator microorganism that is susceptible to the bacteriocin (Kang and Fung, 1998). The diffusion of the bacteriocin from the point application will create a concentration gradient of the bacteriocin within the surrounding agar medium. Growth of the indicator microorganism creates a visible

lawn however a zone of inhibition will evident around the bacteriocin source (Woods and Washington, 1995).

1.2.9.2. Indicator microorganism

Listeria species and LAB are commonly used as indicator microorganisms in determining bacteriocin activity of LAB. Although *Listeria* species have been used in antimicrobial assays Roller and Miller, 2000, recommended the use of LAB instead of acid sensitive Gram-positive bacteria like *Listeria*. Although the culture broth supernatant is neutralized to prevent inhibition due to the presence of acids, the presence of other metabolites may produce zones of inhibition against non-LAB indicator microorganisms. Roller and Miller, 2000, recommended the use of *P. acidilactici* LB42, *Lactobacillus plantarum* NCDO 955, *Leuconostoc mesenteroides* Ly and *Enterococcus facallis* MB1. Their recommendations were based on the acid tolerance and high sensitivity of these LAB to bacteriocins.

Indicator microbes within a same genus may exhibit variation in their sensitivity to bacteriocins. This trend is also evident amongst strain of some species. The age of the indicator microorganism has a bearing on the sensitivity to bacteriocins (Davidson and Parish, 1989; Meghrous, Lacroix and Simard, 1999). Bacteriocins are most active to indicator microorganisms during the logarithmic phase of growth as opposed to any other stage. Indicator microbes are usually applies to the overlaying medium at a population of 10^5 to 10^6 cfu of a freshly grown culture (Roller and Miller, 2000).

1.2.10. Hypotheses and objectives

1.2.10.1.Hypotheses

1. *Pediococcus* species occur in varying numbers as part of the non-starter lactic acid bacteria of South African farm-style cheeses through pre-contamination of cheese milk or post contamination of curd during processing and ripening respectively.
2. Bacteriocin producing *Pediococcus* species that occur in South African cheese may exhibit antimicrobial activity against LAB and pathogenic bacteria through the production of antimicrobial peptides known bacteriocins.

1.2.10.2. Objectives

1. To determine whether *Pediococcus* species occur in South African farm-style cheeses as part of the non-starter lactic acid bacteria.
2. To characterise *Pediococcus* species from SA cheese to species level.
3. To determine bacteriocinogenic activity of *Pediococcus* species isolated from SA farm-style cheese.
4. To determine antimicrobial activity of bacteriocinogenic *Pediococcus* species against *L. monocytogenes* and *B. cereus*.

Chapter II

Isolation and characterisation of *Pediococcus* species occurring in South African farm-style cheese

2.1. Abstract

Pediococci are Gram-positive, non-spore forming lactic acid bacteria, LAB, that are commonly used in the industrial fermentation of vegetables and meat. Species of pediococci may constitute part of the secondary microflora as non-starter lactic acid bacteria, NSLAB, responsible for cheese ripening. In this study different *Pediococcus* species were isolated from South African farm-style cheese and characterised morphologically and physiologically and identified to species level. Eight farm-style cheeses namely pasteurised young Gouda (PYG), pasteurised matured Gouda (PMG) as well as pasteurised matured Parmesan (PMP); un-pasteurised or raw milk cheeses, aged Bouquet (RAB), Barbond (RMB), aged Gouda (RAG) and matured Gouda (RMG) as well as goat cheese Gouda (RGG) were chosen for the isolation of *Pediococcus* species. LAB were cultivated on MRS agar where logarithmic counts of LAB ranged from 6.90 cfu/g to 9.40 cfu/g. Microscopic examination of selected colonies identified 110, 18 % of 606 isolates as Gram-positive, catalase negative presumptive pediococci occurring in pairs, clusters and tetrads. Presumptive pediococci were distributed among five of the eight cheeses namely PYG, PMG, RAB, RAG and RMG in numbers of 33, 21, 28, 12 and 16 respectively. Physiological characterisation of presumptive pediococci isolates was determined under specific growth parameters, temperature, pH and salt (w/v) and these were characterised as *P. acidilactici,* 49, and *P. pentosaceus*, 61, isolates.

2.2. Introduction

Lactic acid bacteria, LAB, naturally occurring in fermented food products have the potential to inhibit spoilage and pathogenic bacteria, which threaten consumers as a result of the consumption of food containing pathogenic microorganisms or their toxins (Boughton, 1990). The use of LAB occurring naturally in fermented foods offers potential in biopreservation through the co-culture of these LAB with starter culture or the use the LAB metabolites to ensure a further hurdle in food preservation (Roller, 1995).

LAB are Gram-positive homofermentative or heterofermentative microorganisms that produce primary and secondary metabolites like organic acids, lactic acid, hydrogen peroxide; lactoperoxidase; diacetyl; exopolysaccharides and bacteriocins that inhibit other undesirable bacteria in food (Vandenbergh, 1993). Apart from their preservative action, LAB improve the sensory characteristics and the commercial value of food (Caplice and Fitzgerald, 1999). In industrial fermentations LAB are commonly used as starter cultures in dairy, meat and vegetable fermentation and naturally occur as a secondary group of contaminants in bacterial ripened cheese. This group is known as the non-starter lactic acid bacteria, NSLAB, and is composed of lactobacilli, pediococci and micrococci species (Crow, Curry, Christison, Heller, Holland and Liu, 2002).

Pediococci are Gram-positive homofermentative LAB commonly used in the industrial fermentation of pickles, green olives, sauerkraut and sausages (Buckenhuskes, 1997; Ricke and Keeton, 1997). In other reports pediococci have been identified as the spoilage microoganisms in beer and wine (Weiss, 1992; Simpson and Taguchi, 1998). Although pediococci have been reported as part or co-culture in cheese processing, pediococci are rarely used as the starter culture in dairy fermentation (Bhowmik, Resterer, Van Boekel and Marth, 1990).

The occurrences of pediococci have been reported in a number of dairy products (Garvie, 1984). Initial reports of pediococci were from New Zealand Cheddar cheese (Darce, 1958). In subsequent reports pediococci have been isolated from Canadian, American and English Cheddar cheese (Fryer and Sharpe, 1966; Elliot and Mulligan, 1968; Litopoulou-Tzanetaki, Graham and Beyatli, 1989a). In these reports pediococci

occurred in higher numbers in English and New Zealand compared to American and Canadian Cheddar.

In Cheddar cheese, pediococci has been shown to occur as part of the NSLAB at levels of 10^7-10^8 cfu/g that may constitute 1 % to 25 % of the LAB (Fryer and Sharpe, 1966; Law, Castanon and Sharpe, 1976; Litopoulou-Tzanetaki *et al.*, 1989). The direct role of pediococci in cheese has not been fully explained. However it is reported that pediococci acts synergistically to effect the typical cheese flavour (Franklin and Sharpe, 1963; Law *et al.*, 1976). The occurrence of pediococci in cheese ripening may be attributed to the survival of thermal tolerant pediococci strains as well as the contamination of the curd by NSLAB during ripening (Fryer and Sharpe, 1963; Litopoulou-Tzanetaki *et al.*, 1989a). Pasteurisation or heat-treatment reduces the competitive flora thus allowing the proliferation of thermal-tolerant pediococci. Elliot and Mulligan, 1968, reported on the higher occurrence of pediococci in heat-treated cheese compared to raw-milk Cheddar cheese

Pediococci has been reported in commercial Cheddar cheese and in farm-style or artesian cheese. Commercial cheese is produced under standard hygienic conditions (Grappin and Beuvier, 1997), while farm-style or artesian cheese is produced from raw or heat-treated bovine or ovine milk using traditional or less commercialised conditions (Cogan, Barbosa, Beuvier, Bianchi-Salvadori, Cocconcell, Fernandes, Gomez, Gomez, Kalantzopoulos, Ledda, Medina, Rea and Rodriguez, 1997). Starter cultures are normally absent and cheese producers usually rely on the adventitious LAB contaminants present in milk for growth and acid production (Boubekri and Ohta, 1996; Cogan *et al*, 1997).

Two species of pediococci have been commonly associated or isolated from cheeses namely *Pediococcus acidilactici* and *Pediococcus pentosaceus* (Garvie, 1984; Tzanetakis and Litopoulou-Tzanetaki, 1992). Although both *Pediococcus* species have been isolated from cheese, *P. pentosaceus* normally occurs at higher levels compared to *P. acidilactici* (Tzanetakis and Litopoulou-Tzanetaki, 1989b; Tzanetakis and Litopoulou-Tzanetaki, 1992). In other reports *P. pentosaceus* was the only species occurring in cheese (Tzanetakis and Litopoulou-Tzanetaki, 1992; Pordromou, Thasitou, Haritonidou, Tzanetakis and Litopoulou-Tzanetaki, 2001)

The occurrence of pediococci in cheese especially pasteurised cheeses may be indicative of the quality of the cheese milk since pediococci may occur in milk as adventitious contaminants; the level of heat treatment and the possibility of lactose fermenting pediococcal strains (Litopoulou-Tzanetaki *et al.*, 1989a). In this study different *Pediococcus* species were isolated from South African farm-style cheese and characterised morphologically and physiologically and identified to species level

2.3. Materials and methods

2.3.1. Bacterial strains and culture media

A pure culture of *P. acidilactici* ST1 was obtained from the department of Wine Biotechnology (University of Stellenbosch; Stellenbosch; South Africa) and used as the positive control for morphological and physiological identification of pediococci isolates from farm-style cheese. The corresponding isolates numbers were used for differentiation of strains of *Pediococcus* species

Table II. Bacteriological media and pediococci strain used as a positive control in morphological and physiological characterisation of pediococci isolates from South African farm-style cheese

Organism	Source	Media	OIT (°C)
P. acidilactici ST1	Institute of Wine Biotechnology, University of Stellenbosch, SA	MRS broth (de Man Rogosa & Sharp, 1960)	30
Pediococci isolates	Farm-style cheeses	MRS	30

OIT=Optimum incubation temperature

2.3.2. Commercial cheese

A pilot survey was conducted to determine and to isolate pediococci species from commercial cheese. Cheddar cheese samples, 500 g, at different ripening stages 1 d, 1, 2 and 3 months (m) were obtained from the Animal Nutrition and Animal Products Institute (ANPI) of the Agriculture and Research Council (ARC) dairy plant in Irene South Africa. The commercial Cheddar cheese was produced under standard hygienic conditions using milk from a single herd of cattle. Cheese milk was pasteurised and subjected to analysis to ensure a good microbial and chemical quality of the milk. Cheese samples at 1d, 1 and 2 m were aseptically cut in half to give a total of seven samples that were vacuum packed and ripened at a temperature of 10 to 15 °C and a relative humidity of 85 % in the department of Food Science (Pretoria, South Africa). LAB were isolated and examined for the presence of *Pediococcus* species at all of the ripening stages (1 d, 15 d, 1, 1 ½, 2, 2 ½, and 3 m). All samples were subjected to microbial and chemical analysis at the respective ripening stages.

2.3.3. Farm-style cheese

Pasteurised and un-pasteurised samples of farm-style cheeses produced by various small-scale farmers were obtained from the annual ANPI farm-style cheese fair (ARC; Irene; South Africa). Un-pasteurised or raw milk cheeses consisted of Barbond (17b), Bouquet (17b), Aged Gouda (17c), Gouda (18) and Gouda (21). Young Gouda (21), Parmesan (1) and Gouda (20) were pasteurised. Gouda (21) cheese was produced from goat milk, all others cheeses were produced from bovine origin. Farm-style cheeses were identified according to the coding scheme in Table II. Farm-style cheeses were kept under refrigeration temperature of 4 ± 2 °C for one month before microbial analysis. All cheeses were subjected to microbial and chemical analysis.

Table III. Identity codes for farm-style cheese (n=8)

Cheese type	Sample #	Code
Pasteurised cheese		
Young Gouda	17a	PYG
Matured Gouda	20	PMG
Matured Parmesan	1	PMP
Un-pasteurised/ raw milk cheese		
Aged Gouda	17b	RAG
Matured Gouda	18	RMG
Matured Barbond	17b	RMB
Aged Bouquet	17b	RAB
Goat milk Gouda	21	RGG

2.3.4. Microbial analysis

2.3.4.1. Isolation of non-starter lactic acid bacteria from commercial cheese

Sampled of cheese, 10 g, were aseptically grated and emulsified in sterile 2 % (w/v) tri-sodium citrate. Serial dilutions were made in peptone saline 0.1 % (w/v) and 0.85 % (w/v) respectively. Serial dilutions of the samples were inoculated and spread plated on duplicate plates of appropriate media (Table II). Plates were incubated at a temperature of 30 °C for 5 d in a gas-pack anaerobic atmosphere (Oxoid, New Hampshire, UK). Total LAB counts were determined and the mean and standard

deviation of the duplicate plates were noted. Twenty colonies were randomly selected from the highest dilution with a colony count of 30 to 300 cfu per plate. These colonies were inoculated in MRS broth and microscopically examined after growth at 30 °C for 24 h. Cultures were purified by streak plating before identification (Jordan and Cogan, 1993). Gram-positive cocci occurring as pair, tetrads, short chains and irregular clusters were stored as presumptive pediococci isolates in Tryptone Soy Broth (Biolab; Midrand; South Africa) 0.6 % agar (w/v) in 2 ml eppendoff tubes at a temperature of –18 °C for two months.

2.3.4.2. Isolation of non-starter lactic acid bacteria from farm-style cheese

The same procedure as that for commercial cheese was used to isolate LAB from the farm-style cheese. However, all colonies occurring on the highest dilution within a range of 50 to 100 cfu per plate were picked and characterised.

2.3.5. Identification of isolates

Physiological identification under different growth parameters of presumptive pediococci isolates was done according to the methods described by Garvie (1986) in the Bergey's Manual. Prior to tests the presumptive pediococci isolates were propagated twice in MRS broth at 30 °C for 24 h.

Growth characteristics were monitored daily at 40 and 50 °C in tubes of MRS broth over a 3 d period. Tolerance of the isolates to salt was assessed after 3 days of incubation at concentrations of 4 and 6.5 % (w/v) NaCl in MRS broth. The initial pH for growth was tested using MRS broth adjusted aseptically with 1 N HCl and 1N NaOH and results were noted after 3 d at 30 °C. *P. acidilactici* ST1 was used as the positive control.

2.3.6. Chemical analysis

Farm-style cheeses were subjected to chemical analysis, salt, pH and moisture. All analyses were done in triplicates; the mean and standard deviation were noted for all samples.

2.3.6.1. Salt determination

A sample of cheese, 2 g, was broken-down in 20 ml of distilled water to form slurry with an additional 10 ml being added to the slurry. Portions of 25 ml of 0.1 N silver nitrate and 10 ml of concentrated nitric acid were added to the slurry; this was followed by the addition of 50 ml of distilled water. The slurry was boiled for 3 h whilst 15 ml of 5 % potassium permanganate was added. Once clear the solution was filtered and cooled to room temperature. Two-millimetres of ferric ammonium sulphate were added as the indicator and the filtrate was titrated against 0.1 N potassium thiosynate until a pale pink colour appeared. The final titre was obtained from subtracting the blank value obtained from using 2 ml of distilled water (Bradley, Arnold, Barbano, Semerad, Smith and Vines, 1992).

2.3.6.2. Moisture determination using the using the Forced-Draft oven

A sample of 3 ± 0.5 g of cheese was weighed into a pre-weighed aluminium dish. The dish was placed in the forced-draft oven at a temperature of 105 °C for 16 ± 0.5 h. The dish was removed and cooled in a desiccator for 30 min and weighed. The moisture content was expressed as percentage of the difference between initial and final weight (Bradley *et al.*, 1992).

2.3.6.3. pH

The pH of the cheese was determined electrometrically using a Sentron pH meter (Sentron Inc: Postbus, The Netherlands).

2.4. Results

2.4.1. Commercial cheese

The logarithmic counts of LAB at the different ripening stages of the commercial Cheddar cheese are presented in Table IV. Logarithmic counts were similar ranging from 7.73 $\log_{10}$ at 1 day (d) to 8.16 $\log_{10}$ at 1 month (m), followed by a log reduction to 7.46 $\log_{10}$ at 1.5 m and an increase to 8.53 $\log_{10}$ at the end of 3 m. Morphological characterisation of the 140 LAB isolates identified Gram-positive rods as "presumptive" lactobacilli and Gram-positive chains as "presumptive" streptococci. No Gram-positive cocci occurring in pairs, tetrads or clusters were identified hence suggesting the absence of pediococci

Table IV. Logarithmic count of lactic acid bacteria, LAB, ($\chi \pm$ SD) of commercial Cheddar cheese and the occurrence of pediococci.

Age of cheese curd	LAB[1]	Pediococci isolates
1 d	7.73 ± 0.74	0
15 d	7.98 ± 0.97	0
1 m	8.16 ± 1.06	0
1.5 m	7.46 ± 0.11	0
2 m	8.36 ± 0.08	0
2.5 m	8.34 ± 0.05	0
3 m	8.53 ± 0.04	0

[1]Mean of three samples

2.4.2. Farm-style cheese

2.4.2.1. Chemical properties of cheese and log counts of NSLAB

The logarithmic counts and physiochemical properties of the eight farm-style cheeses are presented in Table V. Logarithmic counts of LAB counts among the cheese varied and ranged between 6.0 $\log_{10}$ in RMB to 9.4 $\log_{10}$ in RGG. The pH of cheeses ranged from 4.98 in RGG to 5.66 in PYG cheese. Moisture content was lowest in PMP 19.6 % while in other cheese it ranged from 32.4 % to 44.0 %. The salt content ranged from 1.5% to

5.5 % and the salt-in-moisture ratio was high in PMP and RMB 13.5 % and 14.5 % respectively.

Table V. Logarithmic count of lactic acid bacteria (LAB) and chemical properties ($\chi \pm$ SD) of South African Farm-style cheese (n = 8)

	Pasteurised cheese[3]			Un-pasteurised cheese[4]				
Characteristic	PMG	PYG	PMP	RMB	RAB	RAG	RMG	RGG
LAB[1]	8.02 ± 0.09	7.91 ± 0.09	7.57 ± 0.06	6.90 ± 0.08	7.07 ± 0.52	7.92 ± 0.07	7.12 ± 0.16	9.40 ± 0.141
pH[1]	5.49 ± 0.08	5.66 ± 0.29	5.09 ± 0.18	5.21 ± 0.02	5.29 ± 0.02	5.46 ± 0.12	5.43 ± 0.03	4.98 ± 0.05
H_2O[1] %	35.4 ± 0.37	42.7 ± 0.27	19.6 ± 0.80	32.4 ± 0.27	35.3 ± 0.48	37.0 ± 0.61	36.1 ± 0.51	44.0 ± 0.24
NaCl[1] %	1.5 ± 0	2.3 ± 0.14	3.1 ± 0.06	5.5 ± 0.23	3.8 ± 0.04	2.1 ± 0.04	1.4 ± 0.16	2.1 ± 0.00
S: M[1, 2] %	4.2 ± 0.04	5.0 ± 0.00	13.5 ± 0.00	14.5 ± 0.01	9.6 ± 0.00	5.4 ± 0.00	3.6 ± 0.00	4.6 ± 0.00

[1]Mean of three samples

[2] S: M = Salt-in-moisture %: NaCl % / (NaCl % + Moisture %)

[3]PMG: Pasteurised matured Gouda; PYG[3]: Pasteurised young Gouda; PMP[3]: Pasteurised matured Gouda

[4]RMB: raw milk matured Bouquet; RAB[4]: raw milk aged Barbond; RAG[4]: raw milk aged Gouda; RMG[4]: raw milk natured Gouda; RGG[4]: raw milk goat Gouda.

2.4.2.2. Characterisation and distribution of pediococci in farm-style cheese

Morphological and physiological characterisation of LAB isolates is presented in Table VI. Morphological characterisation of a total of 606 isolates obtained from the eight cheeses identified 110, 18 %, isolates as Gram-positive "presumptive" pediococci occurring in pairs, tetrads and clusters. "Presumptive pediococci isolates were further characterised into two species namely *P. acidilactici* and *P. pentosaceus*. Both species grew uniformly at pH, 4.5 and 7, salt, 4 and 6.5 % (w/v), and at 40 °C. However, the two species were differentiated by their growth at 50 °C where *P. acidilactici* grow whilst the growth of *P. pentosaceus* was inhibited.

The two *Pediococcus* species were distributed among five of the eight farm-style cheeses (Table VII). In pasteurised cheese, PYG and PMG, the total number of strains from both *Pediococcus* species was thirty-three and twenty-one respectively. The distribution of the two species between the pasteurised cheese showed the occurrence of twelve strains of *P. acidilactici* and twenty-one *P. pentosaceus* strains in PYG, while PMG had six strains of *P. acidilactici* and fifteen *P. pentosaceus* strains.

Among the un-pasteurised cheeses, RAB, RMG and RAG strains of *Pediococcus* species were distributed as twenty-eight, sixteen and twelve respectively. Among theses cheeses, the number of *P. acidilactici* strains was distributed as twenty-four, fourteen and five in RAB, RMG and RAG respectively, while strains of *P. pentosaceus* were distributed as four, two and seven respectively.

Three of the farm-style cheeses PYG, PMG and RAG had more strains of *P. pentosaceus* compared to *P. acidilactici*; however, more strains of *P. acidilactici* were isolated from RAB and RMG. Among Gouda type cheese the number of strains from both *Pediococcus* species were higher in pasteurised cheese PYG and PMG, thirty-three and twenty-one respectively compared to raw milk cheeses RAG and RMG that had sixteen and twelve respectively.

Table VI. Morphological and physiological characterization of presumptive pediococci from farm-style cheese

	Morphology[1]	Catalse	Gram-stain	Temperature		pH		% NaCl		Species	
Isolate #				40 °C	50 °C	4.5	7	4	6.5	*P. pentosaceus*	*P. acidilactici*
Pasteurised young Gouda (PYG)											
1	S; P	-	+	+	-	+	+	+	+	√	
2	C	-	+	+	+	+	+	+	+		√
3	S; P; C	-	+	+	+	+	+	+	+		√
4	S; P; T	-	+	+	+	+	+	+	+		√
5	S; P	-	+	+	+	+	+	+	+		√
6	P; T; C	-	+	+	+	+	+	+	+		√
7	P; S	-	+	+	+	+	+	+	+		√
8	P; S	-	+	+	+	+	+	+	+		√
9	P; S	-	+	+	+	+	+	+	+		√
10	S; P; T	-	+	+	+	+	+	+	+		√
11	S; P; T	-	+	+	+	+	+	+	+		√
12	S; P; T	-	+	+	+	+	+	+	+		√
13	S; P; T	-	+	+	-	+	+	+	+	√	
14	S; P; T	-	+	+	+	+	+	+	+		√
15	P; C	-	+	+	-	+	+	+	+	√	
16	S; P; T	-	+	+	-	+	+	+	+	√	
17	S; P; T	-	+	+	-	+	+	+	+	√	

18	S; P; T	-	+	+	-	+	+	+	+	√	
19	S; P; T	-	+	+	-	+	+	+	+	√	
20	P; S	-	+	+	-	+	+	+	+	√	
21	S; P	-	+	+	-	+	+	+	+	√	
22	S; P	-	+	+	-	+	+	+	+	√	
23	P; C	-	+	+	-	+	+	+	+	√	
24	P	-	+	+	-	+	+	+	+	√	
25	P; T	-	+	+	-	+	+	+	+	√	
26	P; T	-	+	+	-	+	+	+	+	√	
27	S; P	-	+	+	-	+	+	+	+	√	
28	S; P	-	+	+	-	+	+	+	+	√	
29	S; P	-	+	+	-	+	+	+	+	√	
30	S; P	-	+	+	-	+	+	+	+	√	
31	S; P	-	+	+	-	+	+	+	+	√	
32	S; P	-	+	+	-	+	+	+	+	√	
33	P; C	-	+	+	-	+	+	+	+	√	
Total 33										21	12
Raw aged Gouda (RAG)											
34	P; S	-	+	+	-	+	+	+	+	√	
35	C; P	-	+	+	-	+	+	+	+	√	
37	S; P	-	+	+	-	+	+	+	+	√	
38	P; S	-	+	+	-	+	+	+	+	√	
39	P; S	-	+	+	-	+	+	+	+	√	

40	**P; C; T**	-	+	+	+	+	+	+	+		√
41	**P; C; T**	-	+	+	-	+	+	+	+	√	
42	**P; C; T**	-	+	+	+	+	+	+	+		√
43	**P; C; T**	-	+	+	-	+	+	+	+	√	
44	**P; C; T**	-	+	+	+	+	+	+	+	√	
45	**P; C; T**	-	+	+	+	+	+	+	+		√
47	**S; P**	-	+	+	+	+	+	+	+		√
Total (12)										**7**	**5**
Raw aged Bouquet (RAB)											
49	**S; P**	-	+	+	+	+	+	+	+		√
50	**P**	-	+	+	+	+	+	+	+		√
52	**P; T**	-	+	+	+	+	+	+	+		√
53	**P; T**	-	+	+	+	+	+	+	+		√
54	**P; T**	-	+	+	+	+	+	+	+		√
55	**P; T**	-	+	+	+	+	+	+	+		√
56	**S**	-	+	+	-	+	+	+	+	√	
57	**C; T**	-	+	+	-	+	+	+	+	√	
58	**C; T**	-	+	+	+	+	+	+	+		√
59	**P; T**	-	+	+	+	+	+	+	+		√
60	**S**	-	+	+	+	+	+	+	+		√
61	**P; C**	-	+	+	+	+	+	+	+		√
62	**C**	-	+	+	+	+	+	+	+		√

63	**S; P**	-	+	+	+	+	+	+	+		√
64	**S; P**	-	+	+	-	+	+	+	+	√	
65	**S; P**	-	+	+	+	+	+	+	+		√
66	**S; P**	-	+	+	+	+	+	+	+		√
67	**S; P**	-	+	+	+	+	+	+	+		√
72	**S; P**	-	+	+	+	+	+	+	+		√
73	**S**	-	+	+	-	+	+	+	+	√	
74	**S; P**	-	+	+	+	+	+	+	+		√
75	**S**	-	+	+	+	+	+	+	+		√
76	**S**	-	+	+	+	+	+	+	+		√
77	**S; P; T**	-	+	+	+	+	+	+	+		√
78	**S; P**	-	+	+	+	+	+	+	+		√
79	**S; P**	-	+	+	+	+	+	+	+		√
80	**P; S**	-	+	+	+	+	+	+	+		√
81	**P; T**	-	+	+	+	+	+	+	+		√
Total (28)										**4**	**24**
Pasteurised matured Gouda (PMG)											
83	**P**	-	+	+	+	+	+	+	+		√
84	**S**	-	+	+	+	+	+	+	+		√
85	**S; P**	-	+	+	+	+	+	+	+	√	
87	**S**	-	+	+	+	+	+	+	+		√
88	**P**	-	+	+	-	+	+	+	+	√	

89	S	-	+	+	-	+	+	+	+	√	
90	S; P; T	-	+	+	-	+	+	+	+	√	
91	S; P; T	-	+	+	-	+	+	+	+	√	
92	P; T	-	+	+	+	+	+	+	+		√
93	P	-	+	+	-	+	+	+	+	√	
94	P	-	+	+	-	+	+	+	+	√	
95	S; P; T	-	+	+	-	+	+	+	+	√	
96	P	-	+	+	+	+	+	+	+		√
97	P	-	+	+	-	+	+	+	+	√	
98	S; P	-	+	+	-	+	+	+	+	√	
99	P; S	-	+	+	-	+	+	+	+	√	
100	S; P; T	-	+	+	-	+	+	+	+	√	
101	S; P; T	-	+	+	-	+	+	+	+	√	
103	S; P; T	-	+	+	-	+	+	+	+	√	
104	S; P; T	-	+	+	-	+	+	+	+	√	
106	S; P; T	-	+	+	+	+	+	+	+		√
Total (21)										15	6
Raw matured Gouda (RMG)											
113	S; P; T	-	+	+	+	+	+	+	+		√
114	S; P; T	-	+	+	+	+	+	+	+		√
115	S; P; T	-	+	+	+	+	+	+	+		√
116	S; P; T	-	+	+	+	+	+	+	+		√

117	**P; S; C**	-	+	+	-	+	+	+	+	√	
119	**S; P**	-	+	+	+	+	+	+	+		√
120	**S; P; T**	-	+	+	+	+	+	+	+		√
121	**P; S; C**	-	+	+	+	+	+	+	+		√
123	**S; P; T**	-	+	+	+	+	+	+	+		√
124	**S; P; T**	-	+	+	+	+	+	+	+		√
126	**S; P; T**	-	+	+	+	+	+	+	+		√
127	**S; P; T**	-	+	+	-	+	+	+	+		√
131	**S; P; T**	-	+	+	-	+	+	+	+	√	
137	**S; P; T**	-	+	+	+	+	+	+	+		√
138	**S; P; T**	-	+	+	+	+	+	+	+		√
140	**S; P; T**	-	+	+	+	+	+	+	+		√
Total (16)										**2**	**14**

[1]Morphological structures: S-single cells; P-paired; T-tetrad; C-clustered

+ positive; -negative; √ confirmation of respective species

Table VII. Occurrence and distribution of *Pediococcus* species in farm-style cheese

	Pasteurised cheese[4]			Un-pasteurised cheese[5]				
Characteristic	PMG	PYG	PMP	RMB	RAB	RAG	RMG	RGG
Pediococci isolates	21	33	0	0	28	12	16	0
% pediococci[1]	19	30	0	0	26	11	14	0
P. acidilactici	6[2] (29 %)[3]	12 (40 %)			24 (86 %)	5 (42 %)	14 (88 %)	
P. pentosaceus	15 (71 %)	21 (60 %)			4 (14 %)	7 (58 %)	2 (12 %)	

[1] Percentage of presumptive pediococci isolates per cheese as a percentage of the total of the presumptive pediococci isolates

[2] Number of the respective specie occurring in the cheese

[3] Percentage of the specie occurring in the respective cheese as a total of the number of pediococci in the respective cheese

[4]PMG: Pasteurised matured Gouda; PYG[4]: Pasteurised young Gouda; PMP[4]: Pasteurised matured Gouda

[5]RMB: raw milk matured Barbond; RAB[5]: raw milk aged Bouquet; RAG[5]: raw milk aged Gouda; RMG[5]: raw milk natured Gouda; RGG[5]: raw milk goat Gouda.

2.5. Discussion

2.5.1. Commercial cheese

Among the commercial Cheddar cheese, pediococci was absent among the 140 non-starter culture isolates examined at all the ripening stages, 1 day to 3 months. These reports are similar to those from Irish and United Kingdom Cheddar that identified lactobacilli as the sole constituent of NSLAB and no pediococci (Jordan and Cogan, 1993; Williams and Banks, 1999). Similar reports were found among South African Cheddar cheese produced by the open-vat protocol (Lues and Both, 1997; Lues, Smit and van Zyl, 1999). The absence of pediococci may be attributed to either high or good microbial quality of cheese milk used in the production of commercial Cheddar cheese as well as the hygienic protocols during production and ripening (Litopoulou-Tzanetaki *et al.*, 1989; Grappin and Beuvier, 1997; Williams and Banks, 1997) or to the inability of some pediococci strains to utilise residual lactose as an energy source (Garvie, 1986; Raccach, 1987; Simpson and Taguchi, 1998). The use of milk contaminated with indigenous lactic flora may be a source of adventitious contaminants like pediococci that tend to persist into the ripening of the cheese (Tzanetakis and Litopoulou-Tzanetaki, 1989; Grappin and Beuvier, 1997).

2.5.2. Farm-style cheese

Among the farm-style cheese a total of 110 presumptive pediococci, 18 %, were identified from 606 LAB isolates. In Canadian and American Cheddar cheese pediococci constituted 1 % of the NSLAB isolates (Elliot and Mulligan, 1968; Litopoulou-Tzanetaki *et al.*, 1989). The higher occurrence of pediococci among farm-style cheese compares with New Zealand Cheddar cheese where 25 % of the isolates were pediococci (Dacre, 1963) as well as other farm-style cheeses where similar counts of 33.3 % and 40 % were isolated (Bouton *et al.*, 1996; Gerasi *et al.*, 2003). In cases where heat-treatment is applied to the cheese milk, the high numbers of pediococci among pasteurised farm-style cheese could be attributed to the survival of thermal-tolerant pediococci strains that constitute the milk lactic flora (Franklin and Sharpe, 1966; Elliot and Mulligan, 1968; Tunner *et al.*, 1986). The use of raw milk in the production of farm-style cheese may introduce strains of *Pediococcus* species that occur in raw milk as part of the adventitious lactic contaminates (Lane, Fox, Walsh, Flokertsma, and McSweeney, 1997; Broadbent *et al.*, 2002). Alternatively environmental contamination of the cheese curds during ripening may introduce

Pediococcus species as part of the NSLAB in the farm-style cheeses during ripening (Williams and Bank, 1997; Beresford, 2003).

Pediococci occurred in pasteurised, 49 % and un-pasteurised, 51 %, cheeses. Comparison of the respective cheeses showed that PYG and PMG had a higher number of pediococci, 23 and 21 isolates respectively, compared to RAG and RMG with 12 and 16 isolates respectively. These results are similar to those of Franklin and Sharpe, 1966, who reported a higher number of pediococci in heat-treated Cheddar compared to raw-milk cheese (Elliot and Mulligan 1968; Litopoulou-Tzanetaki *et al*, 1989).

Species of pediococci were not isolated from PMP and two un-pasteurised cheeses namely, RMB and RGG. The absence of pediococci in RGG may not be clearly explained, however, the high LAB count of 9.40 $\log_{10}$ may reflect increased competition among the LAB where pediococci probably failed to grow. Absence of pediococci isolates in RMB and PMP could furthermore be explained by the high salt-in-moisture content 14.5 % and 13.5 % (w/v) respectively. Both species of pediococci isolated from farm-style cheese have an optimal salt tolerance of 10 % (w/v) hence the high salt-in moisture may be inhibitory to pediococci. Among the chemical composition of cheese pH, salt and salt-in-moisture were shown to be determinate factors on the rate and extent of growth of NSLAB (Fox, McSweeney and Lynch, 1998). A high salt-in-moisture above 11.5 % retarded the growth of NSLAB in "Pit" cheese (Gobetti, Folketsma, Fox, Corsetti, Smacchi, de Angelis, Rossi, Kilcawley and Cortini, 1999).

Two *Pediococcus* species *P. pentosaceus* and *P. acidilactici* were isolated from five farm-style cheese. Both species were isolated together and in variable numbers. More isolates were characterised as *P. pentosaceus* compared to *P. acidilactici* in PYG, PMG and RAG. Between the two species, *P. pentosaceus* has been reported to occur more frequently and in larger numbers among milk and dairy products compared to *P. acidilactici* (Garvie, 1984; Tzanetakis and Litopoulou-Tzanetaki, 1989; Tzanetakis and Litopoulou-Tzanetaki, 1992). Although strains of *P. acidilactici* were isolated from farm-style cheese, few reports have characterised this specie among pediococci isolates from cheese. Among these reports *P. acidilactici* has been characterised from Feta (Tzanetakis and Litoupoulou-Tzanetaki, 1992) and traditional "El-Klila" cheese

(Boubekri and Ohta, 1996). The occurrence of *P. pentosaceus* and *P. acidilactici* among the cheeses could be attributed to the close characteristics similarity shared by the two species (Garvie, 1986; Raccach, 1987; Simpson and Taguchi, 1998).

2.6. Conclusions

Pediococci was not isolated from commercial Cheddar cheese, however, both pasteurised and un-pasteurised South African farm-style cheese harboured pediococci. Among the isolates from the farm-style cheeses, *P. acidilactici* and *P. pentosaceus* were characterised as the constituent *Pediococcus* species. Amongst the factor influencing the growth of NSLAB, the growth or occurrence of pediococci seemed to depend on the salt-in-moisture content of cheeses.

Chapter III

Antimicrobial activity of crude pediocin extracts from *Pediococcus* species isolated from South African farm-style cheese against *Bacillus cereus* and *Listeria monocytogenes*

3.1. Abstract

Strains of *Pediococcus acidilactici* and *P. pentosaceus* isolated from South African farm-style cheese were evaluated for the production of antimicrobial peptides, pediocins, and their effect against food pathogens *Bacillus cereus* ATCC 1178 and *Listeria monocytogenes* ATCC 7644. Three techniques, the agar disc, spot and overlay method were evaluated. Comparisons on the sensitivity or susceptibility of two *Lactococcus* strains, *L. diacetilactis* NCDO 176 and *L. lactis* NCDO 605, to the crude pediocin extract from *P. acidilactici* ST1 was assessed. The agar disc assay technique produced more reliable results compared to the other techniques and *L. diacetilactis* NCDO 176 was more susceptible to the crude pediocin extract produced from *P. acidilactici* ST1. A total of fifty-two strains (47 %) from both species, twenty-seven (24 %) *P. acidilactici* and twenty-five (23 %) *P. pentosaceus*, exerted antagonism against *L. diacetilactis* NCDO 176 through the action of pediocins. Among these strains thirteen (13 %) seven (6 %) *P. acidilactici* and six (7 %) *P. pentosaceus* inhibited *B. cereus* ATCC 1178. A total of thirty-seven strains (33 %) seventeen (15 %) *P. acidilactici* and twenty (18 %) *P. pentosaceus* showed inhibition against *L. monocytogenes* ATCC 7644. More strains of pediococci exerted inhibition against *L. monocytogenes* ATCC 7644 compared to *B. cereus* ATCC 1178.

3.2. Introduction

Food poisoning outbreaks involving bacterial pathogens and the survival of some pathogens like *Listeria monocytogenes* and *Bacillus cereus* under refrigeration conditions has increased awareness of the importance of food safety (Marth, 1998). Controlling pathogenic bacteria may reduce cases of food poisoning thereby ensuring consumer of safer, wholesome and nutritious food (El-Adawy, 2001). Antimicrobial substances are used to inhibit the growth of food borne bacteria and to extend the shelf life of processed food. However, consumers are advocating for more natural preservatives owing to the risk associated with the use of artificial chemical preservatives (Abee, Krockel and Hill, 1995).

Lactic acid bacteria are widely used for the industrial fermentation of meat, dairy and vegetables because of their inhibitory activity against undesirable microorganisms (Piva and Headon, 1994). Using LAB or their metabolites as food preservatives is more accepted by consumers as "natural" and "health promoting" (Montville and Winkowski, 1997). Among the antimicrobial agents form LAB; bacteriocins produced by some strains have gained interest as hurdles in natural preservation. Bacteriocins are proteins or peptides that are active against Gram-positive spoilage and pathogenic bacteria (Rodríguez, Martinez, Hom and Dodd, 2003). To date nisin is the only bacteriocin that has been approved by the Food and Drug Administration (FDA) for use in processed food (Food and Drug Administration, 1988)

Pediococci are Gram-positive homofermentative LAB commonly used in industrial fermentation of vegetables and sausages (Weiss, 1992). Their use in dairy fermentation is limited by their inability to utilise lactose (Garvie, 1986). However pediococci occurs in bacterial ripened cheese as adventitious contaminants of the non-starter lactic acid bacteria that contribute towards the typical cheese flavour (Darce, 1958, Franklin and Sharpe, 1966; Litopoulou-Tzanetaki, Graham and Beyatli, 1989a). Some strains of *P. acidilactici*, *P. damanosus* and *P. pentosaceus* produce bacteriocins known as pediocins in addition to other antimicrobial agents (Ray and Daeschel, 1994; Nel, Bauer, Vadamme and Dicks, 2001)

The class of pediocins includes pediocin PA-1 and AcH from *P. acidilactici* that have been characterised and shown to be similar to pediocin A from *P. pentosaceus* and

pediocin DI from *P. damanosus* (Bhunia, Johnson and Ray, 1988; Piva and Headon, 1994; Nel *et al.*, 2001). Primary structure of pediocins is composed of 37 to 60 amino acids, characterised by a common sequence of Try-Gly-Ash-Val-Xaa-Cys and two cysteines forming a disulphide bridge in the N-terminal (Ennahar, Sashiara, Sonomoto and Ishizaki, 2000a). Pediocins are classified as class IIa bacteriocins based on their small size of less than 80 kDa, heat stability and their high activity against *Listeria* species (Klaenhammer, 1993; Piva and Headon, 1994).

Inhibition spectrum of pediocins extends over wide genera of Gram-positive microorganisms that include undesirable bacteria such as spoilage LAB, *Brochothrix* species, *Clostridium* spp., *Bacillus* spp., and *Staphylococcus* spp., (Ray and Daeschel, 1994). No reports have cited the inhibition of Gram-negative bacteria. The mode of action of pediocins against sensitive bacteria occurs through the binding, destabilisation and permeabilization of the cytoplasmic membrane (Montville and Chen, 1998). This results in the dissipation of the membrane potential and the loss of inorganic phosphate that inhibits active processes and eventually leads to the death of the cells (Chen, Shapira, Eisenstein and Montville, 1997).

The objectives of the present study were to identify strains of *Pediococcus* species isolated from farm-style cheese (Chapter II) that produce pediocins and to determine the activity of the crude pediocin extracts, CPE, produced by these strains against food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644.

3.3. Materials and methods

3.3.1. Bacterial strains and culture media

Strains of *P. pentosaceus* and *P. acidilactici* were isolated from five farm-style cheeses as described in Chapter II. A pure culture of pediocin producing *P. acidilactici* ST1 was obtained from the department of Wine and Biotechnology (University of Stellenbosch, Stellenbosch, South Africa) and used as the positive control in the antimicrobial assay. Other bacterial strains, their source, incubation temperature and growth media are presented in Table I.

Table VIII. Bacterial strains and media used in this study

Organism	Source	Media	OIT (°C)
P. acidilactici ST1	Institute of Wine Biotechnology, University of Stellenbosch, SA	MRS broth (de Man Rogosa & Sharpe 1960)	30
P. pentosaceus strains	Farm-style cheese (ARC Irene, South Africa)	MRS broth	30
P. acidilactici strains	Farm-style cheese (ARC Irene, South Africa)	MRS broth	30
Lactococcus lactis subsp *diacetilactis* NCDO 176	Agriculture Research Council (ARC), SA	MRS broth	30
Lactococcus lactis subsp *lactis* NCDO 605	ARC	MRS broth	30
Listeria monocytogenes ATCC 7644	ARC	Tryptone Soya broth (Biolab, Midrand SA)	30
Bacillus cereus ATCC 1178	ARC	Brain Heart Infusion broth (Biolab, Midrand, SA)	37

OIT=Optimum incubation temperature

3.3.2. Evaluation of antimicrobial techniques

Three methods were compared in determining the antimicrobial activity of the crude pediocin extract, CPE, produced from *P. acidilactici* ST1. The methods include the disc assay by Kim, Marshal and Wei, 1995, with modification from El-Adway, 2001; the agar spot method as described by Con, Gökalp and Kaya, 2001 and the overlay method as described by Ray and Miller 2000. The assays were evaluated for the most appropriate or reliable method for the subsequent studies.

In all assays MRS broth was used as a negative control and treated identically as the pediococci culture used in the antimicrobial assays.

3.3.2.1. Agar disc assay (I)

The inhibitory activity of the CPE from *P. acidilactici* ST1 was determined on solid agar media against *L. lactis* NCDO 176 by adding 1.5 % (w/v) agar to MRS broth media using the disc assay technique (Kim *et al.*, according to El-Adawy, 2001) as follows: The pediococci strain was grown for 48 h at 30 °C and centrifuged at 3000 g for 15 min at 4 °C (Skyttä, Haikara and Mattila-Sandholm, 1993). A sterile filter paper disc (Whitman AA, 13.0 mm diameter, Merck, Midrand, SA) was dipped into the cell free supernatant containing the CPE for 30 min, and then applied on MRS agar plates and overlaid with MRS soft agar 0.6% (w/v) agar seeded with 1 x 10^6 cfu/ml of bacterial culture. The plates were incubated overnight at 30 °C and the diameter of the resulting zone of inhibition was measured in mm as the distance form the edge of the paper disc to the edge of the clearing zone. Clear zones extending for 0.5 mm or more were considered as positive for inhibition (Litopoulou-Tzanetakis, Vafopoulou-Mastrojiannaki and Tzanetakis, 1989; El-Adawy, 2001).

3.3.2.2. Agar spot assay (II)

Four samples of 0.5 µl of a 24 h culture of *P. acidilactici* ST1 was spotted on the surface of MRS agar plates and incubated at 30 °C for 24 h under anaerobic conditions. The plates were then overlaid with 7 ml of soft TSAYE 0.7 % (w/v) agar seeded with 1 x 10^6 cfu/ml of the indicator microorganism *L. diacetilactis* NCDO 176. After incubation for 24 h at 30 °C the plates were examined for zones of inhibition surrounding underlying colonies of the pediococci strain.

3.3.2.3. Overlay method (III)

An 18 h culture of *P. acidilactici* ST1 was serially diluted and spread on MRS agar 1.5% (w/v) agar and allowed to dry. The plates were further overlaid with 3 ml of the same MRS agar and incubated at 30 °C for 24 h or longer until small colonies developed. Plates with approximately 100 cfu were overlaid with 5 ml of MRS soft agar 0.7 % (w/v) agar inoculated with 1 x 10^6 cfu/ml of *L. diacetilactis* NCDO 176. The plates were further incubated for 24 h at 30 °C and examined for clear and circular zones on inhibition around the underlying pediococci colonies.

3.3.3. Comparison of the susceptibility of the *Lactococcus* strains to the CPE from *P. acidilactici* ST1

Indicator microoraganisms, *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176, were evaluated for their susceptibility towards CPE from pediocin producing *P. acidilactici* ST1 using the agar disc technique. The most susceptible strain was used in the determination of pediocin production among strains of *Pediococcus* species using the same technique.

3.3.4. Determining antimicrobial activity against *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644

The agar disc diffusion technique (I) was used for the determination of the antimicrobial activity of the CPE produced from strains of *Pediococcus* species against *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644. Modifications to the agar disc method included the neutralisation of the cell free supernatant, CPE, from the 48 h culture broth. The pH of the supernatant was adjusted to a 6.5 using 10M NaOH before the immersion of the paper discs.

The test microorganism *B. cereus* ATCC 1178 or *L. monocytogenes* ATCC 7644 was propagated twice and then grown for 18-24 h in 10 ml of the appropriate growth media (Table VIII). The turbidity of the culture broth was compared with the McFarland tubes to give an estimate of the bacterial population (Harrigan, 1998). A sample of 0.1 ml of 1 x 10^7 cfu/ml was transferred and spread plated on the appropriate media and allowed to dry. Paper discs previously immersed in the CPE for 30 min were aseptically transferred on the agar plates and allowed to dry before

being incubated at the appropriate temperature for the growth of each microorganism for 24 h.

A positive control, *P. acidilactici* ST1, and a negative control, MRS broth, were treated in a similar manner as the pediococci cultures. The zones of inhibition occurring around the paper discs were compared after 24 h incubation.

3.4.Results

3.4.1. Evaluation of antimicrobial techniques

In order to select the most appropriate agar diffusion technique in determining and screening inhibition potential of the crude pediocin extract, CPE, from strains of pediococci against the indicator and food pathogens, three procedures were evaluated. Table IX shows the comparison of techniques I, II and III. Clear zones of inhibition were produced using technique I whilst techniques II and III produced fuzzy zones. Based on these results technique I was chosen as the most appropriate method for antimicrobial determination.

Table IX. Comparison of agar diffusion techniques in the determination of the antimicrobial activity of the crude pediocin extract, CPE, from *P. acidilactici* ST1 against *L. diacetilactis* NCDO 176

Source of CPE	Agar diffusion method[1]		
	Disc (I)	Spot (II)	Overlay (III)
P. acidilactici ST1	+	-/+	-/+
MRS negative control	-	-	-

[1] Mean of three trials

- No inhibition, + inhibition> 1 mm, - /+ Fuzzy zone inhibition

3.4.2. Comparison of the susceptibility of the *Lactococcus* strains to CPE from *P. acidilactici* ST1

The choice of an indicator microorganism was based on the sensitivity between *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176 to the CPE from pediocin producing *P. acidilactici* ST1 (Table X). *L. diacetilactis* NCDO 176 was more sensitive hence it was used as the indicator microorgamism for determining antimicrobial activity of the CPE produced by strains of pediococci isolated from farm-style cheese.

Table X. Comparison of the antimicrobial sensitivity of *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176 to the CPE from *P. acidilactici* ST 1

Source of CPE	Zone size[1] of Indicator strains	
	L. diacetilactis NCDO 176	*L. lactis* NCDO 605
P. acidiclactici ST 1	3.0 (2.5 – 3.3) [2]	2.0 (1.6 – 2.4) [2]
MRS negative control	-	-

[1]Zone size was measured from edge of the disc to the edge of the clearing zone

- No inhibition, 0.5-1.0 mm: low inhibition, 1.1-2.0 mm: medium inhibition, 2.1-3.5 mm: high inhibition

[2]: Mean of three trials.

3.4.3. Antimicrobial activity of the crude pediocin extract produced by strains of pediococci isolated from farm-style cheese against *L. diacetilactis* NCDO 176

Among the isolates of *P. acidilactici*, twenty-seven strains, 24 %, showed antagonism against *L. diacetilactis* NCDO176 (Table XI). Among the isolates nine strains, 8 %, showed low inhibition, fourteen, 12 %, showed medium inhibition and four, 4 %, showed high inhibition against the indicator. Thirty-four isolates, 31 %, did not show antagonism against *L. diacetilactis* NCDO 176.

Twenty-five strains, 23 %, of *P. pentosaceus* exhibited antagonism against *L. diacetilactis* NCDO 176 (Table XII). Among these isolates four strains, 4 %, showed low inhibition, seventeen strains, 15 %, showed medium inhibition and while four strains, 4 %, showed high inhibition (Table XII). Twenty-four isolates, 22 %, did not show antagonism against *L. diacetilactis* NCDO 176.

The number of strains of pediococci exerting antagonism against *L. diacetilactis* was comparable between the two species. Strains from both *Pediococcus* species exerting inhibition zones of 1 mm and less were not used for the subsequent antimicrobial assays. A total of thirty-nine strains, twenty-one *P. pentosaceus* and eighteen *P. acidilactici* were used for subsequent assay.

Table XI. Antimicrobial activity of the crude pediocin extract from strains of *P. acidilactici* (n = 61) against *L. diacetilactis* NCDO 176

	Level of inhibition[1]					
Strain No inhibition	Strain	Low	Strain	Medium	Strain	High
4	40	0.9	3	1.6	2	2.2
5	49	0.6	6	1.9	72	2.3
8	50	0.9	7	1.1	76	2.5
10	52	0.8	9	2.0	78	2.3
11	54	0.5	62	1.8		
12	65	0.5	74	1.3		
14	92	0.9	75	1.1		
42	120	1.0	77	1.8		
44	121	1.0	79	1.8		
45			80	1.1		
47			81	1.5		
53			83	1.3		
55			84	1.9		
58			140	2.0		
59						
60						
61						
63						
66						
67						
87						
96						
106						
113						
114						
115						
116						
119						
123						
124						
126						
127						
137						
138						
Total strains	34	9		14	4	
% of strains	31	8		12	4	
Mean zone size			0.8		1.6		2.3

[1] Mean of three trials

0.5>: No inhibition, 0.5-1.0 mm: low inhibition, 1.1-2.0 mm: medium inhibition, 2.1-3.5 mm: high inhibition

Table XII. Antimicrobial activity of the crude pediocin extract from strains of *P. pentosaceus* (n = 49) against *L. diacetilactis* NCDO 176

	Level of inhibition[1]						
	Strain No inhibition	Strain	Low	Strain	Medium	Strain	High
	15	29	0.9	13	1.9	1	2.5
	17	37	0.9	16	1.3	19	2.1
	18	40	0.9	22	1.1	21	2.5
	20	91	0.8	24	1.3	94	2.5
	23			31	1.5		
	25			38	1.3		
	26			41	1.6		
	27			43	1.8		
	28			56	1.3		
	30			73	1.3		
	32			85	1.4		
	33			90	1.1		
	34			95	1.5		
	35			97	1.1		
	36			98	2		
	39			100	1.1		
	64			101	1.1		
	88						
	89						
	93						
	99						
	103						
	117						
	131						
Total strains	24.0	4.0		17.0		4.0	
% of strains	22	4		15		4	
Mean zone size	0.0		0.9		1.4		2.4

[1] Mean of three trials

0.5>: No inhibition, 0.5-1.0 mm: low inhibition, 1.1-2.0 mm: medium inhibition, 2.1-3.5 mm: high inhibition

3.4.4. Antimicrobial activity of strains of *Pediococcus* species against food pathogens

3.4.4.1. *B. cereus* ATCC 1178

Among the respective strains of the two *Pediococcus* species exerting inhibitory action through CPE, seven strains, 6 %, of *P. acidilactici* and five strains, 5 %, of *P. pentosaceus* showed low inhibition against *B. cereus* ATCC 1178 (Table XIII). One strain, ST 38, 1 %, of *P. pentosaceus* exhibited medium inhibition of *B. cereus* ATCC 1178. A comparable number of strains from both *Pediococcus* species showed low inhibition of *B. cereus* ATCC 1178.

3.4.4.2. *L. monocytogenes* ATCC 7644

Among the respective strains of *Pediococcus* species exerting inhibitory action through CPE, antagonism against *L. monocytogenes* ATCC 7644 was demonstrated by seventeen strains, 15 %, of *P. acidilactici*. amid these strains four, 4 %, showed low inhibition, eight, 7 %, demonstrated medium inhibition and five strains, 4 %, showed high inhibition of *L. monocytogenes* (Table XIV). Twenty isolates of *P. pentosaceus*, 18 %, showed antagonism against *L. monocytogenes* ATCC 7644. Among these strains four, twelve and four exhibited low, 4 %, medium, 10 %, and high, 4 %, antimicrobial activity respectively (Table XIV). The number of *Pediococcus* strains from both species that showed low, medium and high was relatively similar.

3.4.4.3. Comparison of the antimicrobial activity of strains from *Pediococcus* species

Strains 2, 72, 76 and 78, of *P. acidilactici* together with strains 1, 19, 21 and 94 from *P. pentosaceus* were highly inhibitory against *L. diacetilactis* NCDO 176. Further studies against the food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644, showed that none of these strains were found to exert high inhibition against either pathogens.

Strains 9, 62, 77, 79 and 81 from *P. acidilactici* together with strains 13, 56, 100 and 101 from *P. pentosaceus* exerted high inhibition against *L. monocytogenes* ATCC 7644. However, these strains showed only medium inhibition towards *L. diacetilactis* NCDO 176. Among the strains from both *Pediococcus* species, strain 38 from *P. pentosaceus*

was the only strain that showed medium inhibition against *L. diacetilactis* NCDO 176, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644.

Table XIV. Antimicrobial activity of the crude pediocin extracts from strains (n = 39) of *P. acidilactici* and *P. pentosaceus* against *L. monocytogenes* ATCC 7644

	Level of inhibition													
	P. acidilactici							*P. pentosaceus*						
	Strain no inhibition	Strain	Low	Strain	Medium	Strain	High	Strain no inhibition	Strain	Low	Strain	Medium	Strain	High
	80	6	0.5	2	1.3	9	2.4	73	22	0.5	1	1.3	13	2.5
		74	0.5	3	1.3	62	3.3		24	0.5	16	1.3	56	2.5
		75	1	7	1.5	77	2.2		31	0.5	19	1.5	100	2.2
		140	0.5	72	1.8	79	3.8		41	1	21	1.5	101	2.8
				76	1.3	81	2.1				38	1.1		
				78	1.3						43	1.3		
				83	1.7						85	1.8		
				84	1.5						90	1.3		
											94	1.2		
											95	1.7		
											97	1.6		
											98	1.4		
Total strains	1	4		8		5		1	4		12		4	
% of strains	1	4		7		4		1	4		10		4	
Mean zone size			0.6		1.5		2.8			0.6		1.4		2.5

[1] Mean of three trials

0.5mm>: No inhibition, 0.5-1.0 mm: low inhibition, 1.1-2.0 mm: medium inhibition, 2.1-3.5 mm: high inhibition

Table XIII. Antimicrobial activity of the crude pediocin extracts from strains (n = 39) of *P. acidilactici* and *P. pentosaceus* against *B. cereus* ATCC 1178

	Level of inhibition							
	P. acidilactici			*P. pentosaceus*				
	Strain no inhibition	Strain	Low	Strain no inhibition	Strain	Low	Strain	Medium
	2	3	0.7	1	21	0.9	38	1.2
	6	9	0.5	13	38	1.2		
	7	72	0.5	16	43	0.9		
	62	78	0.6	19	85	0.8		
	74	79	1	22	101	0.5		
	75	81	0.9	24				
	76	140	0.5	31				
	77			41				
	80			56				
	83			73				
	84			90				
				94				
				95				
				98				
				100				
Total strains	11	7		15	5		1	
% of strains	11	6		14	6		1	
Mean zone size			0.7			0.9		1.2

[1] Mean of three trials

0.5 mm>: No inhibition, 0.5-1.0 mm: low inhibition, 1.1-2.0 mm: medium inhibition, 2.1-3.5 mm: high inhibition

3.5. Discussion

3.5.1. Evaluation of antimicrobial techniques

The agar disc technique (I) was used for determining the antimicrobial activity of CPE from pediococci as opposed to the Spot (II) and Overlay (III) techniques. Although techniques II and III have been successfully used in the determination of the antimicrobial activity of LAB (Con *et al.*, 2001; Ray and Miller, 2000), in the present study these techniques produced fussy unclear zones that made it difficult to determine the radius of the inhibition zones. In antimicrobial assays it is recommended to measure circular zones as positive for inhibition and neglect any unclear or vague zones (Piddiock, 1990; Ray and Miller, 2000).

3.5.2. Evaluation of indicator microorganism

P. acidilactici ST1 inhibited both *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176. However, *L. diacetilactis* NCDO 176 gave a larger inhibition zone compared to *L. lactis* NCDO 605. As a result *L. diacetilactis* NCDO 176 was used as the indicator microorganism in determining the antimicrobial activity of pediococci strains. The difference in the sensitivity of two strains of *L. lactis* may be attributed to differences in the composition of the membrane of the two strains (Bennik, Verheul, Abee, Naaktgeboren-Stoffels, Gorris and Smid, 1997; Meghrous, Lacroix and Simard, 1999). Strains among species of LAB with highly fluid cell membranes have been reported to be more sensitive to bacteriocin than those with compact membranes (Bennik *et al.*, 1997).

3.5.3. Antimicrobial activity of the crude pediocin extract produced by strains of pediococci isolated from farm-style cheese against *L. diacetilactis* NCDO 176

Among the 606 LAB isolates (Chapter II) from South African farm-style cheese, 52 (8 %) of these isolates exerted antagonism. In previous studies among the 48 000 LAB isolates from dairy products, meat and plant material, 3 % showed antagonism through bacteriocin production (Roller, 1995). The difference in the number of bacteriocin producing LAB could be attributed to differences in the microflora in the environment (Broadbent *et al.*, 2002). At genus level a total of fifty-two, 47 %, strains among the 110 isolates from both *Pediococcus* species exerted antagonism against *L. diacetilactis* NCDO 176. A comparable percentage of pediococci strains, 48 %, from goat milk and cheese were reported to produce inhibitory substances against the LAB

indicator (Litopoulou-Tzanetaki *et al.*, 1989b). Among these isolates from farm-style cheese, twenty-seven strains, 24 %, were from *P. acidilactici* and twenty-five, 23 %, were *P. pentosaceus* strains. Pediococci exert antagonism against other microorganisms primarily through the production of lactic acid. In addition some strains produce antimicrobial peptides known as bacteriocins, pediocins, (Daeschel, 1989). The use of a LAB, *L. diacetilactis* NCDO 176, in the present study eliminates inhibition due to lactic acid production thereby attributing the inhibition of the indicator microorganism to pediocins produced by some strains of the *Pediococcus* species (Ray and Miller, 2000). In some reports pediococci isolated from Cheddar cheese were reported to have no inhibitory action when accessed for pediocin production (Litopoulou-Tzanetakis *et al.*, 1989a). However in other reports strains of *P. pentosaceus* and *P. acidilactici* isolated from goat milk and cheese were reported to exert antagonism through the production of antimicrobial peptides (Litopoulou-Tzanetakis *et al.*, 1989b).

Strains of *Pediococcus* species, thirty-four, 31%, *P. acidilactici* strains and twenty-four, 22 %, strains of *P. pentosaceus* did not exert antagonism against *L. diacetilactis* NCDO 176. The absence of antagonism among these strains may be attributed to the inability of these strains to produce pediocins. The production of pediocins among LAB is a plasmid-linked trait that may be present in some strains of *Pediococcus* species (Daeschel and Klaenhammer, 1985). In other reports the absence of pediocin production among pediococci isolates from cheeses was attributed to the absence of the plasmid that encodes for pediocin production (Litopoulou-Tzanetakis *et al.*, 1989a)

3.5.4. Antimicrobial activity of strains of *Pediococcus* species against food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644

The cell-free supernatant, CPE, from the *Pediococcus* species showed variable antagonism against *L. monocytogenes* ATCC 7644 while showing low inhibition against *B. cereus* ATCC 1178. Twelve strains, 12 %, from both *Pediococcus* species showed low antagonism while *P. pentosaceus* ST 38, 1 %, showed medium inhibition of *B. cereus* ATCC 1178. Previous reports have shown the inhibitory activity of both species against *B. cereus* (Piva and Headon, 1994; Elegado *et al.*, 1997; El-Adawy, 2001). However, it was not possible to find any published reports on the comparison

of the inhibitory activities of strains from either *Pediococcus* species against *B. cereus*. In the present study a comparable number of strains of both *Pediococcus* species showed low inhibition against *B. cereus* ATCC 1178. The similarity in the inhibition level shown by strains from both *Pediococcus* species may be attributed to the similarity in the inhibition spectrum of pediocins from the two species (Ray and Daeschel, 1994). The low activity exerted by strains of both species against *B. cereus* ATCC 1178 could be attributed to the differences in the strain sensitivity of the *B. cereus* used in the different assays (Raccach and Geshell, 1993). Some strains within a species may be less susceptible to bacteriocins compared to other strains (Meghrous *et al.*, 1999; Ray and Miller 2000). Low inhibition of *B. cereus* ATCC 1178 could be attributed to variation in strains susceptibility as determined by the origin of the pathogenic strain. For instance while more reports are yet to confirm, strains of *Pediococcus* species isolated from meat have been reported to exert greater antagonism against bacterial strains that are prevalent in meat products (Nieto-Lozano, Reguera-Useros, Peláez-Martinez and Hardisson dela Torre, 2002). Seemingly strains of *B. cereus* isolated from cheese may be more susceptible to pediocin producing pediococci isolates from cheese.

Antimicrobial activity from strains of pediococci was higher, 33%, against *L. monocytogenes* ATCC 7644 compared to *B. cereus* ATCC 7644, 13 %, as more strains of *Pediococcus* species inhibited *L. monocytogenes* ATCC 7644. The higher antimicrobial activity exerted by strains of *Pediococcus* species could be attributed to the high susceptibility of some strains of *L. monocytogenes* to pediocins (Eijsink *et al.*, 1998; Song and Richard, 1998). Susceptibility of *L. monocytogenes* may be attributed to the differences in the lipid composition between the two pathogens. Pediocins have been shown to increase their affinity for the target cell that have a high lipid composition where the bacteriocins tends to bind to the anionic lipid content (Ennahar *et al.*, 2000a).

3.5.5. Comparison of the antimicrobial activity of strains *Pediococcus* species

A comparable number of strains from both *Pediococcus* species exerted inhibitory activity against *L. diacetilactis* NCDO 176 and *L. monocytogenes* ATCC 7644 at all three inhibitory levels. Likewise a similar number of strains showed low inhibition of *B. cereus* ATCC 1178. Contrary to these findings, the comparison of the antimicrobial

activity between strains from different *Pediococcus* species reported the occurrence of similar inhibitory activity for strains of the same species rather than among strains from different species (Skyttä, Haikara and Mattila-Sandholm, 1993). None of the strains of *P. acidilactici* showed similar level of inhibition across the test microorganisms. Compared to *P. acidilactici* a similar pattern was shown by strains of *P. pentosaceus*. However, *P. pentosaceus* strain 38 exerted medium inhibition across all three-test microorganisms. Different genera differ in their susceptibility to bacteriocins. The different genera of the test microorganism may account for the absence of a constant or similar inhibition level (Carolissen-Mackay, Arendse and Hastings, 1997; Meghrous *et al.*, 1999).

3.6. Conclusion

Strains of *P. acidilactici*, 24 %, and *P. pentosaceus*, 23 %, exerted antimicrobial activity through the production of pediocins against *L. diacetilactis* NCDO 176. Antagonism was further extended by some strains against the food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644. Among these strains inhibition of food pathogens was variable against *L. monocytogenes* ATCC 7644 and low against *B. cereus* ATCC 1178. Comparison of the antimicrobial activity of the two *Pediococcus* species showed similarity in the inhibition pattern, however, more strains of *P. pentosaceus* exerted antagonism against *L. lactis* NCDO 176 and *L. monocytogenes* ATCC 7644 compared to *P. acidilactici*.

Chapter IV

General discussion, conclusion and recommendations

4. General discussion

Pediococci are Gram-positive lactic acid bacteria, LAB, that may occur among the secondary microflora responsible for cheese ripening as part of the non-starter lactic acid bacteria, NSLAB (Dacre, 1958a, b; Fryer and Sharpe, 1966; Beresford, 2003). Some strains of *Pediococcus* species produce bacteriocins known as pediocins. These are classified as class IIa bacteriocins that offer potential as bio-preservatives due to the anti-listerial activity (Ennahar *et al.*, 2000a, b). In addition, unlike other bacteriocins like nisin, pediocins exhibit a relatively narrow inhibition spectrum. As a result pediocins may not kill starter cultures while exerting antagonism against strains of *Listeria* species (O'Sullivan *et al.*, 2002). Isolation of pediocin producing pediococci from cheese offer potential to the use of pediococci as adjunct cultures or the use of pediocin extract in the control of spoilage and pathogenic bacteria in dairy products. This study was aimed at determining and isolating pediocin producing *Pediococcus* species from commercial and farm-style cheese as well as determining the antimicrobial activity of theses species against food pathogens namely, *B. cereus* and *L. monocytogenes*.

4.1. South African commercial cheese

Among the 140 non-starter isolates examined from the seven different ripening stages of commercial Cheddar cheese, no pediococci isolates occurring as Gram-positive cocci in pairs or tetrads were found at any of the ripening stages. The non-starter isolates were characterised as predominately rod shaped "presumptive" lactobacilli and chain shaped "presumptive" streptococci. Similar reports have been found in Cheddar cheese from Ireland, United Kingdom and South Africa. (Jordan and Cogan, 1993; Lues and Botha, 1998; Lues *et al.*, 1999). The absence of pediococci among commercial Cheddar cheese was attributed to either the use of Good Manufacturing Practises (GMP) or to the inability of strains of *Pediococcus* species to utilise lactose as an energy source (Fox *et al.*, 1998)

The use of GMP such as Good Hygienic Practices through adequate pasteurisation of cheese milk ensures the elimination of contaminant LAB. Strains of *Pediococcus* species may occur among the adventitious LAB contaminates in milk (Fryer and Sharpe 1966;

Crow *et al.*, 2002). Under adequate pasteurisation the number of strains of *Pediococcus* species may be reduced or eliminated in pasteurised cheese (Franklin and Sharpe, 1966; Fox *et al.*, 1998; Crow *et al.*, 2002).

Absence of pediococci may be attributed to the absence of strains of *Pediococcus* species that can utilise residual lactose as a source of fermentable sugar (Garvie 1986; Litopoulou-Tzanetaki *et al.*, 1989a). The absence of a fermentable sugar will result in the elimination of un-adaptive LAB that are unable to utilise other substrates as energy sources (Fox *et al.*, 1998).

The absence of *Pediococcus* species at any of the ripening stages is reflective of hygienic processing and environmental conditions since pediococci may contaminate the cheese from either the environment or the equipment (litopoulou–Tzanetaki *et al.*, 1989a).

4.2. South African farm-style cheese

One hundred and ten isolates representing 18 % of all LAB were Gram-positive cocci occurring in pairs, tetrads and clusters. These isolates were morphologically identified as presumptive pediococci (Darce 1958a, b; Elliot and Mulligan, 1968). The isolates were distributed in pasteurised Gouda (PYG and PMG) and un-pasteurised Bouquet (RAB) as well as in aged and matured Gouda (RAG and RMG).

The presence of pediococci in pasteurised farm-style cheese could be attributed to the survival of thermal-tolerant pediococci strains (Tunner *et al.*, 1986; Litopoulou-Tzanetaki *et al.*, 1989a). Strains of pediococci occur as parts of the adventitious contaminate LAB in milk (Franklin and Sharpe, 1963; Crow *et al.*, 2001). These strains may survive during inadequate pasteurisation of the cheese milk and persist during ripening in the cheese curd (Litopoulou-Tzanetaki *et al.*, 1989a; Crow *et al.*, 2002; Wouters, Ayad, Hugenholtz and Simit, 2002).

In raw milk or un-pasteurised cheese, fermentation of the milk is dependent on naturally occurring LAB within the milk (Wouters *et al.*, 2002). The occurrence of pediococci

among un-pasteurised cheese may be attributed to the persistence of adventitious *Pediococcus* species that may occur as part of the LAB in raw milk (Cogan *et al.*, 1997; Lane, Fox, Walsh, Flokertsma and McSweeney, 1997; Kupiec and Revell, 2001; Wouters *et al.*, 2002).

Alternatively the processing of cheese under less commercialised conditions may possibly introduce pediococci into the curd as parts of the adventitious contaminate NSLAB during the ripening and processing of both pasteurised and un-pasteurised cheese (Williams and Bank, 1997). The possible source of pediococci could be the ripening environment or the processing equipment (Fox *et al.*, 1998; Grappin and Beuvier, 1999; Beresford, 2003).

4.2.1. Number of pediococci isolates in pasteurised and un-pasteurised cheese

The numbers of pediococci isolates occurring in heat-treated or pasteurised farm-style cheese was relatively higher compared to the number of isolates in raw milk or un-pasteurised cheese. Both young and matured pasteurised (PYG and PMG) Gouda cheeses possessed thirty-three and twenty-one pediococci isolates respectively, while aged and matured raw milk Gouda cheese (RAG and RMG) had twelve and sixteen isolates respectively. However, Bouquet was the only raw milk cheese (RAB) that had a high number of pediococci isolates i.e. twenty-eight. The occurrence of more strains of pediococci in heat-treated cheese could have been attributed to the proliferation of thermo-tolerant strains of *Pediococcus* species as a result of the destruction of or elimination of competitive microflora (Elliot and Mulligan, 1968; Litopoulou-Tzanetakis *et al.*, 1989a). However, in raw milk cheese the diversity in the LAB may competitively reduce the number of pediococci strains there by accounting for the low number of pediococci isolates in RAG and RMG compared to PYG and PMG (Boukeri and Ohta, 1996; Bouton, Guyot and Grappin, 1998; Podromou. Thasitou, Hatitonnidou, Tzanetakis and Litoulou-Tzanetaki, 2001).

Strains of pediococci were absent in pasteurised matured Parmesan (PMP) and in both un-pasteurised aged Barbond (RMB) as well as goat cheese Gouda (RGG). The absence

of pediococci in (PMP) and (RMB) could be attributed to the physio-chemical properties of these two cheeses. Out of the limiting factors that influence the growth of NSLAB, PMP and RMB exhibited a high salt-in-moisture content of 13.5 % and 14.3 % (w/v) respectively. The inhibitory effect of the high salt-in-moisture content may account for the absence of pediococci in PMP and RMB (Fox *et al.*, 1998; Crow *et al.*, 2002).

While no clear physio-chemical explanation accounts for the absence of pediococci in Gouda goat cheese, it is possible that the high LAB count of 9.40 $\log_{10}$ could have an influence on the occurrence of pediococci. Possibly the unavailability of a readily fermentable substrate, competition for nutrients as well as the production of antimicrobial substances by other LAB could have limited or inhibit the growth of pediococci in RGG (Fleming *et al.*, 1975, Garvie, 1989; Vanderbergh, 1993).

4.2.2. Characterisation of *Pediococcus* species

Pediococci isolates from farm-style cheeses were characterised into two species, *P. acidilactici* and *P. pentosaceus*. This report is similar to work reported from goat milk and cheese as well as in other dairy diary products where both species constituted part of the NSLAB (Garvie, 1984; Litopoulou-Tzanetakis *et al.*, 1989b).

Strains from both *Pediococcus* species occurred among five farm-styles cheeses in variable numbers. More strains were characterised as *P. pentosaceus* compared to *P. acidilactici* in PYG, PMG and RAG. *P. pentosaceus* has been reported to occur more frequently and in higher numbers in milk and among dairy products compared to *P acidilactici* (Garvie, 1984; Tzanetakis and Litopoulou-Tzanetaki 1989a, b; 1992). Although strains of *P. acidilactici* were isolated during this study from South African farm-style cheese, work by Tzanetakis and Litoupoulou-Tzanetaki, 1992 as well as Boubekri and Ohta, 1996 has characterised this specie among pediococci isolates from artisan cheese. Simultaneous occurrence of strains from both *P. pentosaceus* and *P. acidilactici* among the cheeses may be attributed to the close characteristics relationship shared by the two species (Garvie, 1986; Raccach, 1987; Simpson and Taguchi, 1998)

4.3. Comparison of the susceptibility of the *Lactococcus* strains to CPE from *P. acidilactici* ST1

Indicator microorganisms, *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176, were evaluated for their susceptibility towards CPE from pediocin producing *P. acidilactici* ST1 using the agar disc technique. *P. acidilactici* ST1 inhibited both *L. lactis* NCDO 605 and *L. diacetilactis* NCDO 176. However, *L. diacetilactis* NCDO 176 gave a larger inhibition zone compared to *L. lactis* NCDO 605, hence the former was used as the indicator microorganism in determining the antimicrobial activity of strains of *Pediococcus* species. The difference in the sensitivity of two strains of *L. lactis* could be attributed to the variation in the composition of the cell membrane between the two strains (Montlagh, Holla, Johnson, Ray and Field, 1992; Eljsink, Skeie, Middelhoven, Brurberg and Nes, 1998).

4.3.1. Antimicrobial activity of the crude pediocin extract produced by strains of pediococci isolated from farm-style cheese against *L. diacetilactis* NCDO 176

Out of the 606 LAB isolates from farm-style cheeses 52 isolates, 8 %, exerted antagonism through the production of antimicrobial substances. Compared to other reports (Roller, 1995; Litopoulou-Tzanetakis *et al.*, 1989a) the higher number in bacteriocin producing LAB could have been attributed to the variation in the composition of the environmental microflora (Broadbent *et al.*, 2002). Among the pediococci genus where 110 isolates were identified, fifty-two strains, 47 %, from both *Pediococcus* species exerted antagonism against *L. diacetilactis* NCDO 176 (Litopoulou-Tzanetakis *et al.*, 1989b). Within these strains twenty-seven, 24 %, were strains from *P. acidilactici* and twenty-five, 23 %, were *P. pentosaceus* strains. Antagonism of strains from both *P. pentosaceus* and *P. acidilactici* has been reported among isolates from goat milk, Feta and Kaseri cheese as a result of the production of antimicrobial substances (Litopoulou-Tzanetaki *et al.*, 1989b).

Pediococci exert antagonism against other microorganisms primarily through the production of lactic acid, in addition some strains produce antimicrobial peptides known as bacteriocins, pediocins (Daeschel, 1989). The use of a LAB, *L. diacetilactis* NCDO

176, in the present study eliminates inhibition due to lactic acid production thereby attributing the inhibition of the indicator microorganism to pediocins produced by some strains from the *Pediococcus* species (Ray and Miller, 2000).

Strains from *P. acidilactici* and *P. pentosaceus* exerted comparable antagonism at medium inhibition, with fourteen and seventeen strains respectively, while four strains from both species showed high inhibition against *L. diacetilactis* NCDO 176. The similarity in the antagonism shown by the strains from both *Pediococcus* species may be attributed to the similarity in the inhibitory spectrum showed by pediocins produced by strains from both species (Ray and Daeschel, 1994; Ray and Miller, 2000). Antimicrobial spectrum of both *P. acidilactici* and *P. pentosaceus* has been reported to include *L. Lactis* strains among the sensitive indicator to pediocins (Eljsink *et al.*, 1998; Ray and Miller, 2000).

Some strains of both *Pediococcus* species thirty-four, 31 %, *P. acidilactici* strains and twenty-four, 22 %, strains of *P. pentosaceus* did not exert antagonism against *L. diacetilactis* NCDO 176. The absence of pediocin production among pediococci isolates from cheeses was attributed to the absence of the plasmid that encodes for pediocin production (Daeschel and Klaenhammer, 1985; Litopoulou-Tzanetaki *et al.*, 1989a).

4.3.2. Antimicrobial activity of strains of *Pediococcus* species against food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644

A similar number of strains from both *Pediococcus* species showed antagonism against *L. diacetilactis* NCDO 176. Strains exerting inhibition zones of 1 mm and less were not used for the subsequent antimicrobial assays. Thirty-nine strains; twenty- one *P. pentosaceus* and eighteen *P. acidilactici* were used for subsequent assay.

The cell-free supernatant, CPE, of strains from both *Pediococcus* species showed variable antagonism against *L. monocytogenes* ATCC 7644 and low inhibition against *B. cereus* ATCC 1178. Against *B. cereus* ATCC 1178 twelve strains from both *Pediococcus* species showed low antagonism while *P. pentosaceus* ST 38 showed medium inhibition.

The inhibitory spectrum of pediocins from both *Pediococcus* species has been reported to inhibit *B. cereus* (Piva and Headon, 1994; Elegado *et al.*, 1997; El-Adawy, 2001). However, it was not possible to find any published reports on the comparison of the inhibitory activities of strains from either *Pediococcus* species against *B. cereus*. The low activity exerted by strains from both *Pediococcus* species against *B. cereus* ATCC 1178 can be attributed to the differences in the sensitivity of the strains of the *B. cereus* used in the different assays (Raccach and Geshell, 1993; Meghrous *et al.*, 1999; Ray and Miller 2000). Low inhibition of *B. cereus* ATCC 1178 may be further attributed to variation in strains susceptibility as determined by the origin of the pathogenic strain. While more reports are yet to confirm, strains of *Pediococcus* species isolated from meat have been reported to exert greater antagonism against bacterial strains that are prevalent in meat products (Nieto-Lozano, Reguera-Useros, Peláez-Martinez and Hardisson dela Torre, 2002). Presumably strains of *B. cereus* isolated from cheese may be more susceptible to pediocin producing pediococci isolates from cheese.

The antimicrobial activity of strains of pediococci was higher, 33 %, against *L. monocytogenes* ATCC 7644 compared to, 13 %, against *B. cereus* ATCC 7644 as more strains of *Pediococcus* species inhibited *L. monocytogenes* ATCC 7644. The higher antimicrobial activity exerted by strains of *Pediococcus* species may be attributed to the high susceptibility of some strains of *L. monocytogenes* to pediocins (Eljsink *et al.*, 1998; Song and Richard, 1998; Montville and Chen 1998; Ray and Miller 2000). Susceptibility of *L. monocytogenes* may be attributed to the differences in the lipid composition between the two pathogens (Ennahar *et al.*, 2000).

4.3.3. Comparison of the antimicrobial activity of strains *Pediococcus* species

A comparable number of strains of *Pediococcus* species from both species exerted inhibitory activity against *L. diacetilactis* NCDO 176 and *L. monocytogenes* ATCC 7644 at all three inhibitory levels. Likewise a similar number of strains showed low inhibition of *B. cereus* ATCC 1178. Contrary to these findings, the comparison of the antimicrobial activity between strains from different *Pediococcus* species against specific pathogens reported the occurrence of similar inhibitory activity for strains of the same species rather

than among strains from different species (Skyttä, Haikara and Mattila-Sandholm, 1993). Among all strains of *P. acidilactici* and most strains of the *P. pentosaceus*, none showed a similar level of inhibition across all three-test microorganisms. However, *P. pentosaceus* strain 38 exerted medium inhibition against all three microorganisms. Differences in the inhibition pattern or susceptibility of the three genera may be attributed to the variation in the susceptibility of different genera to bacteriocins (Carolissen-Mackay, Arendse and Hastings, 1997; Meghrous *et al.*, 1999).

4.4. Methodology review

The methods used in the research project are discussed in line with the scientific principles and an outline of the advantages, limitations and modifications will be examined.

4.4.1. Evaluation of antimicrobial techniques

Antagonism among strains of *Pediococcus* species against indicator and test microorganisms as a result of the production of pediocins was demonstrated using the agar disc diffusion assay (El-Adawy, 2001). Cultures of *Pediococcus* strains were propagated for 24-48 h to optimise pediocin production. Both strains of *Pediococcus* species have been shown to optimally produce pediocin within the first 48 h of growth (Mattila-Sandholm *et al.*, 1991). Thereafter the culture was centrifuged at pH 6.0-6.5 to maximise adsorption of bacteriocins from the bacterial cells and the supernatant was used as the crude pediocin extract (Yang, Johnson and Ray, 1992).

The agar diffusion technique has several modifications, among these the agar spot, overlay and the disc assay were compared for the most appropriate assay to be used in the antimicrobial assay (Davidson and Parish, 1989). In the agar diffusion assay the principle is based on the diffusion of the antimicrobial substance from the source across the agar thereby creating a concentration gradient that is inversely proportional to the distance from the source. The presence of a zone of no growth of the indicator microorganism demonstrates inhibition around the antimicrobial source (Parish and Davison, 1993).

Comparison of the three techniques showed that the overlay method failed to produce distinct zones of inhibition against the indicator microorganism. Similar results were obtained using the spot assay, however, in cases where inhibition zones appeared; the zones were fuzzy thereby making it difficult to measure the diameter of the inhibition zones. Inhibitions zones of indicator microorganisms should be clear and circular to allow the measurement of the zones (Parish and Davison, 1993; Ray and Muller, 2000). Contrary to these results from the spot and overlay assay, the disc method produced measurable circular inhibition zones.

Although the agar diffusion is the most commonly used assay, this method is limited by some factors. In the case where the inhibition zone extends over a large diameter, the zone may cover the area of less inhibitory microorganism. In other cases where a heavy inoculum size is used, the growth of the bacteria might partially or totally mask the inhibition area of the indicator microorgansm (Kang and Fung, 1997). Another limitation of the agar method is that the test is qualitative. Results from this test categorise microorganisms as being susceptible, intermediate or resistant (Davidson and Parish, 1989)

4.5. Recommendations

Isolation of pediococci from farm-style cheese may be improved through the use of growth factors that are selective for pediococci (pH and salt content) or the choice of a selective media for pediococci. The use of indicator like bromoceresol may be used to distinguish pediococci among the LAB colonies on an appropriate agar (Raccach, 1999).

In the determination of the antimicrobial activity of pediococci isolates, it would be appropriate to optimise the production of pediocins among isolates that exert antagonism against LAB indicator. Optimisation of antimicrobial activity may be done through supplementation of the growth medium with growth stimulants and the establishment of the incubation temperature and time that give optimal production of antimicrobial substances (Carolisen-MacKay *et al.*, 1997).

In the determination of the antimicrobial activity of the isolates it would be recommended to use a wider range or spectrum of test microorganisms. The spectrum should include pathogens isolated from farm-style cheeses or dairy products. Pathogens isolated from dairy products would give an indicator of the antimicrobial potential of pediocin producing isolates against target food pathogens (Raccach and Geshell, 1993; Nieto-Lozano *et al.*, 2002). The selection of the pediococci isolates for use in actual food systems should be based on the broadness of the inhibition spectrum of the isolates (Roller, 1995)

Several modifications of the agar diffusion assay are to be used in determining the ability of LAB to produce bacteriocins. The use of different modifications will allow the choice of a single method that is most appropriate since no single method may be used universally for all LAB. Where possible the use of partially purified or purified solution of the bacteriocin extract may give more distinctive results with clear zones of inhibition (Carolisen-MacKay *et al.*, 1997; El-Adawy, 2001).

4.6. Conclusions

Among the farm-style cheeses NSLAB isolates occurring in pairs, tetrads and clusters were identified as "presumptive" pediococci. A total of 110 'presumptive' pediococci were isolated from the farm-style cheeses. These isolates were further characterised as either *P. acidilactici* or *P. pentosaceus*. The numbers of pediococci isolates were distributed as sixty-one (55 %) of *P. acidilactici* and forty-nine (45 %) of *P. pentosaceus*.

Five of the eight farm-style cheeses harboured strains from both *Pediococcus* species. Among the farm-styles cheeses, two heat-treated or pasteurised cheeses, PYG and PMG and three, raw milk or un-pasteurised cheese RMB, RAG and RMG harboured pediococci as part of their LAB. Strains of *Pediococcus* species were distributed in both pasteurised and un-pasteurised in approximately equivalent numbers of fifty-four and fifty-six isolates respectively.

Strains of *P. pentosaceus* were present in larger numbers compared to *P. acidilactici* in three of the farm-style cheeses, PYG, PMG and RAG. However *P. acidilactici* was present in higher numbers in un-pasteurised cheeses, RMB and RMG. Conversely strains of *Pediococcus* species were absent from three farm-styles cheese, PMP, RAB and RGG.

Antagonism of strains of *Pediococcus* species was demonstrated against the indicator microorganism, *L. diacetilactis* NCDO 176, through the crude pediocin extract, CPE. Among the 110 pediococci isolates fifty-two isolates (47 %) showed antimicrobial activity through the CPE. Antagonism among these isolates was distributed as twenty-seven isolates of *P. acidilactici* and twenty-five isolates of *P. pentosaceus* strains.

Some of the strains of *Pediococcus* species exerting antagonism through the CPE showed variable antagonism against *L. monocytogenes* ATCC 7544 and low inhibition against *B. cereus* ATCC 1178. Inhibition shown by strains of both *Pediococcus* species was more pronounced against *L. monocytogenes* ATCC 1178, compared to *B. cereus* ATCC 1178. Comparison of the number of strains exerting antimicrobial activity between the two *Pediococcus* species showed similarity in the inhibition pattern, however, more strains of *P. pentosaceus* exerted antagonism against *L. lactis* NCDO 176 and *L. monocytogenes* ATCC 7644 compared to *P. acidilactici*.

Although strains of *Pediococcus* species were isolated from farm-style cheeses, the sample size may be small to make conclusion on the occurrence of pediococci in farm-style cheese. Nevertheless, strains of pediococci occurred in both heat-treated and raw milk cheese. Some among the strains of both *P. acidilactici* and *P. pentosaceus* showed antagonism against food pathogens, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644, through the production of antimicrobial peptides known as pediocins.

4.7. Future Research needs

- Identification or characterisation of NSLAB responsible for natural fermentation and ripening of South African farm-style cheese.
- Optimise production of pediocins among strains from *Pediococcus* species.

- Determination of the width or broadness of the inhibitory spectrum of *Pediococcus* species isolated from dairy products.
- Determining the activity of pediococci culture against commercial LAB starter cultures and the activity of pediococci cultures during the fermentation process e.g. changes in pH and acidification rate
- Co-production of two or more different bacteriocins in addition to pediocin production among strains of *Pediococcus* species and the determination of the inhibitory action of two or more bacteriocins against pathogenic bacteria isolated from dairy products