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Application of *Pediococcus* spp. as adjunct cultures in Gouda cheese

By

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Declaration

I declare that the dissertation herewith submitted for the degree Master in Science (MSc) at the University of Pretoria, has not previously been submitted by me for a degree at any other university or institution of higher education.

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Abstract

Application of *Pediococcus* spp. as adjunct cultures in Gouda cheese

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Department: Food Science

Degree : MSc

The commercial significance of the dairy fermentation industry which incorporates the production of cheeses, is well recognised and ranks second only to the production of alcoholic beverages. Consumers today demand greater choice and variety with improved quality standards. Lactic acid bacteria, in particular the genera *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Pediococcus* play a central role in the production of new and exotic flavoured cheeses.

Gurira & Buys (2005) found that the *Pediococcus* species crude pediocin extract that were isolated from South African Gouda cheeses was inhibitory against food pathogens, *Listeria monocytogenes* and *Bacillus cereus*. Selected *Pediococcus* species were evaluated in this study for their activity against available mixed strain mesophilic lactic acid Gouda cheese starter cultures. The commercially available starter cultures, Nizostar 500MT, LL 50C and Dairysafe™ were evaluated. The starter cultures were evaluated for their sensitivity to the *Pediococcus* species crude pediocin extract on the basis of their lactic acid production, pH and growth levels. The mesophilic mixed strain lactic acid Gouda cheese starter culture, Dairysafe™ was identified as the starter culture that was the least sensitive to the *Pediococcus* species crude pediocin extract as the lactic acid production and pH level of Dairysafe™ was the least affected. Thus, starter culture, Dairysafe™ was used in conjunction with live cultures of *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture in the manufacture of Gouda cheese.

Gouda cheese made using Dairysafe™ as the starter culture and live cultures of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture was found to have a chemical composition comparable to that of the cheese, to which no adjunct culture had been added. The addition of the *Pediococcus* species as an adjunct culture in Gouda cheese manufacture had no significant effect on the levels of lipolysis or proteolysis of the cheese during the 45 day ripening period.

However, there were significant differences noted in the sensory attributes of the cheese manufactured using *Pediococcus* species as an adjunct culture and the cheese manufactured without an adjunct culture.

The results of this study indicate that *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 can be used as an adjunct culture in conjunction with a pediocin resistant starter culture, such as Dairysafe™ to produce a cheese that is physico-chemically comparable to Gouda cheese currently on the market but is clearly differentiable from the homogenous products currently available.

Table Of Contents Page

List of tables Page

List of figures Page

University of Pretoria etd – Verachia, W (2005)

CHAPTER I

Introduction and literature review

1.1 Introduction

Gouda is a semi-hard continental cheese that originated in Holland (McSweeney, 2004). Cheese is a fermented milk product that preserves milk, which is highly perishable (Farkye, 2004). During cheese manufacture, the initiation of the fermentation process begins with the addition of a starter culture to the milk. Starter cultures produce a fermented food by accelerating and steering its fermentation process. Mesophilic starters with an optimal growth temperature of about 30 ^{o}C are used (Marilley & Casey, 2004). Gram-positive lactic acid bacteria (LAB), occupy a central role in these processes, and has a long and safe history of application and consumption in the production of fermented foods (Ayad, Verheul, Wouters & Smit, 2002a).

The earliest production of fermented foods were based upon the spontaneous fermentation of the raw material due to the development of microflora naturally present in the raw material, environment and equipment (Leroy & De Vuyst, 2004). Today fermented foods like cheese are manufactured on a large scale from pasteurised milk under a high degree of control using a uniform commercial starter culture. Replacement of the complex native microbial flora in raw milk by uniform commercial starter cultures results in a standardised end-product (Macedo, Tavares & Malcata, 2004).

Standardisation has, however, led to the loss of the typical characteristics that are peculiar to the type of traditional cheese originally manufactured from raw milk (Rehman, Banks, McSweeney & Fox, 2000). Starter cultures are now produced on an industrial scale leading to limited biodiversity, absence of the necessary characteristics for product diversification and a lack of commercially available new interesting starter cultures (Macedo *et. al.*, 2004). Today's consumers appreciate traditionally fermented products, produced by spontaneous fermentation of microflora native to the raw milk due to their outstanding diverse gastronomic qualities (Leroy & De Vuyst, 2004). The introduction of secondary non starter lactic acid bacteria (NSLAB) to the pasteurised milk in conjunction with the commercial starter culture was found to bring about a more intense flavour with improved gastronomic quality (Antonsson, Molin & Ardo, 2003).

Dacre (1958) found that a specific group of LAB namely, pediococci when added as NSLAB enhanced the flavour of cheese manufactured in an experimental cheese factory. The flavour development of the cheese followed the growth of the pediococci more closely than that of the lactobacilli and at the end of the ripening period the pediococci constituted about a quarter of the normal lactic acid flora of the cheese (Dacre, 1958).

Elliott & Mulligan (1968) suggested that the use of some pediococci as an adjunct culture should be encouraged in cheese and even predicted that the day might come when cheese makers deliberately add selected cultures of pediococci to their cheese milk as NSLAB.

Cheese sales represent about 30 % of the total global dairy sales with a predicted increase of 9.8 % between 2003 and 2007 (Farkye, 2004). Since the ability to positively manipulate the flavour of cheese is a crucial selling point for Gouda cheese, flavour modulation by selected NSLAB strains can be used to enhance the positive flavour notes of the cheese and thereby clearly differentiate it from all other homogenous products on the market. This will serve to strengthen the market position of Gouda cheese in a growing cheese market (Franklin & Sharpe, 1963; Crow, Curry & Hayes, 2001).

Lactic acid bacteria are industrially important, as they not only offer technological functionality but also contribute to microbial safety, through the production of anti-microbial substances (Ray & Miller, 2000). Bacteriocins or antibacterial peptides produced by strains of LAB has generated interest as potential food biopreservatives as current consumers have an increasing interest in commercially processed foods that are not preserved with non-food preservatives (Daeschel according to Yang & Ray, 1994). Therefore, a controlled microflora of bacteriocin producing LAB could act as a hurdle to the growth of undesirable spoilage and pathogenic bacteria (McMullen & Stiles, 1996). Bacteriocins from LAB are low molecular mass weight proteins with an antimicrobial mode of action restricted to related Gram-positive bacteria (Cleveland, Montville, Nes & Chikindas 2001). The production of bacteriocins increases the competitiveness of the producer strain against closely related Gram-positive strains in the food matrix and, therefore, could be inhibitory to the Gram-positive LAB used as starter cultures

(Rodriguez, Martinez, Horn & Dodd, 2002). Therefore, currently available LAB starter cultures need to be investigated for their sensitivity to the bacteriocin-producing LAB that may be used as NSLAB.

But in order to achieve these goals by selling the appropriate cultures for the right application, the capacity and limitations of the available NSLAB strains needs to be fully understood. This is the objective of the project.

1.2 Literature review

1.2.1 Gouda cheese

It is thought that the art of cheese making was developed as far back as 8000 years ago in the Fertile Crescent between the Tigris and Euphrates rivers in Iraq but there are now in access of 1000 cheese varieties worldwide (Fox, Law, McSweeney & Wallace, 1993). Gouda cheese, however, originated in South Holland. Initially the primary objective of cheese manufacturing was to extend the shelf life as well as to conserve the nutritional content of milk through fermentation (Beresford, Fitzsimons, Brennan & Cogan, 2001). Presently, approximately a third of the world's milk production is used in cheese manufacturing (Farkye, 2004). Although fermentations have been exploited as a method of food and beverage preservation for thousands of years, it has only been in the recent past that microorganisms were recognised as being responsible for the fermentation process. In 1861 AD when pasteurisation was developed the role of microorganisms in fermentations was first realised (Ross, Morgan & Hill, 2002). High densities of microorganisms present in cheese throughout ripening play a significant role in the maturation process (Antonsson *et. al.*, 2003). According to Ross *et. al.* (2002) the microflora of cheese may be divided into two groups, (i) starter LAB involved in acid production during manufacture and ripening and (ii) secondary microorganisms which do not contribute to acid production during manufacture, but generally play a significant role during ripening. The secondary microflora comprise of NSLAB.

1.2.1.1 Starter lactic acid bacteria

Starter bacteria have the primary function of producing acid during the fermentation process, however, their enzymes are involved in proteolysis and the conversion of amino acids into flavour compounds, therefore, they also contribute to the cheese ripening process (Leroy & De Vuyst, 2004). Starter bacteria could be defined as isolates, which produce sufficient acid to reduce the pH of milk to < 5.3 in 6 h at 30-37 ^{o}C. Either mesophilic or thermophilic starter cultures are used in cheese production but, mesophilic cultures are used in the production of Gouda cheese (Marilley & Casey, 2004).

Another important role of starter bacteria is to provide a suitable environment, with respect to redox potential, pH and moisture content of the cheese, which allows enzyme activity from the rennet and starter culture, as well as growth of the secondary microflora to proceed favourably (Macedo *et*. *al.*, 2004).

Starter cultures make the only significant contribution to the microbial biomass of LAB in the young curd. This relatively high starter biomass represents considerable biocatalytic potential for cheese ripening reactions, which could be modulated through autolysis of the starter cells (Beresford *et*. *al.*, 2001; Antonsson *et*. *al.*, 2003).

1.2.1.2 Secondary microorganisms / non starter lactic acid bacteria

Non starter lactic bacteria are mesophilic lactobacilli or pediococci, which form a significant portion of the microbial flora of most cheese varieties during ripening (Rehman *et*. *al.*, 2000). They do not form part of the normal starter flora, do not grow well in milk and do not contribute to acid production in the cheese vat (Crow *et*. *al.*, 2001). Non starter lactic bacteria are traditionally divided into three groups on the basis of being (a) obligatory homofermentative, (b) facultatively heterofermentative (FHL), or (c) obligatory heterofermentative (El Soda, 1993). The NSLAB regularly encountered in cheese are members of group b, the facultatively heterofermentative (Beresford *et*. *al.*, 2001).

1.2.2 Flavour

Olson (1990) stated that there is a cheese for every taste preference and a taste preference for every cheese. Cheese starts from milk, a substrate that is not entirely flavourless but rather bland (McSweeney, 2004). A cheesemaker can make in excess of 500 varieties of cheese from the same starting substrate with each variety having a unique and characteristic flavour and aroma.

Cheese is a biochemically dynamic product and unlike many processed food products for which stability is the key criteria, cheese undergoes significant changes during its ripening period (Fox & Wallace, 1997). The unripened curds of many cheese varieties

have bland and largely similar flavours. It is only during the ripening period that the flavour compounds, which are characteristic of each variety, are produced (Fox & Law, 1991).

Initially it was thought that cheese flavour was due to a single compound or class of compounds, however, it is now generally accepted that the flavour of most cheeses is the result of the correct balance and concentration of a wide range of sapid and aromatic compounds largely due to the "component balance theory" proposed by Mulder according to Fox, Singh & McSweeney (1995). Cheese flavour is inseparable from the biochemistry of cheese ripening and over 300 different volatile and non-volatile compounds have been implicated in cheese flavour (Fox, Singh & McSweeney, 1995).

The flavour of cheese is amongst its principle attributes influencing quality and preference, thus, flavour is one of the most important selling attributes of Gouda cheese and has, therefore, received much attention (McSweeney & Sousa, 2000; Ayad, Verheul, Wouters & Smit, 2002b). The development of flavour in cheese is a complex process, originating from a combination of microbiological, biochemical and technological aspects (Ayad *et. al.*, 2002b). Microbiological changes to the cheese during ripening include the death and lysis of starter cells and the growth of a secondary flora namely, NSLAB (McSweeney, 2004). The sensory properties of cheese are determined to a great extent by biochemical changes to the curd during ripening (Marilley & Casey, 2004). Biochemical changes can be grouped into (i) primary and (ii) secondary events. Primary events include, lipolysis, proteolysis and metabolism of residual lactose, of lactate and citrate. Secondary events include, metabolism of fatty acids and amino acids (McSweeney, 2004). Enzymes in the milk, coagulant, cultures and indigenous microflora cause these catabolic activities. The amount of proteolysis, lipolysis and glycolysis is also influenced by chemical conditions such as water activity, pH of the cheese and release of microbial intracellular enzymes during autolysis (Bachman, Butikofer & Meyer, 1999). The biochemical changes leading to the formation of flavour compounds according to Marilley & Casey (2004) is shown in Figure (Fig.) 1.

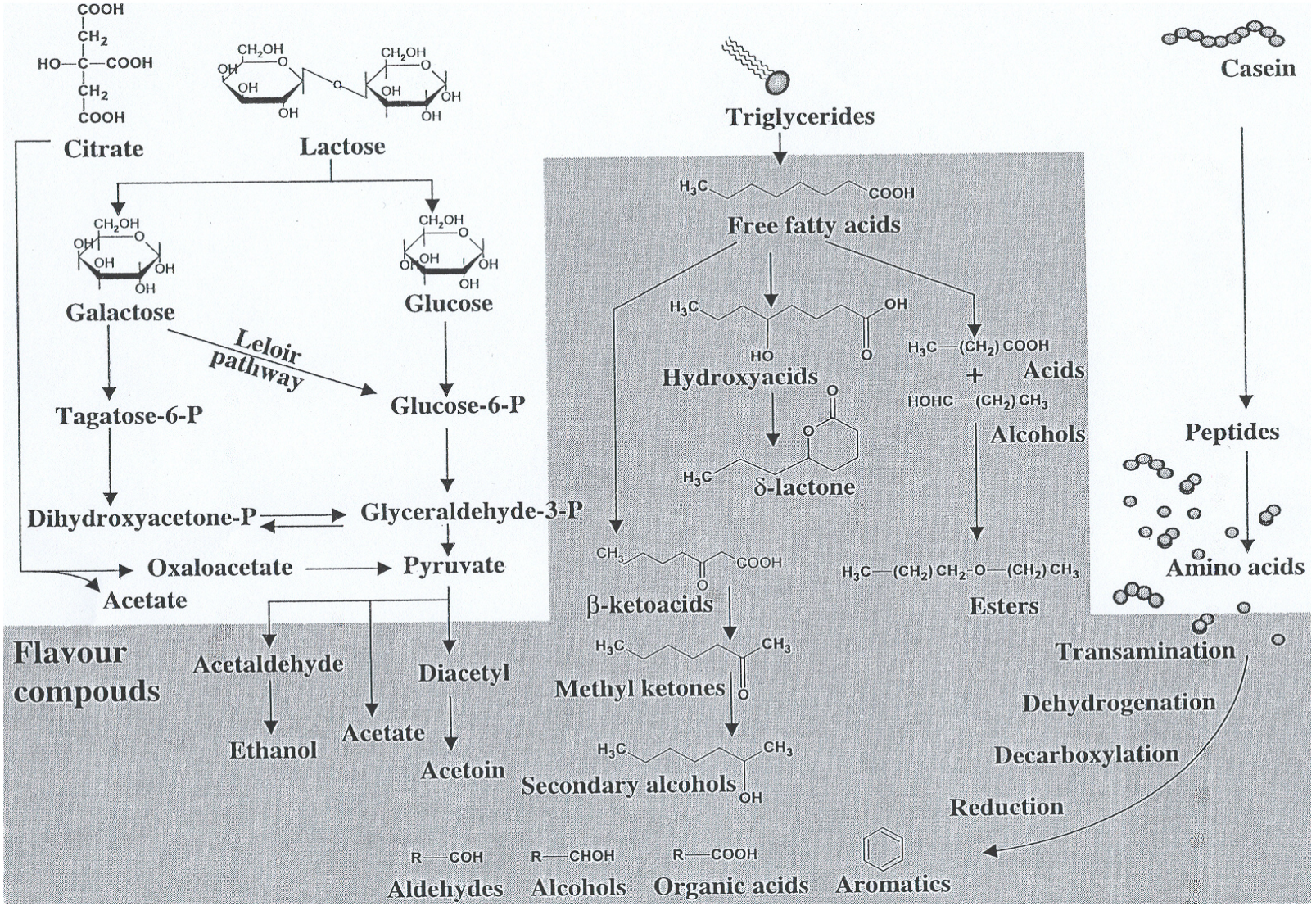

Figure 1. Biochemical pathways leading to the formation of flavour compounds. The grey surface indicates compounds with a flavour note (Marilley & Casey, 2004).

University of Pretoria etd – Verachia, W (2005)

1.2.2.1 Primary biochemical events

1.2.2.1.1 Metabolism of residual lactose

Lactic acid bacteria used as starter cultures metabolise lactose to lactate leading to the fermentation of milk during cheese manufacture (McSweeney, 2004). The pH of cheese curd is determined by the rate and extent of acidification during manufacture as well as the buffering capacity of the cheese curd. The pH indirectly affects the texture and flavour of the cheese by affecting the activity of the enzymes important for ripening as well as the retention of enzymes during manufacture (Holmes, Duersch & Ernstrom, 1977). Most of the lactose in milk is lost in the whey as lactose or lactate during cheese manufacture. Low levels of lactose, however, remain in the curd after manufacture (Huffman & Kristofersen, 1984). Complete fermentation of lactose is important as it avoids the development of undesirable secondary microflora (Marilley & Casey, 2004). Residual lactose is quickly metabolised to L-lactate during the early stages of ripening at a rate largely determined by action of the starter culture, temperature and the salt-in-moisture levels of the curd. Lactose unfermented by the starter culture is probably metabolised by the NSLAB, forming D-lactate or DL-lactate by racemisation of the L-lactate (Turner & Thomas, 1980).

1.2.2.1.2 Metabolism of lactate

Lactate is the result of lactose fermentation produced during the growth of the starter culture and is an important substrate for a range of reactions that occur in cheese during ripening (McSweeney, 2004). Lactose can be directly transformed into D-lactate by starter LAB or by the racemisation of L-lactate (Fox & Wallace, 1997). The rate at which the L-lactate is racemised depends on the composition of the NSLAB flora. Pediococci racemise faster than lactobacilli (Turner & Thomas, 1980). Increased levels of lactate favour the growth of NSLAB (McSweeney & Fox, 2004). Lactate can be oxidised by LAB in cheese to products including acetate, ethanol, formate and CO_2 as shown in Fig. 2 (McSweeney, 2004).

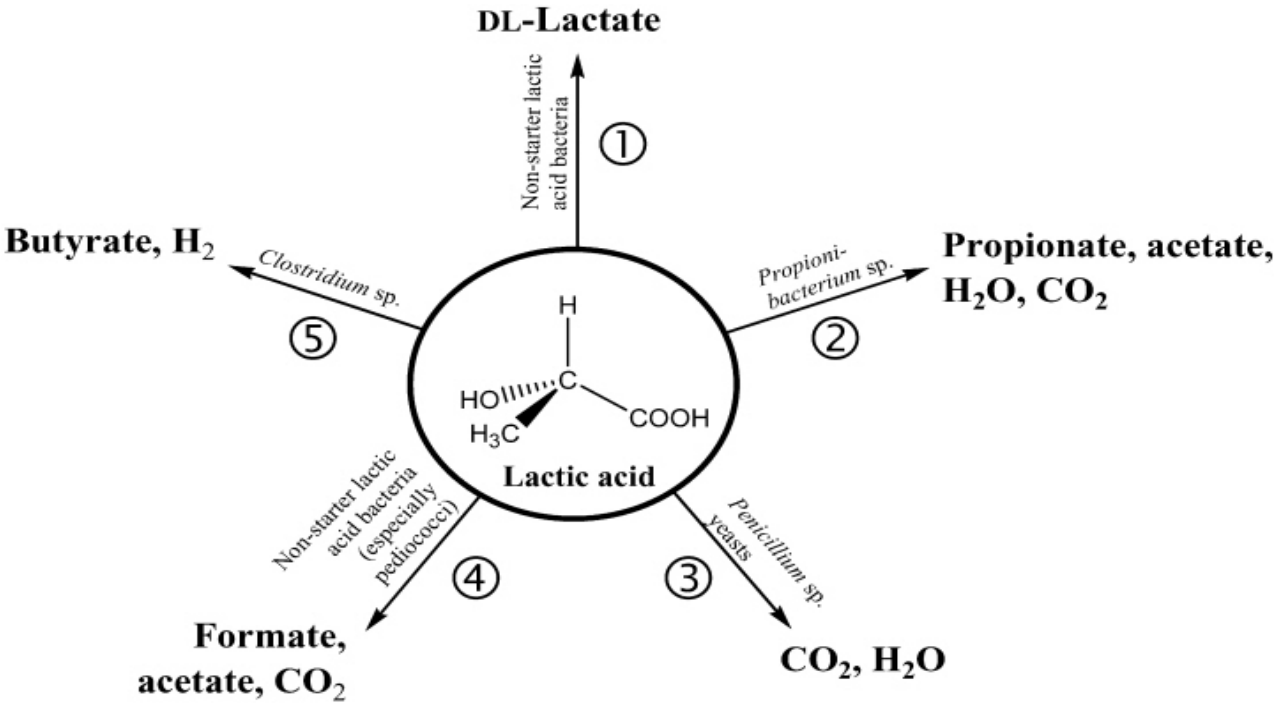

Figure 2. Pathways by which lactate is metabolised in cheese during ripening (McSweeney, 2004).

1.2.2.1.3 Metabolism of citrate

Milk contains approximately 1750 mg citrate per litre, much of which is in the soluble phase (Fox *et. al.*, 1993). Citrate is an important precursor for flavour compounds in varieties of cheeses made using mesophilic starter cultures (Cogan & Hill, 1993). Citrate may also be metabolised by some strains of NSLAB. The products of citrate metabolism include CO_2, which are responsible for the small eyes often found in Dutch type cheeses as well as important flavour compounds, acetoin, acetate and particularly diacetyl (McSweeney, 2004).

1.2.2.1.4 Lipolysis

Lipids, specifically triglycerides in cheese varieties undergo hydrolysis by lipolytic enzymes of indigenous, endogenous and /or exogenous lipases (McSweeney & Sousa, 2000). This results in the liberalisation of free fatty acids (FFA) in the cheese during ripening. The FFA's, especially the short and intermediate-chain FFA's released, directly contribute to the cheese flavour (Collins, McSweeney & Wilkinson, 2003). The

triglycerides of ruminant milk fat are rich in short chain FFA's, which when liberated, have low flavour thresholds that contribute significantly to the flavour of many cheese varieties (Huffman & Kristofersen, 1984). Low levels of lipolysis contribute to the ripening and, thus, flavour of Gouda cheese, whereas excessive levels of lipolysis are undesirable as they lead to rancidity (Collins *et. al.*, 2003). Lipolytic agents in cheese generally originate from the milk, the coagulant and cheese microflora of starter and NSLAB / adjunct microorganisms. Gouda cheese is an internally bacterially ripened variety but when made from pasteurised milk, does not have any strongly lipolytic agents. Although lipolysis progresses during ripening, it is as a result of the action of enzymes from the starter LAB and NSLAB (Marilley & Casey, 2004). As starter LAB and NSLAB are present in the cheese in high numbers, the enzymes from these organisms are responsible for the liberation of significant levels of FFA's during the ripening period of internally bacterially ripened cheeses (Collins *et. al.*, 2003). Lactic acid bacteria contain intracellular lipolytic enzymes, which are released into the cheese matrix upon cell lysis (McSweeney & Sousa, 2000). In addition to their direct contribution of cheese flavour, FFA's are also important precursors for the production of volatile flavour compounds (McSweeney, 2004).

1.2.2.1.5 Proteolysis

Proteolysis is the most complex and in most cheese varieties, the most important contributor to flavour from all the primary biochemical events that occur in cheeses during ripening (Fox & Wallace, 1997).

Proteolysis contributes to cheese ripening in at least four ways according to Fox (1989):

1. Direct contribution to flavour via amino acids and peptides, some of which may cause off-flavours such as bitterness, or indirectly via catabolism of amino acids to amines, thiols and thioesters,
2. Greater release of sapid compounds during mastication,
3. Changes in pH via the formation of ammonia (NH_3),
4. Changes in texture from the breakdown of the protein network, increase in pH and greater water binding by the newly formed amino acids and carboxyl groups.

Although the ripening of cheese is dominated by lipolysis and proteolysis, in Dutch type cheeses, proteolysis is the major biochemical event during ripening, as there is a high correlation between intensity of flavour and free amino acid concentration (Fox, Singh & McSweeney, 1995).

The general steps of proteolysis according to Farkye (2004) are as follows:

1. Initial hydrolysis of caseins by residual coagulation and plasmin to large peptides,
2. Breakdown of large peptides by starter proteinases and peptides into medium and small peptides,
3. Further hydrolysis of the medium and small peptides by starter peptidases into dipeptides, tripeptides and free amino acids.

The action of peptidases found in LAB is shown in Fig. 3:

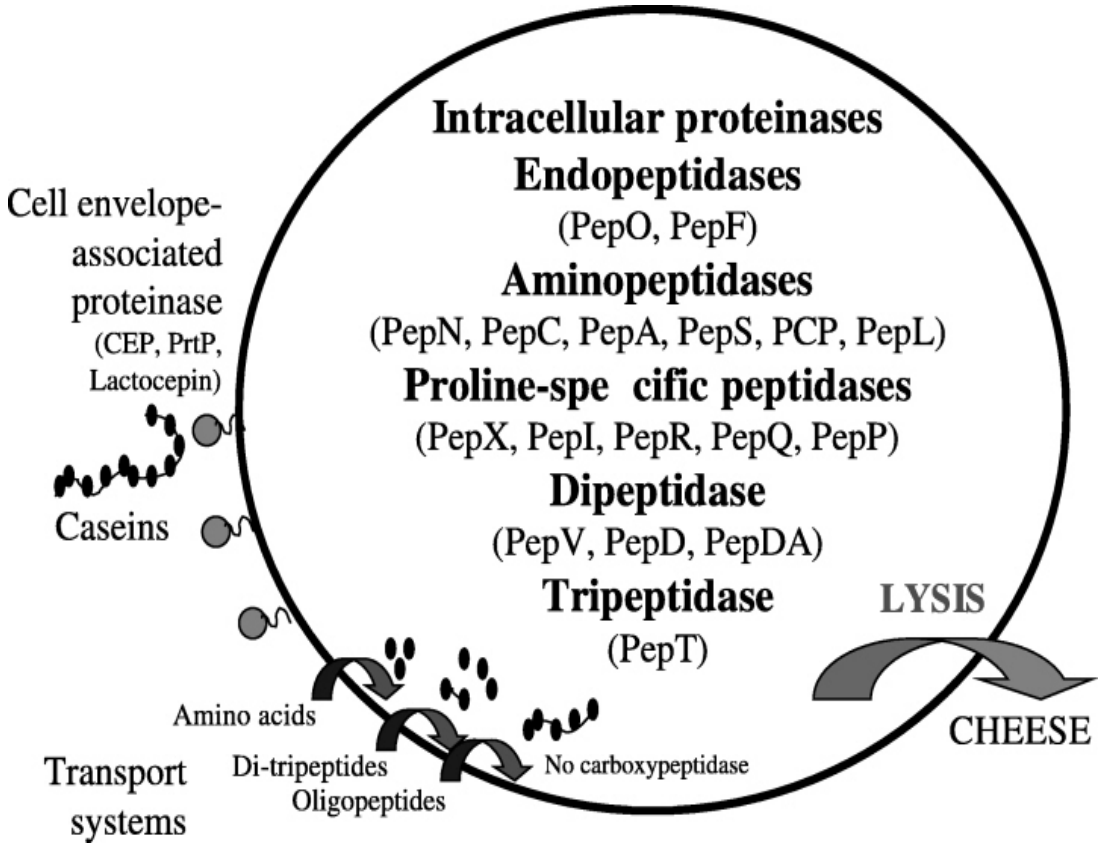

Figure 3. Schematic representation of the action of peptidases found in lactic acid bacteria (McSweeney, 2004).

The proteinases and peptidases that catalyse proteins in cheese during ripening originate from six primary sources, namely, coagulant, milk, starter LAB, NSLAB, secondary starters and exogenous proteinases or peptidases added to the milk or curd (McSweeney, 2004). A major source of proteolytic enzymes in many cheese varieties is the residual coagulant, often chymosin that remains trapped in the curd. Up to 30 % of the coagulant activity added to milk remains active in the curd depending on factors such as enzyme type, cooking temperature and pH at whey drainage (McSweeney, 2004).

The peptides produced by the action of the residual coagulant and plasmin are often either tasteless or bitter and does not directly contribute to the typical cheese flavour (Fox, 1989). However, the mixture of small peptides and amino acids directly influence the taste and mouthfeel of cheese as proteolysis contributes to the softening of the cheese texture during ripening due to hydrolysis of the casein matrix of the curd and a decrease in the water activity (a_w) of the curd brought about by changes in the water binding capacity by the new carboxylic and amino groups formed during hydrolysis (Law & Haandrikman, 1997). The free amino acids may also be further catabolised into flavour compounds that are unique for each cheese variety and are dependant on the types of enzymes and microorganisms available, particularly NSLAB (Farkye, 2004). Flavour compounds in cheese are concentrated in the water-soluble fraction, hence, the methods for studying the proteolysis and fractionation of characterising water-soluble nitrogen (WSN) are important in cheese research and technology (Kuchroo & Fox, 1982).

1.2.2.2 Secondary biochemical events

1.2.2.2.1 Metabolism of free fatty acids

Whilst short-chain fatty acids contribute directly to the cheese flavour, FFA's also indirectly contribute to cheese flavour by acting as precursors for the production of volatile flavour compounds through a series of reactions known collectively as the metabolism of fatty acids (Collins *et. al.*, 2003). Pathways for the metabolism of FFA's in cheese during ripening are summarised in Fig. 4.

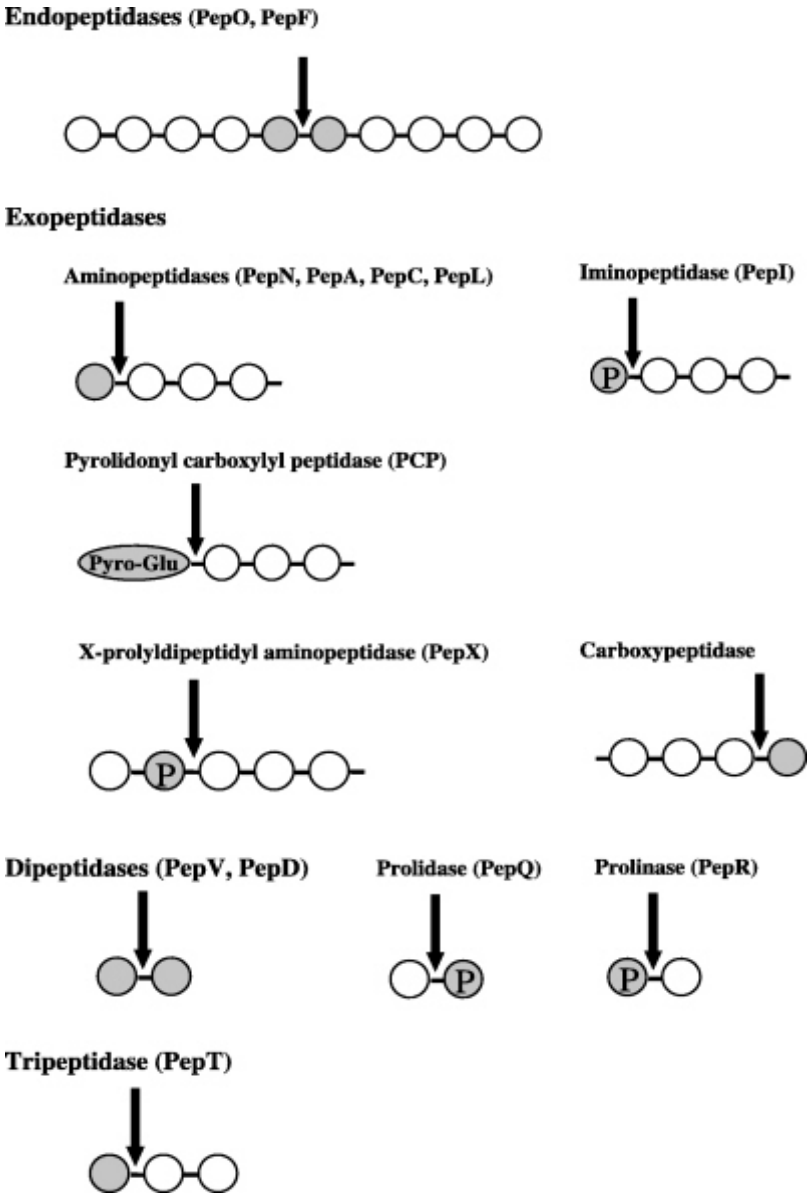

Figure 4. Pathways for the production of flavour compounds from fatty acids during cheese ripening (McSweeney, 2004).

In many cheese varieties the reaction of a FFA with an alcohol produces an ester. While methyl, propyl and butyl esters have been found in cheese, the most common alcohol available for this reaction is ethanol and, hence, ethyl esters are the dominant esters in many cheeses (McSweeney & Sousa, 2000). Ethanol is the limiting reactant in the production of ethyl esters, as this alcohol is derived from the fermentation of lactose or amino acid catabolism. Thioesters are compounds formed by the reaction of FFA's with

sulphydryl compounds, usually methanethiol (Fox & Wallace, 1997). Lactones are cyclic compounds from hydroxyacids following intracellular esterification. Both γ- and δ-lactones have been found in cheeses, but the levels of their precursor compounds, hydroxyacids, limit the production of lactones during ripening (Collins *et*. *al*., 2003).

1.2.2.2.2 Metabolism of free amino acids

Pathways for the catabolism of free amino acids during ripening produce many flavour compounds (McSweeney, 2004). It is now thought that the principal contribution of proteolysis to the development of cheese flavour is through the liberation of amino acids, which act as precursors for catabolic reactions (McSweeney, 2004). The amino acids in cheese appears to be catabolised by one of two major paths initiated by the action of an aminotransferase or a lyase, although other catabolic pathways also occur, as shown in Fig. 5.

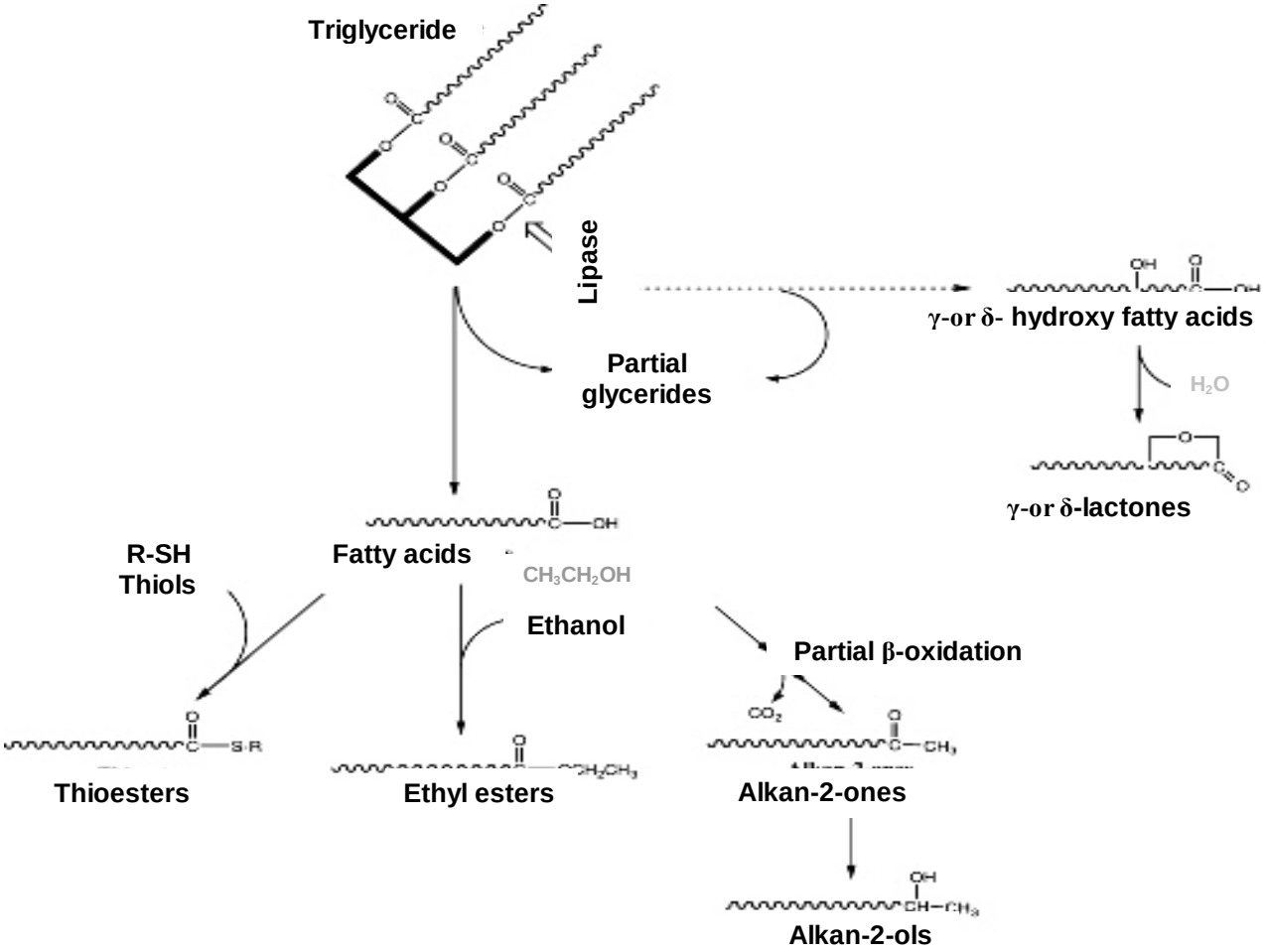

Figure 5. Schematic diagram of pathways for amino acids and some chemical reactions occurring in cheese during ripening (McSweeney, 2004).

The first pathway is initiated by the action of aminotransferases, which convert an amino acid to the corresponding α-ketoacid (McSweeney & Sousa, 2000). The ketoacids produced are then degraded to a range of other compounds by enzyme-catalysed pathways or by chemical reactions. The α-ketoacids produced by the action of aminotransferases, are further broken down by cheese-related microorganisms to volatile flavour compounds (Curtin & McSweeney, 2004).

Amino acids may also be degraded by deamination reactions involving the action of dehydrogenases (which use NAD^+ as the electron acceptor and produces α-ketoacids and ammonia) or oxidases (which use oxygen as the electron acceptor and forms aldehydes and ammonia). Ammonia produced by deamination contributes to the flavour of the cheese (McSweeney, 2004). The rate of production of amines in cheese depend on the concentration of precursor amino acids and, more importantly, the cheese microflora, which in turn may be affected by factors such as ripening temperature, pH and salt concentration (Curtin & McSweeney, 2004). As starter strains with high decarboxylase activities are not used, non starter strains have been implicated in the production of high levels of biogenic amines in most cheese varieties (McSweeney & Sousa, 2000). Ayad *et. al.* (2002a) found that new strains of LAB have the ability to produce flavours distinctly different from those produced by industrially produced starter cultures in model systems.

Dacre (1958) found that the growth curve of the pediococci rather than that of the lactobacilli followed the development of the flavour of the cheese, therefore, the *Pediococcus* culture, when added to cheese milk along with the starter culture, enhanced the flavour of the cheese. The concentration of the lactic acid starter culture gradually disappeared over the ripening period, whilst the pediococci population gradually increased to a final concentration of about a quarter of the flora of the ripened cheese. Elliott & Mulligan (1968) suggested that the use of pediococci should be encouraged in cheesemaking and that cheese makers should deliberately add selected pediococci to their cheese milk.

1.2.3 *Pediococcus* species

Pediococci are a diverse group of LAB (Cintas, Casaus, Fernandez & Hernandez, 1998). Lactic acid bacteria are recognised as playing an important role in food fermentation and preservation either as part of the natural microflora or as a starter culture that has been added under controlled conditions (Leroy & De Vuyst, 2004). The link between fermentation and preservation is biopreservation, which refers to the extension of the shelf life and improvement of food safety using microorganisms and/or their metabolites (Marilley & Casey, 2004). Lactic acid bacteria strains produce several antimicrobials, including organic acids (lactic acid, acetic acid, formic acid, phenyllactic acid and caproic acid), carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin and reutericyclin, which can inhibit or reduce undesirable pathogenic bacteria such as *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria* (*L.*) *monocytogenes* found in food products (Leroy & De Vuyst, 2004).

For the fermentation of milk, meat and vegetables the genera most commonly used as starter cultures are, *Lactococcus* (*Lc.*), *Lactobacillus* (*Lb.*), *Leuconostoc* (*Leuc.*) and *Pediococcus* (*P.*) species (spp.) (Cintas *et. al.*, 1998). Pediococci are harmless bacteria and in general have not been implicated in food poisoning and, therefore, are generally regarded as safe (Raccach, 1987; Schillinger, Geisen & Holzapfel, 1996). The genera used in fermentation processes ensure and enhance the development of the desired changes in texture, flavour, colour, digestibility and nutritional qualities of the fermented products (Buyong, Kok, & Luchansky, 1998).

Dacre (1958) found that cheese which contained pediococci actually had their flavour enhanced during the early stages of ripening. Cheese produced at the National Institute for Research in Dairying (NIRD) in an open vat containing pediococci as the preponderant non starter flora, were found by workers to have a better flavour than the cheeses that did not contain the pediococci cultures (Franklin & Sharpe, 1963).

The International Committee on Systematic Bacteriology officially recognises seven spp. of the genus *Pediococcus, P. damnosus, P. acidilactici, P. pentosaceus, P. halophilus, P. parvulus, P. urinae-equi* and the unnamed Gunther and White Group III (Raccach, 1987).

Raccach (1987) additionally identified two other spp., *P. inopinatus* and *P. dextrinicus*. Gunther & White according to Raccach (1987) characterised pediococci as follows, Gram-positive cocci, 0.36 to 1.43 μ in diameter, that divide in two planes and, thus, may appear in pairs, tetrads and other formations. No endospores or motility was observed.

The food environment, in which pediococci grow and produce pediocins, does not always provide optimal growths conditions. Environmental factors may either inhibit or stimulate the metabolic activity of the pediococci (McMullen & Stiles, 1996).

Pediococci do not reduce nitrate and cannot grow on ammonium salts as a source of nitrogen (Raccach, 1987). They also require almost all of the amino acids, biotin, folinic acid, pantothenic acid, pyridoxine and riboflavin for growth as found by Oumer, Garde, Gaya, Medina & Nunez (2001). Under anaerobic conditions pediococci are homofermentative, they transport glucose using phosphoenolpyruvate (PEP) via the phosphotransferase system (PTS), and metabolise it via the Embden-Meyerhof-Parnas (EMP) pathway shown in Fig. 6 (Rodriguez, Gonzalez, Gaya, Nunez, & Medina, 2000). Lactose fermentation by pediococci is strain dependent. Pediococci are capable of fermenting pentoses to equimolar amounts of lactate and acetate (Ray & Miller, 2000).

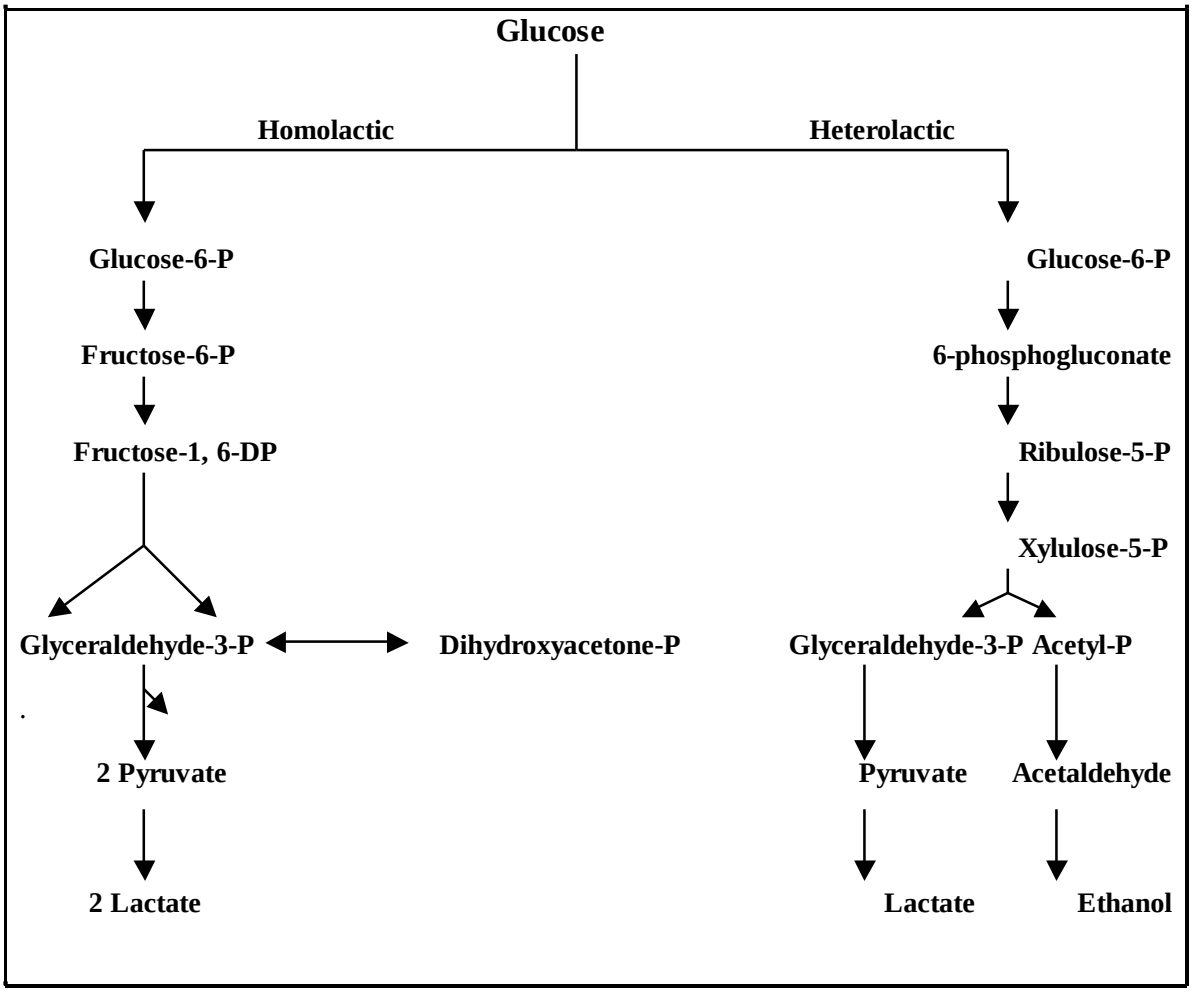

Figure 6. Scheme for the fermentation of glucose by lactic acid bacteria (Caplice & Fitzgerald, 1999).

Growth on D xylose was observed at an appreciable rate under aerobic conditions when the medium has been supplemented with low levels of glucose (0.15 % w/v) (Schillinger, *et. al.*, 1996). Raccach (1987) found that pediococci do not anaerobically ferment glycerol or sorbitol. The capacity to oxidise glycerol was under adaptive control and subject to repression by the "glucose effect", which is the inhibition of inducible enzymes by glucose or other readily metabolisable substrates (Tzanetakis & Litopoulou-Tzanetaki, 1989). The by products of this process are, lactic acid, acetic acid, acetoin and CO_2 at a ratio of 1:1:1:3, respectively.

The following steps in the aerobic metabolism of glycerol starting from the pyruvate (oxidation of glycerol) is shown in Fig. 7 (Bachman *et. al.*, 1999):

Pyruvate + 2H → Lactate

Pyruvate + ½ O_2 → Acetate + CO_2 + H_2O

2 Pyruvate → Acetoloactate + CO_2

Acetolactate → Acetoin + CO_2

Acetoin is reduced to yield diacetyl (Dobrogosz & Stone according to Raccach, 1987).

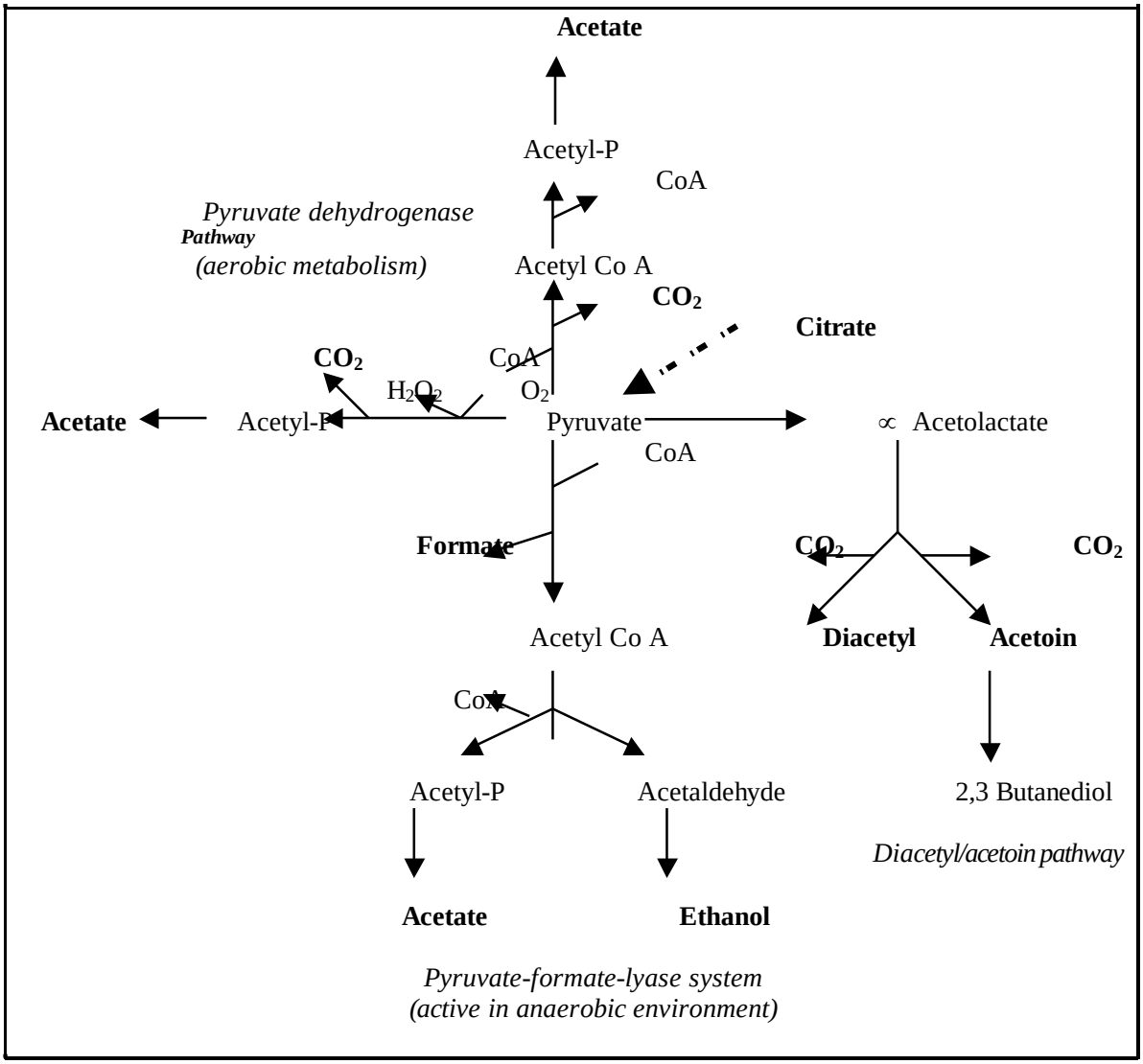

Figure 7. Scheme for the formation of important metabolic products from pyruvate in lactic acid bacteria (Caplice & Fitzgerald, 1999).

1.2.3.1 Pediocins

The term bacteriocin, comprise of a large and diverse group of ribosomally synthesised antimicrobial proteins or peptides, some of which undergo post-translational modifications (Nez & Eijsink according to Rodriquez *et*. *al*., 2002). Although bacteriocins may be found in numerous Gram-positive and Gram-negative bacteria, those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives (Blom, Katla, Hagen & Axelsson, 1997). Most LAB bacteriocins are small (<6 kDa), cationic, heat stable, amphiphilic, membrane-permeabilising peptides (Rodriquez *et*. *al*., 2002). They are degraded by the proteolytic enzymes of the gastrointestinal tract and seem to be neither non-antigenic nor non-toxic and so are generally regarded as safe (GRAS) (Guerra, Rua & Pastrana, 2001).

Bacteriocins are proteinaceous substances that exhibit bactericidal activity often towards other bacteria that are closely related to the bacteriocin-producing strains (Daba, Lacroix, Huang, Simard & Lemieux, 1994). Pediocins produced by pediococci are promising in this respect as they have a wide spectrum of bactericidal activity against a broad range of Gram-positive bacteria, including both spoilage and pathogenic microorganisms (Bhunia, Johnson, Ray & Kalchayanand, 1991). Therefore, pediocins are suitable candidates for use as food biopreservatives (Strasser de Saad & Manca de Nadra, 1993).

There are in principal two ways in which to use a bacteriocin, either by addition of the bacteriocin preparation or by use of a starter culture that produces a bacteriocin, which has the wanted inhibitory spectrum (Stiles according to Blom *et*. *al*., 1997).

Nisin is undoubtedly the most well known and characterised bacteriocin and the only one to have realized widespread commercial use (Caplice & Fitzgerald, 1999). Nisin is composed of 34 amino acids and has a pentacyclic structure with one lanthionine residue (ring A) and four β- methyllanthionine residues rings B, C, D and E. (Ross *et*. *al*., 2002). In Gram-positive bacteria, nisin has been shown to act on energized membrane vesicles to disrupt the proton motive force (PMF), inhibit uptake of amino acids and cause release

of accumulated amino acids (Abee, Krockel & Hill, 1995). As reported by Ross *et. al.* (2002), nisin has unusually high specific activity when compared to eukaryotic derived peptides such as meganin. Nisin has been demonstrated to inhibit peptidoglycan biosynthesis and has been found to cause pore formation in the membrane of sensitive bacteria. In dairy technology, nisin could be used to prevent losses in hard and semi-hard cheeses (Bouksaim, Lacroix, Audet & Simard, 2000). But the use of nisin in cheese can be expensive and results in inhibitory effects against the suitable acidifying or aroma-producing starter cultures, decreasing growth and acidification (Roberts according to Bouksaim *et. al.*, 2000). To overcome these disadvantages, Biswas, Ray, Johnson & Ray (1991) followed the approach of training the starter culture bacteria, lactococci, lactobacilli and propionic acid bacteria to grow in the presence of nisin, so that they can be used for cheese making. This approach is very difficult, since it requires regular control and maintenance of nisin resistance of strains in the culture, in addition to the other vital characteristics of starter cultures (Cintas, Rodriguez, Fernandez, Sletten, Hes, Hernandez & Holo, 1995). Therefore, the use of bacteriocin-producing LAB has gained interest as starter cultures to improve the safety of fermented foods (Leroy & De Vuyst, 2004).

According to Ennahar, Sashihara, Somoto & Ishizaki (2000), four classes of bacteriocins have been defined on mainly structural common characteristics:

- Class I : lantiobiotics,
- Class II : bacteriocins (small heat-stable non-lanthionine-containing peptides being the most abundant and thoroughly studied),
- Class III : large heat-labile bacteriocins,
- Class IV : bacteriocins with lipid and carbohydrate moieties.

1.2.3.1.1. Class I: lantiobiotic family

Generally small bacteriocins composed of one or two peptides approximately 3 kDa in size. Lantiobiotics were originally divided into two groups (Loessner, Guenther, Steffan & Scherer, 2003):

Group A: includes the elongated flexible molecules that have a positive charge and acts via membrane depolarisation. An example of a group A lantiobiotic is, nisin.

Group B: are globular in structure and interfere with cellular enzymatic reactions. Examples of which are mersacidin.

1.2.3.1.2 Class II: bacteriocins

Generally small unmodified peptides, less than 5 kDA in size, which are subdivided into two groupings (Ross *et*. *al.*, 2002).

Class IIa: listeria active peptides characterised by an YGNGV N-terminus, synthesised with a leader peptide attached that is removed by proteolytic processing, usually after a double glycine residue. Examples include bacteriocins such as pediocin PA-1 (Holck, Axelsson, Birkeland, Aukrust & Blom according to Ross *et*. *al.*, 2002).

Group IIb: bacteriocins composed of two separate peptides. The two-component non-modified bacteriocins include lacticin F (Murisna & Kleanhammer according to Ross *et*. *al.*, 2002).

1.2.3.1.3 Class III: large heat-labile proteins

This is the least well-characterised group consisting of heat-labile proteins generally greater than 30 kDa in size. Helvetin J produced by *Lb. helveticus* is an example of this group (Cintas *et*. *al.*, 1995).

1.2.3.1.4 Class IV: bacteriocins with lipid and carbohydrate moieties

These include bacteriocins, which have a carbohydrate or lipid moiety for activity (Montville & Chen, 1998).

1.2.3.2 Mechanistic action of pediocins

According to Bhunia *et*. *al.* (1991) an understanding of the mechanistic action of pediocins against susceptible bacterial strains will be important for the effective application of pediocins in food preservation. The cytoplasmic membrane of the sensitive cell is the biological target of a bacteriocin (Maisner-Patin, Forni & Richard, 1996). The addition of these membrane active peptides to vegetative cells causes rapid and non-

specific efflux of small molecular weight compounds (Montville, Winkowski & Ludescher, 1995). The pediocin activity is plasmid encoded and the mode of action is based on binding the active compound to the surface of the sensitive, Gram-positive strain. The binding site is the lipoteichoic acid layer in the bacterial cell wall shown in Fig. 8 (Bhunia *et. al.*, 1991).

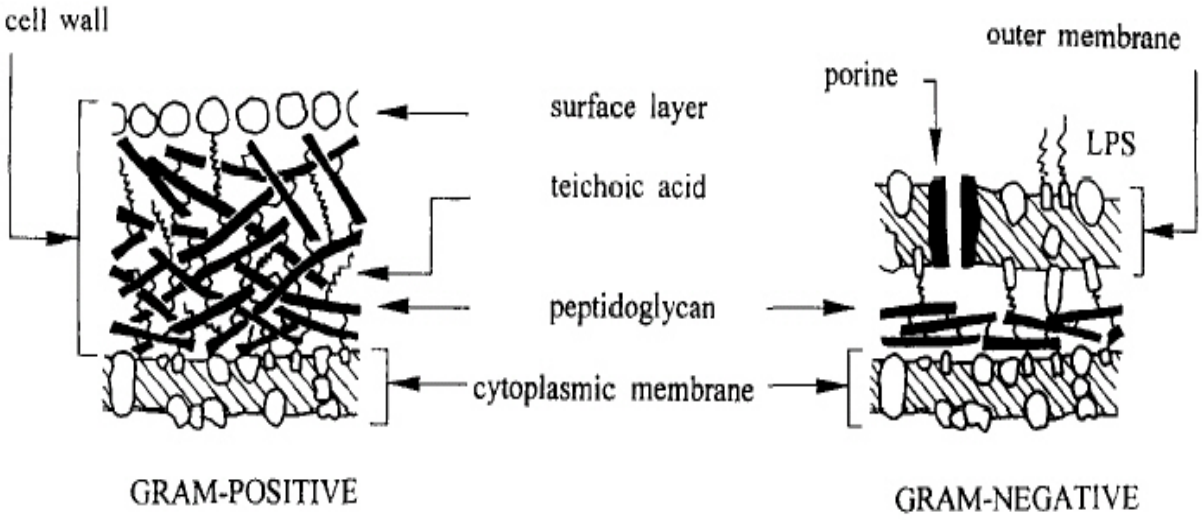

Figure 8. Schematic representation of the cell envelope of Gram-positive and Gram-negative bacteria (Abee, Krockel & Hill, 1995).

Pediocins result in the formation of pores in the cytoplasmic membrane of the sensitive cells leading to changes in the membrane permeability causing leakage of the cellular contents (Chikindas, Garcia-Garcera, Driessen, Ledeboer, Nissen-Meyer, Nes, Abee, Konings & Venema, 1993). The changes to the sensitive cells membrane permeability induced by the pediocin decrease or deplete the PMF of the sensitive cells. Proton motive force is an electro-gradient composed of a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH) (Schved, Lalazar, Lindner & Juven, 1994). These gradients serve as the driving force for many vital energy-dependent cellular processes (Montville *et. al.*, 1995). It was found by Montville *et. al.* (1995) that the ΔpH component of the PMF was depleted first by low levels of the pediocins, whilst higher concentrations are required to decrease the $\Delta\psi$ component of the PMF. Based on the pediocins amphiphilic characteristics, there are at least two different mechanisms, which may explain their membrane permeabilisation

action (Abee *et*. *al*., 1995). Pediocins may act by a poration complex in which bacteriocin monomers bind, insert, and oligomerise the cytoplasmic membrane to form a pore with the hydrophobic residues facing inwards, towards the interior membrane. Alternately, pediocins may destabilise the integrity of the cytoplasmic membrane in a detergent-like fashion (Montville *et*. *al*., 1995).

1.3 Hypotheses and objectives

1.3.1 Hypotheses

1.3.1.1 Gram-positive lactic acid bacteria are sensitive to pediocins produced by *Pediococcus* species, therefore, pediocins may inhibit Gram-positive lactic acid cheese starter cultures.

1.3.1.2 *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 added as an adjunct culture during Gouda cheese production may affect the quality of Gouda cheese during ripening, as non starter lactic acid bacteria have been shown to improve the sensory properties of cheese.

1.3.2 Objectives

1.3.2.1 Determine the effect of selected *Pediococcus* species crude pediocin extract on the lactic acid production, growth and pH levels of lactic acid Gouda cheese starter cultures.

1.3.2.2 Determine the effect of *Pediococcus* species added as an adjunct culture on the physico-chemical composition and sensory properties of Gouda cheese.

University of Pretoria etd – Verachia, W (2005)

CHAPTER II

Research

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2.1 Phase 1: Effect of pediocin-producing *Pediococcus* species on the activity of mesophilic Gouda cheese starter cultures

2.1.1 Abstract

The effect of *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract, previously isolated from South African farm-style cheese, on the activity of commercially available mesophilic Gouda cheese starter cultures was investigated. The commercially available mesophilic Gouda cheese starter cultures, LL 50C, Dairysafe™ and Nizostar 500MT were evaluated in the study. The effect of *P. acidilactici* ST 79 and *P. pentosaceus ST* 13 crude pediocin extract on the mixed strain starter cultures was determined by the lactic acid production, pH and growth levels of the starter cultures. The lactic acid production and pH level of starter culture Dairysafe™ was the least affected by the addition of *P. acidilactici* ST 79 and *P. pentosaceus ST* 13 crude pediocin extract. This is most probably due to Dairysafe™ consisting of nisin resistant *Lactobacillus acidophilus* and *Lactococcus lactis* subspecies *cremoris*, which are not affected by the crude pediocin extract. Therefore, from the three mixed strain mesophilic Gouda cheese starter cultures evaluated, Dairysafe™ is recommended for use in conjunction with *P. acidilactici* ST 79 and *P. pentosaceus ST* 13 as an adjunct culture in Gouda cheesemaking.

Key words: Gouda cheese, pediocin, *Pediococcus* species, adjunct culture

2.1.2 Introduction

The production of fermented milk products is based on the use of starter cultures such as LAB that initiate rapid acidification of raw milk (Leroy & De Vuyst, 2004). Lactic acid bacteria are of industrial importance as they contribute to microbial safety as well as offer organoleptic, nutritional and health advantages (Caplice & Fitzgerald, 1999).

Initial selections of commercial starter cultures were based upon rapid acidification, but these starters were not very flexible with regard to the desired properties and functionality of the end product (Frank & Hassan, 1998). Moreover, the daily propagation of defined mixtures of specific strains has resulted in the disappearance of certain strains thereby leading to a limited biodiversity of commercial starter cultures (Leroy & De Vuyst, 2004). This limited biodiversity leads to a loss of uniqueness and characteristics of the original product, which made the product popular, therefore, consumers appreciate traditionally fermented products for their gastronomic qualities (Ayad *et*. *al*., 2002a).

Traditionally fermented products obtained their flavour intensity from the NSLAB such as pediococci, which do not form part of the normal starter flora but develops in the product, particularly during maturation, as secondary flora (Beresford *et*. *al*., 2001). These NSLAB are the natural, wild type LAB that originates from the raw materials, process apparatus, or the environment (Leroy & De Vuyst, 2004). Wild LAB strains need to withstand the competition of other microorganisms in order to survive their hostile natural environments, so they often produce antimicrobials such as bacteriocins (Raccach, 1987).

Gurira & Buys (2005) isolated pediococci from South African farm-style Gouda cheese. The crude pediocin extract (CPE) of isolated *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 was found to be inhibitory to food pathogens *L. monocytogenes* ATCC 7644 and *Bacillus* (*B.*) *cereus* ATCC 1178 as well as to closely related lactic acid strains, *Lc. lactis* subspecies (subsp.) *diacetylactis* NCDO 176 and *Lc. lactis* subsp. *lactis* NCDC 605.

Pediocins are bacteriocins produced by pediococci and have a wide spectrum of bactericidal activity against a broad range of Gram-positive bacteria (Strasser de Saad & Manca de Nadra, 1993). Bacteriocins are small (<6 kDa), cationic, heat stable, amphiphilic, membrane-permeabilising proteinaceous substances that exhibit bactericidal activity often towards other bacteria that are closely related to the bacteriocin-producing strains (Klaenhammer according to Daba *et*. *al*., 1994).

Starter cultures commercially available in South Africa commonly contain *Lc*. *lactis* subsp. *diacetylactis* and *Lc*. *lactis* subsp. *lactis*. Herein lies the potential problem with using the *Pediococcus* spp. as an adjunct culture in cheese making, as the pediocins produced by the pediococci may inhibit the growth of the strains constituting the starter culture (Raccach, 1987).

Therefore, the objective of the experiment was to determine the effect of *Pediococcus* spp. CPE on selected lactic acid Gouda cheese starter cultures.

2.1.3 Materials and methods

2.1.3.1 Commercial Gouda cheese starter cultures

The cultures used in the study were LL 50C, Nizostar 500MT and Dairysafe™. The mixed strain culture LL 50C was obtained from Anchor Biotechnologies (Johannesburg, South Africa) in a freeze-dried form and consisted of *Lc. lactis* subsp. *lactis* and *Lc*. *lactis* subsp. *cremoris*. The mixed strain culture of Nizostar 500MT was obtained from CSK Food Enrichment (Leeuwarden, Netherlands) in a freeze-dried form and consisted of *Lc*. *lactis* subsp. *lactis*, *Lc*. *lactis* subsp. *diacetylactis*, *Leuc. mesenteroides* subsp. *cremoris* and *Streptococcus* (*S*.) *thermophilus*. The mixed strain starter culture Dairysafe™ was obtained (CSK Food Enrichment) in a freeze-dried form and consisted of nisin resistant *Lb. acidophilus* and *Lc. lactis* subsp. *cremoris*.

The cultures were activated by growing them in (10 % w/v) sterile reconstituted skim milk powder at 30 ^{o}C for 24 h before use.

2.1.3.1.1 Source of *Pediococcus* species

The different pediococci strains used in the study were isolated and characterised from South African farmhouse-style Gouda cheese by Gurira & Buys (2005). Of the strains evaluated in the Gurira & Buys (2005) study, *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 CPE were most inhibitory towards pathogens *L. monocytogenes* and *B. cereus* and were, therefore, selected for the study.

2.1.3.1.2 *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract

Pediococcus cultures were grown in sterile MRS (De Man, Rogosa & Sharpe, 1960) broth for 72 h at 30 ^{o}C. These cultures were then propagated twice in MRS broth for 24 and 48 h respectively at 30 ^{o}C to ensure pediocin production, Gurira & Buys (2005). The cultures were then centrifuged at 3000 rpm for 15 min and the pellet containing the cells discarded. The supernatant containing the pediocin was then added to the sterile reconstituted skim milk powder at a 1 % (v/v) of the milk.

2.1.3.1.3 Inoculation of milk samples with *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract

A 1 % inoculum (v/v) of the milk of each starter culture was added to 300 ml sterile reconstituted skim milk powder. Each 300 ml milk was inoculated by one of the selected starter cultures which was then divided into three equal portions, one portion was used as the control – no CPE was added, to the second portion a 1 % [(v/v) of the milk] of centrifuged *P. acidilactici* ST 79 CPE was added and to the last portion a 1 % [(v/v) of the milk] of centrifuged *P. pentosaceus* ST 13 CPE was added. The initial inoculation level of the cultures in the milk was determined immediately after the addition of the starter culture and *Pediococcus* spp. CPE. The inoculated milk was then incubated at 30 ^{o}C for 18 h. The analysis was repeated three times for each starter culture, with duplicate samples analysed at each repeat.

2.1.3.2 Effect of *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract on the activity of the mesophilic Gouda cheese starter cultures: LL 50C, Nizostar 500MT and Dairysafe™

2.1.3.2.1 Microbiological analysis

The inhibitory effect of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 CPE on the starter cultures was determined by the number of colony forming units (cfu/ml) after 0, 2 and 18 h of incubation at 30 ^{o}C. For viable counts, suitable dilutions, made in a buffered peptone water solution, were plated in duplicate by the spread plate method using MRS agar. Plates were incubated at 30 ^{o}C for 48 h. The analysis was repeated three times for each starter culture, with duplicate samples analysed at each repeat, n = 18.

2.1.3.2.2 Titratable acidity

The lactic acid production was determined by titration with 0.1 N sodium hydroxide (NaOH) and expressed as percentage lactic acid (Bradley, Arnold, Barbano, Semerad, Smith & Vines, 1993). Lactic acid production was measured after 0, 2 and 18 h of incubation at 30 ^{o}C. The analysis was repeated three times for each starter culture, with duplicate samples analysed at each repeat, n = 18.

2.1.3.2.3 pH

The pH level was measured at 0, 2 and 18 h of the incubation period at 30 ^{o}C. The pH was determined electrometrically using a Sentron pH meter (Sentron Inc.: Postbus, The Netherlands). The analysis was repeated three times for each starter culture, n = 9.

2.1.3.3 Statistical analysis of data

Differences in the growth and activity of the starter cultures in the treated and control milk samples at each incubation period were analysed by the Wilcoxon Mann Whitney test of the Statistical Analysis System (SAS, 1999). The viable counts and lactic acid production of the starter cultures were calculated at a 5 % significance level.

2.1.4 Results

2.1.4.1 Effect of *Pediococcus acidilactici* ST 79 crude pediocin extract on commercial starter cultures: LL 50C, Nizostar 500MT and Dairysafe™

There were no significant differences ($P > 0.05$) found for the levels of lactic acid production, pH or starter cell growth rate between the milk samples to which the *Pedicocccus* spp. CPE had been added and the control samples. This was found for starter cultures LL 50C, Nizostar 500MT and Dairysafe™, (Table 1).

Figure 9 indicates the level of lactic acid production (% w/w) of all the starter cultures with and without the addition of *P. acidilactici* ST 79 CPE after 0, 2 and 18 h of incubation. The lactic acid production (% w/w) was greater for all the starter cultures + *P. acidilactici* ST 79 CPE as compared to the control. Dairysafe™ + *P. acidilactici* ST 79 CPE showed the greatest increase in level of acid production after the 2 and 18 h incubation period when compared to the other two starter cultures.

Figure 10 indicates the pH levels of all the starter cultures with and without the addition of *P. acidilactici* ST 79 CPE after 0, 2 and 18 h of incubation. The pH levels for LL 50C and Dairysafe™ + *P. acidilactici* ST 79 CPE decreased over the entire 18 h incubation period. However, Nizostar 500MT + *P. acidilactici* ST 79 CPE only exhibited a lower pH level until after the 2 h incubation period, after which there was an increase noted in pH levels as compared to the control milk sample. After 18 h of incubation Dairysafe™ + *P. acidilactici* ST 79 CPE had the lowest pH levels when compared to the control milk sample.

Figure 11 indicates the growth rates of all the starter cultures with and without the addition of *P. acidilactici* ST 79 CPE at 2 and 18 h of the incubation period. The growth rate of Nizostar 500MT and Dairysafe™ + *P. acidilactici* ST 79 CPE was lower than the control milk sample throughout the entire 18 h incubation period. The growth rate of LL 50C + *P. acidilactici* ST 79 CPE was also lower than that of the control milk sample after 2 h of incubation but this trend was reversed after 18 h.

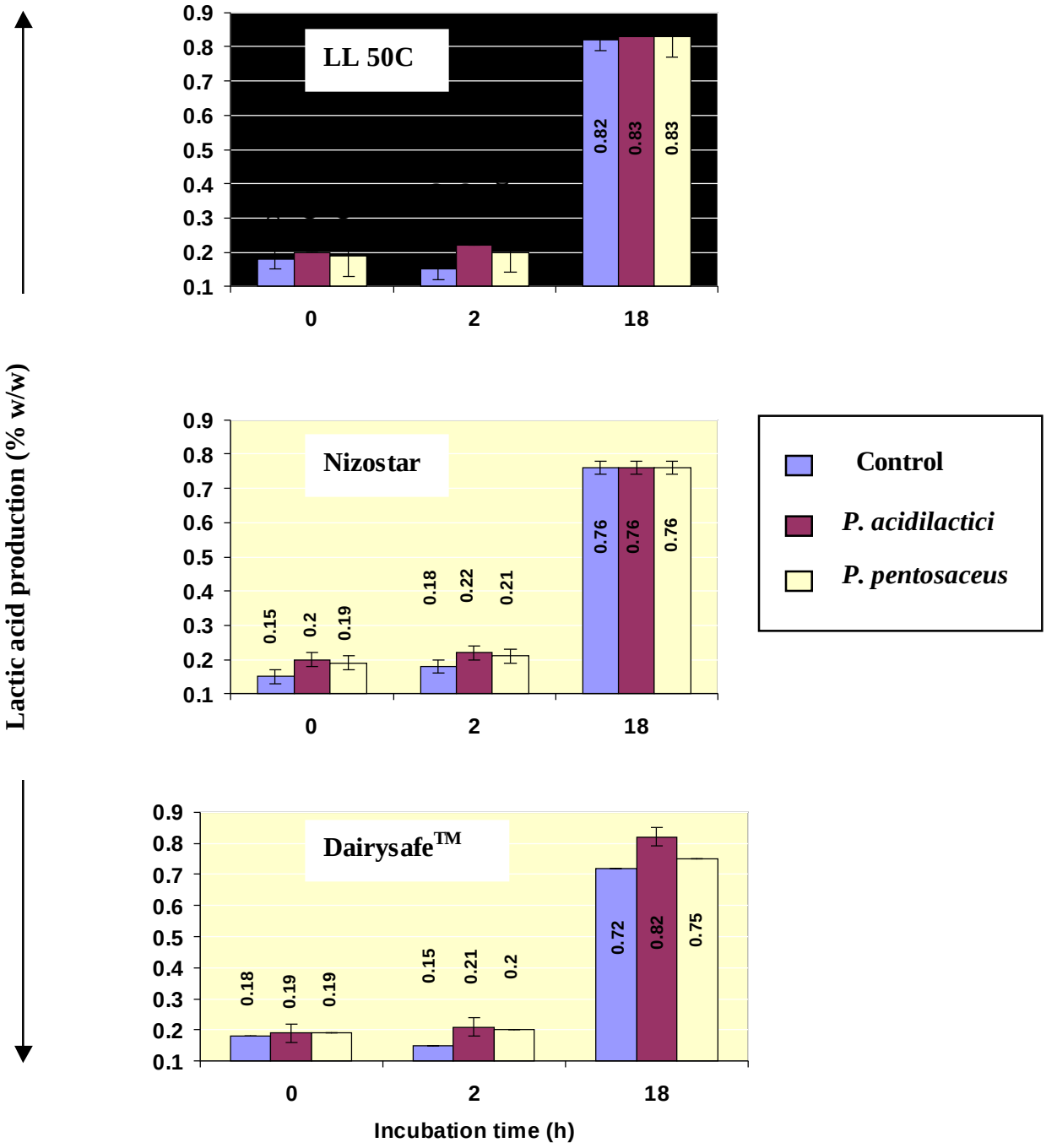

Figure 9. Lactic acid production of starter cultures: LL 50C, Nizostar 500MT and Dairysafe™ with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract after 18 h at 30 ^{O}C, (n = 18).

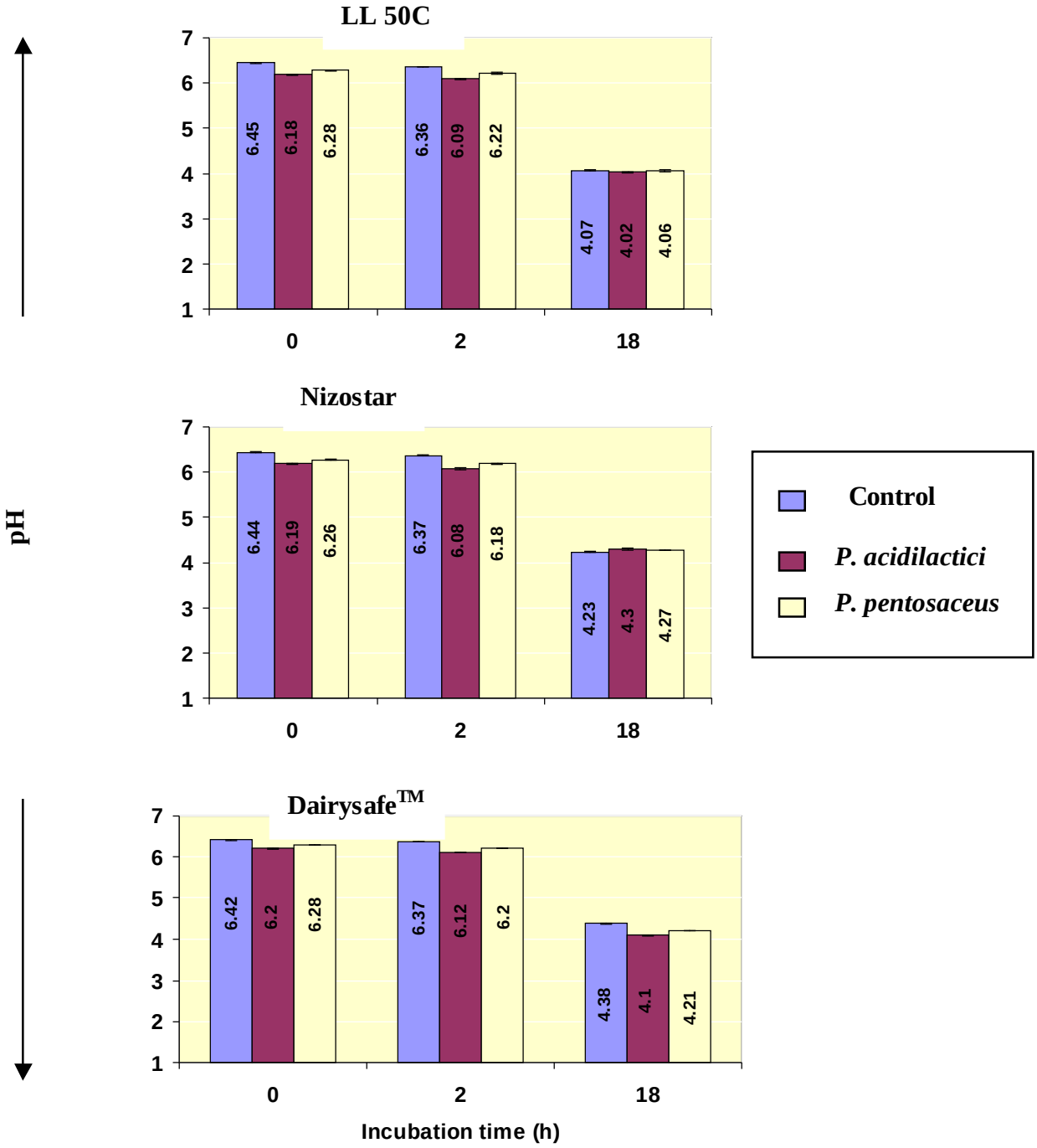

Figure 10. pH of starter cultures: LL 50C, Nizostar 500MT and Dairysafe™ with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract after 18 h at 30 ^{O}C, (n = 9).

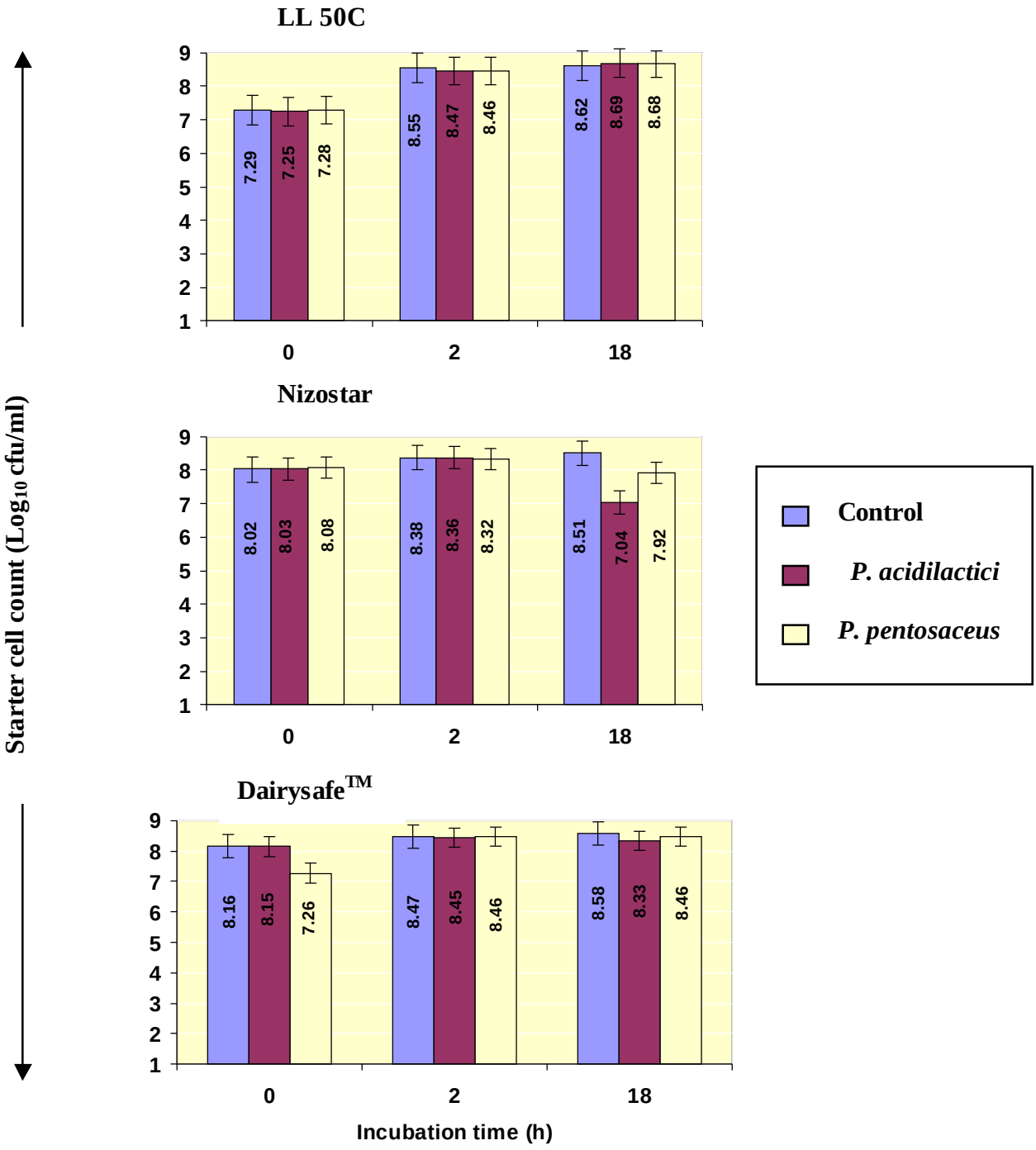

Figure 11. Cell growth of starter cultures: LL 50C, Nizostar 500MT and Dairysafe™ with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract after 18 h at 30 ^{O}C, (n = 18).

Table 1. Statistical data for pH, titratable acidity and growth of starter cultures: LL 50C, Nizostar 500MT and Dairysafe™ with and without 1 % *Pediococcus acidilactici* ST 79 crude pediocin extract

Starter culture	Statistical parameter	Titratable acidity	pH	Growth
LL 50C	**Degrees of freedom**	1	1	1
	P-value	0.82	0.72	0.97
	F value	0.05	0.13	0.002
Nizostar 500MT	**Degrees of freedom**	1	1	1
	P-value	0.81	0.74	0.97
	F value	0.06	0.11	0.002
Dairysafe™	**Degrees of freedom**	1	1	1
	P-value	0.68	0.60	0.14
	F value	0.18	0.28	2.21

2.1.4.2 Effect of *Pediococcus pentosaceus* ST 13 crude pediocin extract on commercial starter cultures: LL 50C, Nizostar 500MT and Dairysafe™

There were no significant differences ($P > 0.05$) noted for the levels of lactic acid production, pH or starter cell growth rate between the milk samples to which the *Pedicocccus* spp. CPE had been added and to the milk samples to which no *Pedicocccus* spp. CPE had been added. This was noted for all starter cultures LL 50C, Nizostar 500MT and Dairysafe™, (Table 2).

Figure 9 indicates the level of acid production (% w/w) of all the starter cultures with and without the addition of the *P. pentosaceus* ST 13 CPE after 0, 2 and 18 h of the incubation period. The levels of lactic acid production for LL 50C and Dairysafe™ + *P. pentosaceus* ST 13 CPE were greater than the control milk sample throughout the 18 h incubation period. However, Nizostar 500MT + *P. pentosaceus* ST 13 CPE only exhibited a greater level of lactic acid production (% w/w) as compared to the control milk sample until after the 2 h incubation period. After the 18 h incubation period the

increase in level of lactic acid production (% w/w) was equal to that of the control milk sample.

Figure 10 indicates the pH levels of all the starter cultures with and without the addition of *P. pentosaceus* ST 13 CPE after 0, 2 and 18 h of the incubation period. The pH levels of LL 50C and Dairysafe™ + *P. pentosaceus* ST 13 CPE decreased over the entire 18 h incubation period, which is concurrent with the increase in levels of lactic acid (% w/w). However, Nizostar 500MT + *P. pentosaceus* ST 13 CPE only exhibited a pH level lower than that of the control milk sample until the 2 h incubation period, after which the increase in pH levels was equal to that of to the control milk samples. After 18 h of incubation Dairysafe™ + *P. pentosaceus* ST 13 CPE had the lowest pH levels of all three starter cultures.

Figure 11 indicates the growth rates of all the starter cultures with and without the addition of the *P. pentosaceus* ST 13 CPE after 0, 2 and 18 h of the incubation period. The growth rates of Nizostar 500MT and Dairysafe™ + *P. pentosaceus* ST 13 CPE was lower than the control milk sample throughout the entire 18 h incubation period. The growth rate of LL 50C + *P. pentosaceus* ST 13 CPE was also lower than that of the control milk sample after the 2 h incubation period but this trend was reversed after the 18 h incubation period.

Table 2. Statistical data for pH, titratable acidity and growth of starter cultures: LL 50C, Nizostar and Dairysafe™ with and without 1 % *Pediococcus pentosaceus* ST 13 crude pediocin extract

Starter Culture	Statistical parameter	Titratable acidity	pH	Growth
LL 50C	**Degrees of freedom**	1	1	1
	P-value	0.88	0.85	0.98
	F value	0.02	0.04	0.001
Nizostar 500MT	**Degrees of freedom**	1	1	1
	P-value	0.87	0.82	0.98
	F value	0.03	0.05	0.001
Dairysafe™	**Degrees of freedom**	1	1	1
	P-value	0.82	0.73	0.14
	F value	0.05	0.12	2.2

2.1.5 Discussion

There was a general increase in level of lactic acid production (% w/w) for all three starter cultures throughout the 18 h incubation period. This result was not limited to the addition of either one of the pediococci cultures but was in fact true for the addition of the CPE of both *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 to all the starter cultures. Lactic acid production (% w/w) was the greatest for Dairysafe™ + *P. acidilactici* ST 79 CPE.

Whilst the pH levels for LL 50C and DairysafeTM + *Pediococcus* spp. CPE decreased throughout the 18 h incubation period, the decrease in pH level was the greatest for starter culture Dairysafe™ + *P. acidilactici* ST 79 CPE. Nizostar 500MT + *Pediococcus* spp. CPE also had a decrease in pH levels after the 2 h incubation period after which the increase in pH was equal to that of the control milk sample.

What Gurira & Buys (2005) found can explain the trends exhibited by each of the starter cultures. The pediocins produced by the *Pediococcus* spp. used as an adjunct culture inhibited the following LAB strains, *Lc. lactis* subsp. *diacetylactis* NCDO 176 and *Lc. lactis* subsp. *lactis* NCDC 605.

Nizostar 500MT is partially composed of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *diacetylactis*, whilst LL 50C is partially composed of *Lc. lactis* subsp. *lactis*. Dairysafe™ does not contain any of the strains affected by the *Pediococcus* spp. CPE.

Thus, the lactic acid production and pH levels of starter culture Nizostar 500MT was the most affected whilst Dairysafe™ was the least affected by the addition of the *Pediococcus* spp. CPE.

Another reason for Dairysafe™ being the least affected by *Pediococcus* spp. CPE is that Dairysafe™ is composed of nisin resistant *Lb. acidophilus* and *Lc. lactis* subsp. *cremoris*. Nisin is a proteinaceous substance like the pediocins produced by *Pediococcus* spp., therefore, if the starter culture is nisin resistant, the probability of the culture also being resistant to the pediocin increases.

The growth rate of LL 50C and Dairysafe™ + *Pediococcus* spp. CPE was lower than that of the control milk samples throughout the 18 h incubation period. This trend was the same for Nizostar 500MT but only until the 2 h incubation period, after which there was an increase in the growth rate of Nizostar 500MT + *Pediococcus* spp. CPE as compared to the control milk sample.

P. acidilactici ST 79 and *P. pentosaceus* ST 13 CPE had a different effect on the growth rate of the starter culture than on the acid production. The effect of the pediocin produced by the *Pediococcus* spp. on the growth of the starter culture as measured by colony count could be different from the acid production and pH levels due to the different single strains that each mixed starter culture consists of and also the ratio of each different single strain making up the mixed starter culture (Scott, Robinson, & Wilbey, 1998).

If one of the strains making up the starter culture do not considerably contribute to acid production but does constitute the majority of the biomass of the starter culture, is sensitive to the *Pediococcus* spp. CPE, the growth of that starter culture will be affected. However, if another strain, making up the starter culture that is the major acid producer, is resistant to the *Pediococcus* spp. CPE, then the acid production will not be affected. Thus, if a mixed starter culture consists of a higher proportion of the resistant single strain culture and a lower proportion of the sensitive single strain, the growth of the mixed strain starter culture will be inhibited more than the acid production (Guirguis & Hickey, 1987). However, further studies on single strain LAB strains are needed.

Differences between the levels of lactic acid production, pH and growth rates of the starter cultures to which *Pediococcus* spp. CPE were added and control milk samples were not significant ($P > 0.05$). Even though the difference in levels of lactic acid production (% w/w) and pH were not significant during cheesemaking, this difference is of practical significance. The titratable acidity needs to be between the narrow margin of 0.19 – 2.0 (% w/w) for the coagulation of the milk proteins and the formation of the curd and whey (Scott *et. al.*, 1998). Therefore, even if the differences in the levels of titratable acidity are not significant, this difference is of significant importance in practical cheesemaking.

2.1.6 Conclusion

Introduction of *P. acidilactici* ST 79 and *P. pentosaceus ST* 13 CPE to the mixed strain lactic acid starter cultures LL 50C, Nizostar 500MT and Dairysafe™ did not have a significant effect on their lactic acid production, pH or growth levels. Therefore, either one of the three mixed strain mesophilic Gouda cheese starter cultures evaluated may be used in conjunction with *P. acidilactici* ST 79 and *P. pentosaceus ST* 13 as an adjunct culture in Gouda cheesemaking. However, from a practical cheesemaker's perspective, Dairysafe™ was the least affected by the addition of *Pediococcus* spp. CPE, therefore, it is recommended that Dairysafe™ be used in conjunction with the *Pediococcus* spp. as an adjunct culture in the cheesemaking process.

2.2 Phase 2: Quality aspects of Gouda cheese manufactured using *Pediococcus* species as an adjunct culture

2.2.1 Abstract

The physico-chemical, microbiological and sensory properties of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture was investigated over a ripening period of 45 days. Cheese made using *P. acidilactici* ST 79 and *P. pentosaceus* ST 13, as an adjunct culture had an insignificantly higher lactic acid bacterial count, as compared to the cheese that did not contain an adjunct culture. The addition of an adjunct culture did not significantly affect the gross chemical composition of the Gouda cheese or the levels of proteolysis and lipolysis during the 45 day ripening period. However, significant differences ($P < 0.05$) in the overall sensory attributes were observed between the Gouda cheeses made with and without an adjunct culture. Sensory panellists indicated that Gouda cheese made with an adjunct culture had a more intense initial flavour with a creamier and sharper overall flavour.

2.2.2 Introduction

Gouda is a semi-hard cheese that forms part of the continental cheese segment (McSweeney, 2004). The manufacture of cheese is a form of preservation of a highly perishable commodity. Approximately a third of the milk produced worldwide is used in cheese production (Farkye, 2004). As flavour is one of the most important selling attributes of Gouda cheese it has received much attention (Ayad *et*. *al.*, 2002b). The global sales value of cheese represents about 30 % of total dairy product sales with a forecast of a 9.8 % growth in cheese sales between 2003 and 2007, therefore, the ability to positively manipulate the flavour of cheese presents a commercial advantage to the dairy industry (Farkye, 2004). Flavour modulation is possible with the aid of selected NSLAB strains, which can be used to accentuate the positive flavour notes of the cheese and thereby clearly differentiate it from similar products already on the market (Franklin & Sharpe, 1963; Crow *et*. *al.*, 2001).

Dacre (1958) found that pediococci cultures, when added to cheese milk along with the starter culture as an adjunct culture, enhanced the flavour of the cheese during the early ripening stages. Dacre (1958) also found that the microbial population of the pediococci cultures, rather than that of the lactobacilli starter cultures, followed the development of the flavour of the cheese during ripening. The pediococci population gradually increased over the ripening period and became the dominant microbial population of the cheese constituting about a quarter of the total microbial population. Elliott & Mulligan (1968) repeated the trials that Dacre (1958) undertook and also found that the flavour of the cheese was enhanced due to the addition of the pediococci culture along with the starter culture to the cheese milk.

Therefore, the objective of the study was to determine the effect of selected *Pediococcus* spp. on the sensory, physico-chemical and microbiological aspects of Gouda cheese when used as an adjunct culture.

2.2.3 Materials and methods

2.2.3.1 Source of *Pediococcus* species

Pediococcus spp. were isolated and characterised from locally produced farmhouse Gouda cheese by Gurira & Buys (2005) at the University of Pretoria, Department of Food Science, South Africa. *Pediococcus acidilactici* ST 79 and *P. pentosaceus* ST 13 used in this study were selected on the basis that they were found by Gurira & Buys (2005) to be the most inhibitory against pathogens *B. cereus* and *L. monocytogenes*.

2.2.3.2 Preparation of *Pediococcus* species for use as an adjunct culture in cheesemaking

Selected *Pediococcus* spp. were stored in a frozen state between –18 and –20 °C in sterile MRS broth. The frozen pediococci cultures, were resuscitated in sterile MRS broth for 72 h at 30 °C to obtain the required adjunct culture. The resuscitated cultures, were then propagated in MRS broth for another 24 h. The 24 h culture was frozen at –75 °C in sterile MRS broth until required. When the *Pediococcus* spp. was required for cheesemaking, the cultures were removed from storage at –75 °C and allowed to defrost at room temperature. An inoculum of 1 ml of *Pediococcus* spp. were then inoculated into 200 ml of sterile reconstituted skim milk powder and incubated at 30 °C for 18 - 24 h.

2.2.3.3 Source and treatment of milk samples

Raw milk for the cheesemaking experiment was obtained from the experimental farm, University of Pretoria, South Africa. The cows were milked according to standard procedures. The 20 l of milk was aseptically collected in stainless steel milk cans during a single milking session. The milk was delivered to the pilot plant of the Department of Food Science, University of Pretoria within 1 h of milking.

2.2.3.4 Cheesemaking

The Gouda cheese was manufactured in the pilot plant of the Department of Food Science using the method described by Lombard (1976). The 20 l of milk was pasteurised in a batch pasteuriser [Anderson Engineering Food and Dairy Equipment (Pty) Ltd,

Pietermaritzburg, South Africa] for 30 min at 72 °C. The milk was then cooled to 30 °C and separated into two equal 10 l batches. To each of the 10 l batches of cooled milk, 5 ml of (40 % w/v) food grade calcium chloride (Merck Laboratory Supplies Pty Ltd., Midrand, South Africa), and 1 % (v/v) of starter culture Dairysafe™ (CSK Food Enrichment, Leeuwarden, Netherlands) were added. Dairysafe™ was chosen as the starter culture due to Dairysafe™ being the least affected by the selected pediococci CPE (see section 2.1.5). To one of the 10 l milk samples a 1 % (v/v) of both *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 was added as an adjunct culture while the other 10 l milk did not receive any adjunct culture and was used as a control. After 15 min, 0.6 ml rennet (diluted 1:10; CSK Food Enrichment) was added to each sample. The remainder of the cheesemaking process was carried out according to Lombard (1976). After draining off the brine and drying each 1kg block of cheese, each of the 1kg blocks of cheese was cut into 6 equal blocks (~167 g) to be used for physical, chemical, microbiological and sensory analysis at days 1 and 45. The 1kg block of cheese was divided into 6 equal blocks in order to ensure that the amount required for each analysis was separately vacuum sealed and to ensure that ripening was not influenced by different sizes of each block of cheese. Each of these blocks of cheese were vacuum packed and placed in a cheese curing room for 6 weeks at a temperature of 10 °C and relative humidity of 85 %. The cheese making experiment was repeated three times. Samples for analysis were randomly taken from each batch at days 1 and 45 of ripening.

2.2.3.5 Sampling of cheese for physical, chemical and microbiological analysis

From the six equal blocks of cheese of each batch, a block of cheese was randomly selected at each day of analysis. The whole block of cheese was aseptically grated using a sterile cheese grater. From the grated cheese, samples for microbiological analysis were aseptically transferred to sterile universal bottles. The remaining grated cheese was used for the chemical analysis. Both chemical and microbiological tests were done on days 1 and 45 of ripening. All tests were done in duplicate except for the pH measurements.

2.2.3.6 Chemical composition

2.2.3.6.1 pH measurement

The pH of the cheese samples was measured using a penetration electrode (Sentron Integrated Sensor Technology, Sentron Inc., USA).

2.2.3.6.2 Fat analysis

The fat content of the milk and cheese were determined by the method described by the British Standards Institution (BSI, 1969).

2.2.3.6.3 Salt analysis

The salt content of the cheese was determined using the International Dairy Federation Standards 179 of 1997 (IDF 179:1997).

2.2.3.6.4 Total solids analysis

The total solids content of the cheese samples was determined by drying the samples in a forced draft oven at 100 ± 2 °C for 16.5 ± 0.5 h (Bradley *et*. *al.*, 1993).

The solids-not-fat content of the cheese was calculated by subtracting the percentage fat from the percentage total solids of the cheese. Cheese yield after pressing was measured by weighing the cheese blocks on an analytical balance and was expressed as kg dry matter 100 l^{-1} of milk.

2.2.3.6.5 Measurement of nitrogen fractions

2.2.3.6.5.1 Total nitrogen analysis

The total nitrogen (TN) of the cheese samples were determined according to the Dumas principle (IDF, 2000) using a Leco FP-528 Nitrogen/Protein Analyser (Leco Corporation, Michigan, USA). The percentage protein content of the cheese samples was determined by multiplying the TN by the factor, 6.38.

2.2.3.6.5.2 Water-soluble nitrogen analysis

The degree of proteolysis in the cheese samples was monitored by measuring the WSN (Kuchroo & Fox, 1982). The nitrogen fractions were determined as stated for TN. The percentage of the nitrogen fraction over the TN was used as indices of proteolysis.

2.2.3.6.6 Free fatty acid analysis

The level of lipolysis in the cheese samples was determined by measuring the total FFA content of the cheese samples as described by Nuñez, García-Aser, Rodriguez-Martin, Medina & Gaya (1986) at days 1 and 45 of ripening.

2.2.3.6.7 Microbiological analysis

Ten-fold serial dilutions of cheese samples were made by aseptically transferring 11g samples of cheese into 99 ml of sterile 2 (% w/w) trisodium citrate solution, which was heated to 40 °C to give a 10^{-1} dilution (White, Bishop & Morgan, 1993). Samples were then homogenised for 2 min using a Stomacher 400 laboratory blender (Seward Ltd, UK). Further 10-fold dilutions of up to 10^{-8} were made by transferring 11ml of the successive serial dilutions into universal bottles containing 99ml of sterile buffered peptone water solution. Lactic acid bacteria in the cheese samples were determined using MRS agar after incubation at 30 °C for 48 h.

2.2.3.6.8 Sensory analysis

Sensory difference tests were performed at the end of the ripening period (45 days) using the triangle test. Each of the three batches was judged by 50 untrained panellists. Thus, a total number of 150 panellists participated in the overall sensory evaluation. The panellists were given three randomly generated coded samples, two of which were the same and one different from the other two samples. They assessed the overall taste of the samples. The cheese samples were grated and presented in polystyrene cups, which were covered with plastic lids at room temperature. The sensory session was conducted in the sensory evaluation area, which was an air-conditioned room under red light in separated booths at the Department of Food Science, University of Pretoria. Correct responses were

counted and significant differences were determined using probability tables for the triangle test (Roessler, Pangborn, Sidel & Stone, 1978).

2.2.3.6.9 Statistical analysis of data

The data for the chemical composition and microbiological counts were analysed using the Wilcoxon Mann Whitney test (SAS, 1999). The mean values of each variable of the cheese made with an adjunct culture (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13) were compared with the respective mean values of the cheese made without an adjunct culture at each analysis time. Significant differences were calculated at a 5 % significance level. The data for the sensory analysis was analysed using the binary logistic with class variables, batch number and treatment with the statistical model of Responses = Batch number and Treatment (SAS, 1999). The mean values of each variable of the cheese made with an adjunct culture (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13) were compared with the respective mean values of the cheese made without an adjunct culture at each analysis time. The sensory data was then correlated with the chemical data using PROC CATMOD, with the chemical data as a variable with the statistical model of Correct or Incorrect response = Treatment and chemical data (SAS, 1999).

2.2.4 Results

2.2.4.1 Growth of lactic acid bacteria during ripening of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture

The LAB counts of the Gouda cheese made using *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture (experimental cheese) and the control Gouda cheese were not significantly different (P = 0.182) over the 45 day ripening period. The LAB counts of experimental Gouda cheese had an insignificant higher LAB count throughout the 45 day ripening period, as compared to that of the control Gouda cheese. The LAB counts decreased for both the control and experimental Gouda cheeses over the ripening period (Table 3).

Table 3. Lactic acid bacterial growth during ripening of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as adjunct culture (n = 6 ± SD)

	Cheese type	Ripening time (days)	
		1 (± SD)	45 (± SD)
Lactic acid bacteria (Log_{10} cfu/ml)	**C**	10.0 ± 0.24	8.6 ± 0.29
	E	11.2 ± 0.68	10.2 ± 0.51

E = Experimental cheese (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13 used as an adjunct culture);
C = Control cheese (no adjunct culture added);
SD = Standard deviation.

2.2.4.2 Chemical composition of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture

No significant differences (P > 0.05) were observed in the moisture, protein, fat and salt contents between the experimental and control Gouda cheeses throughout the 45 day ripening period as seen in Table 4. Similarly no significant differences (P > 0.05) were observed in the fat in dry matter and salt in moisture contents between the experimental and control Gouda cheeses throughout the 45 day ripening period. An increase in protein, fat and salt contents were observed at the end of the 45 day ripening period for both the experimental and control cheeses (Table 4). This increase in protein, fat and salt contents was paired with a concomitant decrease in the moisture content for both the experimental and control cheeses over the 45 day ripening period.

Table 4. Gross chemical composition of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture (n = 6 ± SD)

Parameter	Cheese type	Ripening time (days)	
		1 (± SD)	45 (± SD)
Moisture (%)	C	55.2 ± 2.40	52.3 ± 0.18
	E	55.6 ± 1.26	52.5 ± 0.35
Protein (%)	C	20.6 ± 0.36	21.2 ± 0.53
	E	20.3 ± 0.80	21.7 ± 0.28
Fat (%)	C	24.5 ± 0.73	28.0 ± 0.26
	E	25.2 ± 1.56	25.8 ± 0.75
Fat in dry matter (%)	C	55.0 ± 1.63	58.4 ± 0.49
	E	54.0 ± 3.67	57.6 ± 1.46
Salt (%)	C	3.6 ± 0.02	3.9 ± 0.02
	E	2.9 ±0.06	3.3 ± 0.02
Salt in moisture (%)	C	7.9 ± 0.05	8.4 ± 0.10
	E	6.5 ± 0.08	6.9 ± 0.08

E = Experimental cheese (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13 used as an adjunct culture);
C = Control cheese (no adjunct culture added);
SD = Standard deviation.

2.2.4.3 Levels of proteolysis and lipolysis in Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture

Changes in the proteolytic and lipolytic patterns during the ripening period of the experimental and control cheese are shown in Table 5. The level of proteolysis was measured by determining the WSN levels in both the experimental and control cheeses. The level of lipolysis was measured by the FFA levels in both the experimental and control cheeses. The levels of WSN and FFA increased over the 45 day ripening period but no significant differences ($P > 0.05$) were noted between the experimental and control cheeses. The WSN levels in the experimental cheese increased by 83 % whilst the increase in WSN in the control cheese increased by 82 % over the 45 day ripening period.

In contrast, the FFA levels only increased by 2.1 % and 0.4 % in the experimental cheese and control cheese, respectively, over the 45 day ripening period.

Table 5. Changes in the water-soluble nitrogen and free fatty acid levels during ripening of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture (n = 6 ± SD)

Parameter	Cheese type	Ripening time (days)	
		1 (± SD)	45 (± SD)
WSN (% TN)	C	1.3 ± 0.08	7.4 ± 0.06
	E	1.4 ± 0.03	7.9 ± 0.14
FFA[a]	C	2.3 ± 0.06	2.3 ± 0.09
	E	2.2 ± 0.06	2.3 ± 0.07

E = Experimental cheese (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13 used as an adjunct culture);
C = Control cheese (no adjunct culture added);
SD = Standard deviation;
WSN = Water-soluble nitrogen;
TN = Total nitrogen;
FFA = Free fatty acid;
a = Free fatty acids are expressed as milliequivalent 100 g^{-1} fat.

2.2.4.4 Sensory properties of Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture

It was found that the repetition of each batch of cheese manufactured on the different days as well as the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture played a significant role in the assessors correctly identifying which cheeses they were presented with (Pr > ChiSq less than 0.1) (Table 6). The addition of an adjunct culture to each repetition of cheese made on the different days contributed to the experimental and control cheeses having significantly different overall sensory properties. Thus, there were significant differences noted in the overall sensory attributes for repetitions 1, 2 and 3 between the experimental and control cheeses during the sensory evaluation test sessions. Whereas the order of which the samples were presented to the assessors during the sensory evaluation session did not play a significant role in the assessors correctly identifying which cheeses they were presented with (Pr > ChiSq greater than 0.1), (Table 6). This shows that the samples were randomly presented and

that the sensory test was designed objectively and without prejudice. The order of the samples presented to the assessors during the evaluation sessions played no role in the overall sensory attributes of the experimental and control cheeses being significantly different.

Table 6. Statistical data for the overall sensory difference test between Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture for batches 1, 2 and 3

Parameter	Pr > ChiSq
Batch number/ Repetition of cheese	0.0097
Addition of *Pediococcus* spp. as an adjunct culture	0.0066
Order of samples presented during sensory evaluation	0.1031

The combined sensory evaluation results revealed that there were significant differences ($P < 0.05$) when comparing the overall sensory attributes of the experimental and control Gouda cheeses (Table 7).

Fifty consumers separately evaluated the cheese from each repetition. During the first evaluation only 19 assessors correctly detected the difference in the overall sensory attributes between the experimental and control cheeses, thus, the difference in sensory evaluation was not significantly different ($P = 0.29$) (Table 7). However, 28 % of the 19 assessors who correctly detected differences between the two cheese types commented that the experimental cheese had more of an intense initial flavour with an overall sharper flavour than the control cheese.

For the second evaluation, 28 assessors correctly detected the difference in the overall sensory attributes of the experimental cheese and the control cheese, thus, the difference in overall sensory profile was significantly different ($P = 0.001$) (Table 7). Of the 28 assessors who correctly detected differences between the two cheese types, 34 % commented that the experimental cheese had an overall sharper flavour than the control cheese and that the control cheese was bland tasting.

The third evaluation, like the second, revealed significant differences ($P < 0.05$) in overall sensory profiles of the experimental cheese and the control cheese, as 34 assessors correctly detected the difference in the overall sensory attributes. Of the 34 assessors who correctly detected differences between the two cheese types, 50 % commented that the experimental cheese had had an overall sharper more characteristic / distinctive / interesting and creamier flavour that was more mature tasting than the control cheese whilst the control cheese was bland and ordinary tasting with a flavour that was not long lasting (Table 7).

Table 7. Sensory difference test between Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture for repetitions 1, 2 and 3

Repetition of Cheese	Number of assessors	Correct responses	Incorrect responses	P-value	Comments	
					Experimental cheese	Control
1	50	19	31	0.29	▪ More intense initial flavour (10 %)* ▪ Overall sharper (20 %)*	
2	50	28	22	0.001	▪ Overall sharper flavour (22 %)*	▪ Bland flavour (14 %)*
3	50	34	16	0.00	▪ Overall sharper (30 %)* ▪ More mature tasting (10 %)* ▪ More characteristic / distinctive / interesting flavour (15 %)* ▪ Creamier tasting (17 %)*	▪ Flavour not long lasting (7 %)* ▪ Bland & ordinary tasting (10 %)*
Total	**150**	**81**	**69**	**0.00**		

E = Experimental cheese (*P. acidilactici* ST 79 and *P. pentosaceus* ST 13 used as an adjunct culture);
C = Control cheese (no adjunct culture added);
* = Percentage of evaluators making specific comment, calculated as (number of evaluators making comment / total number of assessors x 100)

2.2.4.5 Statistical correlation between sensory difference test and levels of free fatty acids and water-soluble nitrogen for Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture for batches 1, 2 and 3

No correlation was found between the results of the sensory evaluation for each repetition of cheese manufactured being significantly different and the FFA's levels at day 45 of ripening (Pr > ChiSq greater than 0.1), (Table 8). As noted in Table 6, there was a statistical correlation found between the repetitions of the cheese manufactured on the different days as well as the addition of an adjunct culture to each repetition of cheese made on the different days. Thus, cheese manufactured on different days as well as the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture played a significant role in the assessors correctly identifying which cheeses they were presented with.

Table 8. Statistical data for the correlation between the sensory difference test and free fatty acid level for Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture for batches 1, 2 and 3

Parameter	Pr > ChiSq
Batch number/ Repetition of cheese	0.0905
Addition of *Pediococcus* spp. as an adjunct culture	0.0056
Free fatty acids	0.5532

No correlation was found between the results of the sensory evaluation for each repetition of cheese manufactured being significantly different and the WSN levels at day 45 of ripening (Pr > ChiSq greater than 0.1), (Table 9). As noted in Table 6, there was a statistical correlation found between the cheeses manufactured on the different days as well as the addition of an adjunct culture to each repetition of cheese made on the different days. Thus, cheese manufactured on different days as well as the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture played a significant role in the assessors correctly identifying which cheeses they were presented with.

Table 9. Statistical data for the correlation between the sensory difference test and water-soluble nitrogen level for Gouda cheese made with and without *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 as an adjunct culture for batches 1, 2 and 3

Parameter	Pr > ChiSq
Batch number/ Repetition of cheese	0.0051
Addition of *Pediococcus* spp. as an adjunct culture	0.0051
Water-soluble nitrogen	0.4316

2.2.5 Discussion

The control cheese had a lower initial LAB count than the experimental cheese due to the addition of the *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture. The trend of a higher LAB count in the experimental cheese continued throughout the 45 day ripening period. However, the difference in the LAB counts between the experimental and control cheeses were not significant. This finding is consistent with the report of Gomez, Gaya, Nuñez & Medina (1996) that found no significant differences in LAB counts between semi-hard cheese made from pasteurised cows milk to which NSLAB was added and the control semi-hard cheese to which no NSLAB was added.

No significant differences in fat, protein, salt and moisture contents were observed between the experimental and control cheese, which is in agreement with previous reports. Goodwins, Manoury, Schuck, Pellerin, Skowera & Mornet (1993) did not find significant differences in fat in dry matter and salt in moisture contents between Cheddar cheese made using a lactic acid adjunct culture and a control cheese, to which no lactic acid adjunct culture was added. An increase in fat, salt, protein, fat in dry matter, and salt in moisture contents were observed at the end of the ripening period. This increase may be due to the decrease in moisture content of the cheese samples and a concurrent increase in the percentage of these components.

The level of proteolysis in the cheese as measured by the WSN level, which increased over the 45 day ripening period for both the experimental and control cheeses. The WSN levels of the experimental cheese were higher than the WSN levels of the control cheese. However, the difference noted in the WSN levels between the experimental and control cheese was not significant. Rehman *et*. *al.* (2000) showed that NSLAB had little effect on the production of WSN in cheddar cheese made from pasteurised milk to which a NSLAB was added. In Gouda cheese, proteolysis is brought about mainly by the action of the starter enzymes, which are released upon death and lysis of the starter cultures (Visser, 1977; Fox & Law, 1991). Since there were no significant differences noted in the LAB counts between the experimental and control cheese throughout the 45 day ripening period, the level of proteolysis which is brought about mainly by the action of the starter enzymes, was also not significantly different. Proteolytic enzymes of the starter culture are intracellular and as the starter culture used during cheesemaking are resistant to the pediocin-producing NSLAB, there would be no increase in starter cell lysis in the experimental cheese as compared to the control cheese (Fox & Law, 1991). This may also play a role in the levels of proteolysis between the experimental and control cheeses being insignificantly different.

The level of lipolysis in cheese during ripening as determined by the FFA level is dependant on the activity of the intracellular peptide hydrolysing esterolytic enzyme (Bhowmik & Marth, 1989). All *Pediococcus* spp. do not exhibit an active esterase band. The FFA levels of both the experimental and control cheeses increased throughout the 45 day ripening period. The FFA levels of the experimental cheese was higher than that of the control cheese, however, this difference was not significant at the end of the 45 day ripening period. Bhowmik & Marth (1989) found that strains of *P. acidilactici* examined had no esterolytic activity as no active esterase band was detected and, therefore, did not contribute to the level of lipolysis during ripening. Bhowmik & Marth (1989) also found that some of the strains of *P. pentosaceus* exhibited low levels of esterase activity as a weak esterase band was detected, which is consistent with FFA levels of the experimental cheese being insignificantly higher than the control cheese after the 45 day ripening period. Bhowmik & Marth (1989) concluded that the esterases of *Pediococcus* spp.

produce FFA's only in minute quantities, therefore, the FFA levels of the experimental cheese is not significantly different from levels of the control cheese after the 45 day ripening period.

Of the 150 assessors participating in the sensory session, 81 assessors correctly detected differences in the overall sensory attributes between the experimental and control cheeses. The results of the sensory evaluation revealed significant differences (P = 0.00) in the overall sensory attributes of the experimental and control cheeses. The significant differences in sensory properties observed during the sensory evaluation test between the experimental and the control cheeses can be attributed to the cheeses being produced on different days as well as the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture (Pr > ChiSq less than 0.1). The order of the samples presented to the assessors during the sensory evaluation test, however, did not have any significant effect on the assessors correctly identifying which cheeses they were presented with (Pr > ChiSq greater than 0.1).

In accordance with the statistical analysis results, the 50 assessors evaluating batch 1 could not correctly detect the differences in the overall sensory attributes between the experimental and control cheeses, whereas the 100 assessors evaluating batch 2 and 3 could correctly detect the differences in the overall sensory attributes between the experimental and control cheeses. The batch of cheese evaluated had an affect on the response of the assessor (Pr > ChiSq less than 0.1). The assessors that correctly detected the differences between the experimental and control cheeses of batch 1, noted that the experimental cheese had a more intense initial flavour, whilst the assessors correctly detecting the differences in batch 2 noted that the experimental cheese was more mature tasting, and that the control cheese was bland and ordinary tasting. The assessors correctly detecting the differences in batch 3, noted the experimental cheese had a more characteristic / distinctive / interesting flavour that overall was sharper than the control cheese while, the control cheese was noted as having a flavour that was not long lasting. There was no statistical correlation found between the results of the sensory evaluation

for the experimental and control cheeses being significantly different and the WSN or the FFA levels at day 45 of ripening (Pr > ChiSq greater than 0.1).

2.2.6 Conclusion

The addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture had no effect on the physico-chemical properties of the cheese but the sensory properties were significantly influenced. The pediococci spp. had an effect on the sensory quality of Gouda cheese making it distinguishable from the control cheese, to which no adjunct culture was added.

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CHAPTER III

General discussion and conclusion

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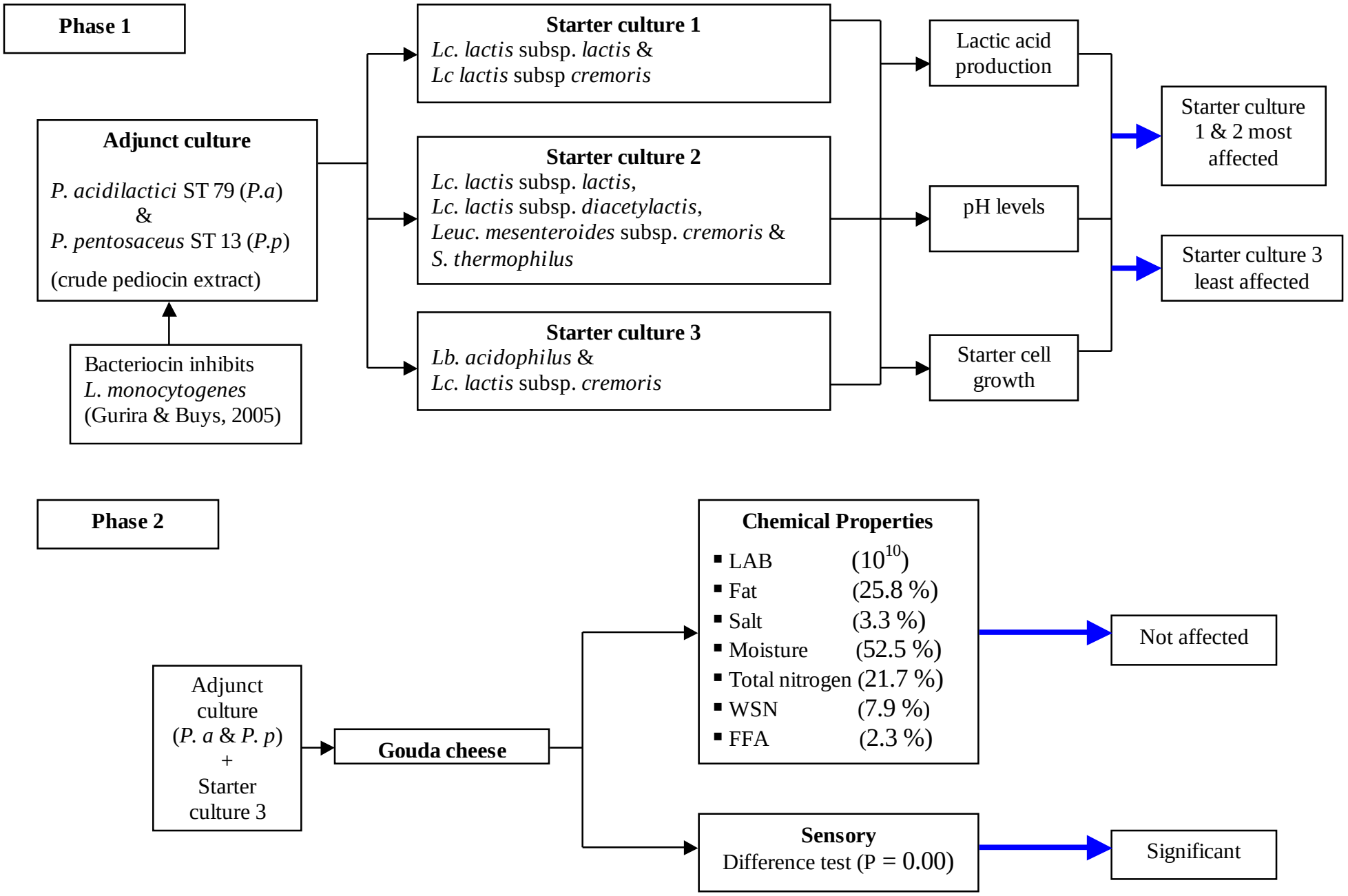

Figure 12. Proposed model for the application of *Pediococcus* species as an adjunct culture in Gouda cheese

3.1 General Discussion

The present study as shown in the proposed model (Fig. 12), quantified the effect of the selected pediocin-producing *Pediococcus* spp. on commercially available mesophilic lactic acid Gouda cheese starter cultures and the quality of Gouda cheese manufactured with and without the pediocin-producing *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture.

3.1.1 Effect of *Pediococcus acidilactici* ST 79 and *Pediococcus pentosaceus* ST 13 crude pediocin extract on commercially available mesophilic lactic acid Gouda cheese starter cultures

The experiment on the commercially available mesophilic lactic acid Gouda cheese starter cultures was aimed at determining the effect of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 CPE on the levels of lactic acid production, pH, starter cell survival and growth rate of the commercially available mesophilic lactic acid Gouda cheese starter cultures. The following starter cultures were evaluated, LL 50C, Nizostar 500MT and Dairysafe™. The results of the study revealed that there were insignificant differences noted in sensitivity to the inhibitory effect of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 CPE for all three starter cultures evaluated.

The levels of lactic acid production (% w/w) increased whilst the pH levels correspondingly decreased for LL 50C, Nizostar 500MT and Dairysafe™ + *Pediococcus* spp. CPE throughout the 18 h incubation period. However, the increase in lactic acid production (% w/w) and decrease in pH levels of all the starter cultures with and without the addition of *Pediococcus* spp. CPE after 18 h of incubation were all insignificant, which is in agreement with O'Sullivan *et. al.* (2003), that found that the inclusion of a pediocin-producing NSLAB did not compromise lactic acid production (% w/w) and pH levels of the starter LAB and consequently, cheese was successfully manufactured. The increase in lactic acid production (% w/w) and decrease in pH level was the greatest for Dairysafe™ + *P. acidilactici* ST 79 CPE. Thus, the lactic acid production and pH levels

of starter culture Dairysafe™ were the least affected by the addition of the *Pediococcus* spp. CPE.

The survival and growth rate of the commercially available mesophilic lactic acid Gouda cheese starter cultures (LL 50C, Nizostar 500MT and Dairysafe™) were not adversely affected by the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 CPE. The differences noted between the growth of the starter culture with and without the addition of *Pediococcus* spp. CPE as measured by a colony count after 18 h of incubation, were insignificant.

The *Pediococcus* spp. CPE had a greater effect on the growth rate of the mixed strain starter cultures than that of their acid production. This difference might be attributed to the different single strains that each mixed starter culture consists of and also the ratio of each different single strain making up the mixed starter culture (Scott *et. al.*, 1998).

It was found by Gurira & Buys (2005) that the pediocins produced by the *Pediococcus* spp. used as an adjunct culture in this specific study inhibited the following LAB, *Lc. lactis* subsp. *diacetylactis* NCDO 176 and *Lc. lactis* subsp. *lactis* NCDC 605.

Nizostar 500MT is partially composed of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *diacetylactis*, whilst LL 50C is partially composed of *Lc. lactis* subsp. *lactis* and Dairysafe™ does not contain any of these strains. Instead, Dairysafe™ is composed of nisin resistant *Lb. acidophilus* and *Lc. lactis* subsp. *cremoris*.

Lactococcus lactis subsp. *lactis* is the major lactic acid producer amongst the lactic acid starter cultures used for cheesemaking (Scott *et. al.*, 1998). Thus, if a mixed starter culture is composed of higher proportion of the sensitive spp. *Lc. lactis* subsp. *lactis*, the acid production of that mixed culture will be more inhibited than their growth (Seifu, Buys & Donkin, 2004).

Thus, the lactic acid production and pH levels of starter culture Nizostar 500MT was the most affected whilst Dairysafe™ was the least affected by the addition of an adjunct culture. Thus, Dairysafe™ was used in conjunction with the *Pediococcus* spp. as an adjunct culture in the cheesemaking process.

3.1.2 Effect of *Pediococcus* species as an adjunct culture on the quality aspects of Gouda cheese

The experiment on cheese was aimed at assessing the suitability of the live cultures *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture in the manufacture of Gouda cheese and to determine its effect on the physico-chemical, microbiological and organoleptic properties of Gouda cheese over a ripening period of 45 days.

No significant differences in moisture, protein, fat, fat in dry matter, salt and salt in moisture contents were observed between the experimental and control cheese. This finding is in line with reports by Macedo *et. al.* (2004) who found no significant differences in moisture, percentage (dry matter basis) of fat, total nitrogen and salt contents. The fat, protein and salt contents of both the experimental and control cheeses had increased when measured at the end of the ripening period, whereas the moisture content of both the experimental and control cheeses had decreased.

No significant difference in the chemical composition was observed between the experimental and control cheeses, which might be attributed to the use of the pediocin resistant starter culture (Dairysafe™) as a starter culture for cheesemaking. Thus, it can be concluded that Gouda cheese of satisfactory composition can be manufactured using *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture.

Although there was an increase in the WSN and FFA levels noted over the 45 day ripening period, the levels of proteolysis and lipolysis in the experimental cheese as measured by the WSN and FFA levels respectively, was comparable to that of the control cheese. The same observation was noted by Fox & McSweeney (1996).

Proteolysis in cheese is brought about by the proteolytic enzymes derived from the rennet, milk used, starter bacteria and non starter bacteria (McSweeney & Sousa, 2000). Rennet is responsible for the breakdown of casein to large peptides during the early stages of ripening, but in Gouda cheese, proteolysis is mainly due to the action of starter enzymes (Fox & Law, 1991). Fox & McSweeney (1996) found NSLAB contribute little to proteolysis in cheese, as they do not lyse as readily as the starter culture.

The level of lipolysis in cheese during ripening as determined by *Pediococcus* spp. is dependant on the activity of the intracellular peptide hydrolysing esterolytic enzyme. All *Pediococcus* spp. do not exhibit an active esterase band (Bhowmik & Marth, 1989). In support of Bhowmik & Marth (1989) findings, Collins *et*. *al.* **(**2003) found evidence of a relationship between the extent of starter cell autolysis and the level of lipolysis during cheese ripening. Collins *et*. *al.* **(**2003) also found that the levels of FFA increased significantly when made with a starter culture, thus, suggesting that starter enzymes are mainly responsible for lipolysis. The starter culture used in the present study is resistant to the pediocin-producing adjunct culture, which partly explains the reason for the absence of significant differences in the levels of proteolysis and lipolysis between the experimental and control cheeses.

Contrary to the physico-chemical results being insignificant the overall sensory attributes of the experimental and control cheeses were significantly different. The significant differences in sensory properties observed during the sensory evaluation test between the experimental and the control cheeses can be attributed to the repetitions of cheeses produced on different days as well as the addition of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture. The order of the samples presented to the assessors during the sensory evaluation process did not have any significant effect on the response of the assessors correctly identifying which cheeses they were presented with. Neither was there any statistical correlation found between the results of the sensory evaluation for each repetition of cheese manufactured and the WSN or FFA levels at day 45 of ripening.

In summary, the experiment on cheese revealed the suitability of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture in the manufacture of Gouda cheese when used in conjunction with a pediocin resistant starter culture. The appropriate starter culture was identified in phase 1 and its importance reflected in phase 2 by the absence of significant differences in the chemical composition and total LAB counts of the experimental and control cheeses. The addition of the *Pediococcus* spp. as an adjunct culture had no adverse effect on the physico-chemical properties of Gouda cheese and satisfies the objective set in the study, to determine the effect the addition of *Pediococcus* spp., as an adjunct culture may have on the chemical composition of Gouda cheese. The findings of this study also supports the hypothesis that addition of the *Pediococcus* spp. as an adjunct culture had an effect on the sensory parameters of Gouda cheese as significant differences were noted in the overall sensory attributes of the experimental and control cheeses.

In general, the results of the study highlights the potential use of live cultures of pediocin-producing NSLAB for the improvement of the sensory properties of Gouda cheese made from milk to which *Pediococcus* spp. are added as an adjunct culture. The value of cheese sales represents about 30 % of total dairy products and the Society of Dairy Technology forecasts a growth of 9.8 % for global cheese manufacture in the following three years (Farkye, 2004). The growth in the cheese sector is mostly due to an increasing interest in speciality and exotic cheeses. This could be of significance to Gouda cheese producers as presently a Gouda cheese with that can be differentiated from Gouda cheese currently on the market will serve to strengthen the market position of Gouda cheese in a growing cheese market.

3.2 Conclusion

Dairysafe™ was chosen from three commercially available mesophilic lactic acid Gouda cheese starter cultures to be used in conjunction with the live cultures of *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 as an adjunct culture in Gouda cheesemaking.

P. acidilactici ST 79 and *P. pentosaceus* ST 13 added as an adjunct culture in conjunction with starter culture Dairysafe™ did not adversely affect the physico-chemical properties of Gouda cheese as all levels were comparable to levels in the control cheese.

During the sensory evaluation tests the experimental cheese was found to be significantly different from the control cheese, which is representative of the Gouda cheese commercially available in the market place.

3.3 Future research needs

- In the study, an adjunct culture used during cheesemaking comprised of both *P. acidilactici* ST 79 and *P. pentosaceus* ST 13. It is, therefore, recommended that each of the pediococci cultures be used separately as individual adjunct cultures to determine the affect of each pediococci strain on cheese quality.

- During the course of the study, it was observed that the CPE produced by the *Pediococcus* spp. used as an adjunct culture had varying inhibitory effects on the commercially available mixed strain mesophilic lactic acid Gouda cheese starter cultures evaluated. Thus, detailed studies aimed at investigating the inhibitory effect of the pediocins produced by *P. acidilactici* ST 79 and *P. pentosaceus* ST 13 on individual strains of LAB constituting common commercially available mixed strain mesophilic lactic acid Gouda cheese starter cultures.

- Genetic improvements of commercially available starter cultures with respect to industrially important traits such as pediocin resistance may help to solve the problem of first having to screen the starter cultures for pediocin sensitivity before using *Pediococcus* spp. as an adjunct culture in conjunction with commercially available starter cultures in Gouda cheese manufacturing.

- The sensory evaluation in the study comprised of difference testing. It is, therefore, recommended that a descriptive panel be included in the sensory evaluation to gain a more detailed flavour profile of the cheeses manufactured with and without an adjunct culture, which can be used to develop a preference map.

- It is recommended that *L. monocytogenes* and *B. cereus* be included in cheesemaking and determine the effect of the *Pediococcus* spp. CPE on the pathogens *in situ* in the cheese.

- Include other chemical analysis not included in the scope of the study to help determine the differences in the chemical properties of the cheeses manufactured with and without an adjunct culture, which can be used to explain the difference in the overall sensory attributes of the cheeses.

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CHAPTER IV

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