PRESERVATION OF RED MEAT WITH NATURAL ANTIMICROBIAL PEPTIDES PRODUCED BY LACTIC ACID BACTERIA

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entity or in part, been submitted at any university for a degree.

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ABSTRACT

Red meat has a limited shelf-life at refrigerated temperatures, where spoilage is mainly due to the proliferation of bacteria, yeast and moulds, acquired during the dressing process. In addition, almost a fifth of food-borne disease outbreaks, caused by micro-organisms such as *Escherichia coli* 0157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* are associated with red meat. To improve the microbiological quality of red meat, systems such as HACCP, GHP and GMP are currently practiced; however, these practices are not able to extend the shelf-life of these products. At present suitable food-grade preservatives are recommended, but the use of some of these preservatives is increasingly being questioned with regard to their impact on human health. Additionally, food service customers demand high quality products that have a relatively long shelf-life, but still prefer the appearance of minimally processed food. All these factors challenge the food manufacturing industry to consider more natural means of preservation.

Antimicrobial metabolites of food grade bacteria, especially lactic acid bacteria, are attracting increasing attention as food preservatives. Bacteriocins are antimicrobial peptides (3 to 10 kDa) with variable activity spectra, mode of action, molecular weight, genetic origin and biochemical properties that are bacteriostatic or bactericidal to bacteria closely related and bacteria confined within the same ecological niche.

Micro-organisms were isolated from beef, lamb and pork, obtained from four commercial retailers. The number of viable cells three days after the sell-by date at 4ºC ranged from 80 $cfu.g^{-1}$ to 1.4×10^8 $cfu.g^{-1}$. Fifty-three percent were Gram-negative bacteria, 35% Gram-positive and 12% yeast. The microbial population of the meat was greatly influenced by the origin, i.e. the retailer. Bacteriocins produced by *Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 were screened for activity against bacteria isolated from the different meat samples. Sixteen to 21% of the isolates, identified as members of *Klebsiella*, *Shigella*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus* and *Bacillus* were sensitive to the bacteriocins.

Curvacin DF 38, plantaricin 423 and caseicin LHS (2.35 to 3.4 kDa) had the broadest activity range and inhibited species of *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Listeria*, *Bacillus*, *Clostridium* and *Propionibacterium*. The bacteriocins remained stable at 121ºC for 20 min, in buffers with a pH ranging from 2 to 10 and in NaCl concentrations of between 0.1 and 10% (m/v). Like most peptides, they were

sensitive to proteolytic enzymes. Curvacin DF 38 is sensitive to amylase, suggesting that the bacteriocin might be glycosylated.

To assess the efficiency of curvacin DF 38, plantaricin 423 and caseicin LHS as meat preservatives, they were partially purified by ammonium sulphate precipitation and separation in a Sep Pak C_{18} cartridge. The shelf-life of pork may be extended by up to two days. Meat samples treated with bacteriocins were darker than the control (untreated) sample. Descriptive sensory evaluation by a seven-member panel indicated that there were significant differences ($P \leq 0.05$) regarding the aroma, sustained juiciness, first bite and metallic taste attributes of the control and the 4 day-treated samples. The control and 2 day-treated samples and the 2 day- and 4 day treated samples did not differ significantly regarding these attributes. There were no significant differences regarding the initial juiciness, residue and pork flavour attributes.

Concluded from the results obtained in this study, bacteriocins produced by *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS effectively extended the shelf-life of pork loins by up to 2 d at refrigerated temperatures with no drastic changes on sensory characteristics. In edition, the stability of these bacteriocins broadens their application as preservatives in many foods.

UITTREKSEL

Die rakleeftyd van rooivleis by yskastemperature is beperk, waar bederf hoofsaaklik deur die vermenigvuldiging van bakterieë, giste en swamme veroorsaak word. Die meeste van hierdie kontaminante is afkomstig van die slagtingsproses. Byna ’n vyfde van alle uitbrake van voedselvergiftigings wat deur organismes soos *Escherichia coli* 0157:H7, *Listeria monocytogenes* en *Staphylococcus aureus* veroorsaak word, word met rooivleis geassosieër. Die praktyke HACCP, GMP en GHP word tans toegepas om die mikrobiologies kwaliteit van vleis te handhaaf, maar is egter nie voldoende om die rakleeftyd van rooivleis the verleng nie. Die preserveermiddels wat huidiglik aanbeveel word vir dié doel, word toenemend bevraagteken aangaande die invloed daarvan op die menslike gesondheid. Hierby is daar ’n aanvraag na hoë kwaliteit, ongeprosesseerde produkte met ’n verlengde rakleeftyd. Gevolglik word die voedsel vervaardigings industries aangemoedig om meer natuurlike vorms van preservering the oorweeg.

Die aandag word tans op die anti-mikrobiese metaboliete van voedselgraad microbes, veral melksuurbakterieë, gevestig. Bakteriosiene is anti-mikrobiese peptiede (3 tot 10 kDa) met verskeie aktiwiteitsspektra, werkswyse, molekulêre massa, genetiese oorsprong en biochemiese eienskappe. Bakteriosiene is meestal bakterie-dodend of -staties teen taksonomies naby geleë organismes en organismes vanuit dieselfde ekologiese nis.

Mikroörganismes is geïsoleer vanuit bees-, skaap- en varkvleis, verkry vanaf vier supermarkte. Die aantal lewensvatbare selle per gram (cfu.g^{-1}) het drie dae na die “verkoop”-datum by 4ºC vanaf 80 cfu.g^{-1} tot 1.4 × 10^{8} cfu.g^{-1} gevarieër. Drie en vyftig persent van die isolate is as Gram-negatief, 35% as Gram-positief en 12% as giste geïdentifiseer. Die sensitiwiteit van hierdie isolate teen bakteriosiene wat deur *Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 en *Pediococcus pentosaceus* ATCC 43201 geproduseer is, is vervolgens getoets. Tussen 16% en 21% van die isolate was sensitief teen die bacteriosiene en is onder andere as *Klebsiella*, *Shigella*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus* en *Bacillus* geïdentifiseer.

Die bakteriosiene met die wydste aktiwiteitsspektrum, naamlik, curvacin DF 38, plantaricin 423 en caseicin LHS is verder ondersoek. Hierdie antimikrobiese peptiede (2.35 tot 3.4 kDa) toon aktiwiteit teen spesies van *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Listeria*, *Bacillus*, *Clostridium* and *Propionibacterium*. Die bakteriosiene is stabiel by 121ºC vir 20 min, in buffers met ‘n pH-reeks van tussen 2 en 10 en

soutkonsentrasies vanaf 0.1% tot 10%. Soos die geval by meeste peptiede is hierdie bakteriosiene sensitief vir proteolitiese ensieme. Curvacin DF 38 is ook sensitief vir amylase, wat daarop dui dat hierdie bakteriosien moontlik geglikosileer is.

Die effektiwiteit van curvacin DF 38, plantaricin 423 en caseicin LHS as preserveermiddel in voedselsisteme is getoets deur dit te suiwer (ammonium sulfaat presipitasie en Sep Pak C_{18} kolom) en op vark lendestukke aan te wend. Mikrobiese analise het bewys dat die rakleeftyd van vark met sowat 2 dae verleng kan word. Volgens die vleiskleurevaluering was die bakteriosien behandelde vark donkerder as die kontrole. Die aroma-, sappigheid-, tekstuur- en metaalgeur-eienskappe van die kontrole en die 4-dag behandelde monster het volgens ‘n opgeleide sensoriese paneel betekenisvol verskil ($P \leq 0.05$). Die kontrole en die 2-dag behandelde en die 2-dag behandelde en die 4-dag behandelde monsters het nie betekenisvol verskil nie. Daar was geen betekenisvolle verskil aangaande die aanvanklike sappigheid-, residu- en varkgeur-eienskappe nie. Hierdie sensoriese eienskappe is belangrik ten opsigte van die verbruiker se aanvaarding van die produk.

Vervolgens kan uit hierdie resultate afgelei word dat die bakteriosiene wat deur *Lb. curvatus* DF 38, *Lb. plantarum* 423 en *Lb. casei* LHS geproduseer word voldoende is om die rakleeftyd van vark lendestuk by 4ºC met 2 dae te verleng met min of geen effek op die sensoriese persepsie van die vleis. Hierdie bakteriosiene is ook stabiel onder verskeie kondisies wat die toepassing as preserveermiddel aansienlik verbreed.

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CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts with each chapter an individual entity. Repetition between chapters is thus, unavoidable.

CHAPTER 1

INTRODUCTION

Red meat is a perishable food product with a relatively short shelf-life at refrigerated temperatures (Anon., 2002). Spoilage of red meat at refrigeration temperatures is due to the proliferation of bacteria, yeast and moulds on the meat surface (Jensen, 1954), which are mostly acquired during the dressing process (Jensen, 1954; Borch *et al*., 1996; Merck, undated). The numbers and types of micro-organisms initially present and their subsequent growth, determines the shelf-life of meat. Only about 10% of the initial micro-organisms present are able to grow at refrigerated temperatures, and the fraction that may cause spoilage, is even smaller (Borch *et al*., 1996). Spoilage for the meat industry is generally defined as the presence of a specified maximum microbial level or an unacceptable off-flavour, off-odour or appearance (ICMSF, 1986; Borch *et al*., 1996).

Escherichia coli 0157:H7, *Listeria monocytogenes* and *Staphylococcus aureus* are micro-organisms that frequently cause food-borne diseases associated with red meat. The majority of *E. coli* 0157:H7 outbreaks have been associated with the consumption of ground meat (Abee *et al*., 1995) and present a serious risk because of the low infectious level (Työppönen *et al*., 2003). *Listeria monocytogenes*, in contrast, is a psychrotrophic food-borne pathogen that grows rapidly at refrigeration temperatures and is normally associated with dairy, poultry and meat products (Abee *et al*., 1995). Young children, pregnant woman, immuno-compromised individuals and the elderly are especially at risk to this type of infection (Työppönen *et al*., 2003). Food poisoning associated with *S. aureus* leads to severe symptoms and is usually associated with dairy and meat products. In this case food poisoning results after the production of a toxin (0.2 - 1.0 μg) present in the contaminated food (Kennedy *et al*., 2000).

Systems such as HACCP (Hazard Analysis Critical Control Point) (Kennedy *et al*., 2000), good hygiene practice (GHP) and good manufacturing practice (GMP) (Panisello *et al.*, 2000) are currently used by the meat industry to minimize the health risk of potential pathogens and spoilage micro-organisms, to improve the microbiological quality of food and to prevent recontamination of food. These practices are unfortunately not enough to extend the shelf-life of food products. The National Food Processor Association (NFPA) in the USA recommended the incorporation of a suitable preservative to extend the shelf-life of foods (Kennedy *et al*., 2000). A successful preservative must be effective in small quantities and have a wide spectrum of anti-

microbial activity, but it should not lower the quality of the food and it should be harmless to the consumer (Kennedy *et al*., 2000).

The use of chemical preservatives, currently employed to limit the number of micro-organisms capable of growing in foods, including sulphites, sulphur dioxide, sodium chloride, phosphates, hydrogen peroxide, nitrates, nitrites, Na-diacetate, β-propiolactone, benzoic acid and benzoates, fumaric acid, parabens and therapeutic antibiotics, are increasingly being questioned with regard to their impact on human health (Magnuson, 1997; Kennedy *et al*., 2000). These type of questions challenges the food manufacturing industry to look at more natural means of preservation as food service customers demand high quality products that have a relatively long shelf-life, but still prefer the appearance of minimally processed food (Hugas *et al*., 2002; Ross *et al*., 2002).

There are important interactions between microbes in the normal meat ecosystem. These include competition for nutrients and the production of metabolites with anti-microbial activity, including organic acids (lactic, acetic and formic), diacetyl, CO_2, hydrogen peroxide, aldehydes and antibiotics (Abee *et al*., 1995; Borch *et al*., 1996; Ross *et al*., 2002; Magnusson *et al*., 2003).

A number of micro-organisms are able to produce anti-microbial peptides (3 to 10 kDa) or bacteriocins (Montville & Winkowski, 1997) that have a variable activity spectrum, mode of action, molecular weight, genetic origin and biochemical properties. Bacteriocins are bacteriostatic or bactericidal to other bacteria, especially those closely taxonomically related, but also bacteria confined within the same ecological niche. The producer strain is usually immune to the produced bacteriocin (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002).

Bacteriocins produced by food-associated micro-organisms such as lactic acid bacteria, in particular, are attracting increasing attention as food preservatives (Abee *et al*., 1995; Montville & Winkowski, 1997). These bacteriocins are readily degraded by the protease-enzyme in the human gastrointestinal tract and most bacteriocin-producing lactic acid bacteria have GRAS (generally regarded as safe) status. Therefore, bacteriocins may be considered as natural bio-preservatives (Vandenbergh, 1993; Abee *et al*., 1995; Schillinger *et al.*, 2001; Aymerich *et al.*, 2000).

Bacteriocins may also be used as part of a multiple hurdle preservation system (Cleveland *et al*., 2001), the bacteriocin produces may be applied as bacteriocinogenic cultures to non-fermented foods or even be used as starter culture for fermented foods to facilitate the improvement of quality and safety and to control spoilage or pathogenic organisms (Stevens *et al*., 1991; Zhang & Mustapha, 1999; Nilsson *et al*., 2000).

Several bacteriocins have been reported to have potential in the food industry when used at the recommended conditions (Cleveland *et al*., 2001). However, it is important that applied studies be done to confirm the effectiveness of the addition of bacteriocins to food systems as it has been shown that they are not effective in all food systems (Gänzle *et al.*, 1999; Cleveland *et al*., 2001).

Patented applications of bacteriocins as food preservatives include the use of a combination of nisin (produced by *Lactococcus lactis* subsp. *lactis*), a chelating agent and a surfactant, to inhibit both Gram-positive and Gram-negative micro-organisms in meat, eggs, cheese and fish (Blackburn *et al.* 1998). *Streptococcus* and *Pediococcus*-derived bacteriocins in combination with a chelating agent were successfully used to protect food against *Listeria* (Wilhoit 1996). The number of *Listeria monocytogenes* cells in Manchengo cheese inoculated with a bacteriocin-producing strain of *Enterococcus faecalis* decreased by six log-cycles in only 7 days. However, the survival of *L. monocytogenes* in cheese made with a commercial starter cultures was not affected (Nuñez *et al.*, 1997), but Campanini *et al.* (1993) found that the inoculation of the bacteriocin producer *Lb. plantarum* into a naturally contaminated salami sausage led to a decrease in the number of surviving *Listeria monocytogenes* cells. In 1995, Vedamuthu patented a yoghurt product with increased shelf-life containing a bacteriocin derived from *Pediococcus acidilactici*. The plasmid-encoding pediocin expressed in *L. lactis,* was used as a starter culture for the production of cheddar cheese to aid the preservation of the cheese and to ensure the microbial quality of the fermentation process (Buyong *et al.*, 1998). Pediocin PA-1 was also expressed in "*Streptococcus thermophilus*", which is an important organism in the dairy fermentation industry (Coderre & Somkuti, 1999).

In this study the inhibitory effect of bacteriocins produced by *Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 will be tested against micro-organisms that will be isolated from beef, pork and lamb from four retailers. The three most active bacteriocins will be characterised and evaluated for their effectiveness as a preservative on pork. The bacteriocin-treated pork will be compared with a control pork sample regarding microbial survival, meat colour and sensory deterioration.

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CHAPTER 2

LITERATURE REVIEW

A. RED MEAT SPOILAGE

Red meat, together with poultry, fish, dry beans, eggs, vegetables, fruit and nuts, form an integral part of a nutritious and well-balanced diet (Anon., 2002a). Unfortunately, red meat does not have a long shelf-life at refrigerated temperatures (0º to 4ºC). Beef has a shelf-life of approximately 10 to 14 d, lamb between 7 and 10 d and pork about 4 d. When packaged and stored in an air and moisture proof container at -18ºC, beef has a shelf-life of about 10 months, lamb about 8 months and pork between 4 and 6 months (Anon., 2002b).

Spoilage of chilled beef at refrigeration temperatures is due to the proliferation of various bacteria, yeasts and moulds on the meat surface (Jensen, 1954; Borch *et al*., 1996; Merck, undated). The numbers and types of micro-organisms initially present and their subsequent growth, determines the shelf-life of the meat (Borch *et al.*, 1996). *Enterobacteriaceae, Pseudomonas, Shewanella putrefaciens, Alcaligenes, Achromobacter*, *Flavobacterium, Acinetobacter, Moraxella, Psychrobacter, Brochothrix thermosphacta, Lactobacillus, Carnobacterium, Streptococcus, Leuconostoc, Micrococcus, Staphylococcus*, coryneforme bacteria, *Bacillus, Clostridium*, yeasts and moulds are some of the micro-organisms that are frequently found in beef, mutton, lamb, pork and poultry (Merck, undated). More than 99% of the initial contamination occurs during the dressing process (Jensen, 1954; Borch *et al*., 1996; Merck, undated). Only about 10% of the initial microbes present are able to grow at refrigerated temperatures, and the fraction of microbes that are able to cause spoilage, are even smaller. Environmental factors including temperature, gaseous atmosphere and salt content will select for specific microbes and will consequently influence their growth rate and activity (Borch *et al*., 1996).

Micro-organisms which are the primary cause of meat spoilage and are able to proliferate at refrigerated temperatures include *Enterobacteriaceae, Shewanella putrefaciens, Micrococcus, Achromobacter*, *Aeromonas, Pseudomonas, Acinetobacter, Moraxella, Psychrobacter, Brochothrix thermosphacta, Staphylococcus,* coryneforme bacteria*, Lactobacillus*, *Leuconostoc* and *Weissella* (Jensen, 1954; Borch *et al*., 1996; Merck, undated). *Mycotorula, Candida, Geotrichoides, Blastodendrion* and *Rhodotorula* are the most commonly found yeasts, while the moulds include *Penicillium, Mucor,*

Cladosporium, Alternaria, Sporotrichum and *Thamnidium* (Jensen, 1954). Pathogenic and toxinogenic micro-organisms include *Salmonella* spp., *Yersinia enterocolotica, Campylobacter jejuni*, pathogenic *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus* and *Clostridium perfringens* (Merck, undated).

Spoilage can be defined as a specified maximum microbial level or an unacceptable off-flavour/off-odour or discolouration (Borch *et al*., 1996). Most food products, including meat, have to adhere to legally set microbiological standards. The maximum acceptable level of bacteria allowed during storage is 10^7 to 10^9 cfu.cm^{-2} (Borch *et al*., 1996). According to Merck (undated), spoilage generally occurs at about 1 $\times$ 10^6 cfu.cm^{-2}. The ICMSF (International Commission for the Microbiological Specifications of Foods) (1986), recommended a general viable count of less than 1 $\times$ 10^7 cfu.g^{-1} and that *Salmonella* should not be detected in more than one out of five 25 g samples of meat (ICMSF, 1986). Alternatives to microbial monitoring are the use of chemical indicators of bacterial spoilage such as levels of D-lactate, tyramine, pH changes and the composition of headspace gas (Borch *et al*., 1996).

The packaging of meat determines the environmental factors, which affects the shelf-life. Currently, three different types of packaging are used for red meat, which includes air, vacuum and modified atmosphere (different levels of oxygen and carbon dioxide, balanced with inert nitrogen). In aerobically stored meat, *Pseudomonas* spp. may dominate, and as a result of their rapid growth rate, the shelf-life is only a matter of days (Borch *et al*., 1996). In previous studies conducted on vacuum-packed chill-stored beef, *Lactobacillus curvatus*, *Lb. sakei*, *Carnobacterium piscicola* and *C. divergens*, were found to be the most predominant spoilage bacteria (Sakala *et al*., 2002). Vacuum-packed beef has a longer shelf-life than pork, even though lactic acid bacteria dominate in both types of meat. In pork, the glycogen and glucose decreases faster than in beef which leads to earlier initiation of amino acid degradation. In contrast, members of the family *Enterobacteriaceae* develop faster on pork than on beef (Borch *et al*., 1996).

Spoilage and pathogenic micro-organisms not only cause off-odours and off-flavours, but also discolouration, slime and gas production and a decrease in pH. There are also important interactions between bacteria in the meat ecosystem, including competition for nutrients and the production of antimicrobial substances such as hydrogen peroxide, lactic acid and bacteriocins (Borch *et al*., 1996).

Pseudomonas spp. produces ethyl esters that cause sweet and fruity odours during the early stages of spoilage. Sulphur-containing compounds, including hydrogen sulphide (*Enterobacteriaceae*) and dimethyl sulphide (*Pseudomonas* spp.), are mainly responsible for sulphury and putrid odours. Cheesy odours are generally associated with

acetion/diacetyl and 3-methylbutanol formation by *Enterobacteriaceae, Brochothrix thermosphacta* and homofermentative *Lactobacillus* spp. (Borch *et al*., 1996).

Enterobacteriaceae, Pseudomonas and *Brochothrix thermosphacta* are mainly responsible for deterioration, *Lactobacillus* and *Brochothrix* for acidification and *Shewanella putrefaciens* and *Aeromonas hydrophila* for greening (Merck, undated). Green sulphmyoglobin is formed from a reaction between myoglobin and hydrogen sulphide produced by the bacteria. Cysteine can be converted to hydrogen sulphide when glucose sources are limited. *Lactobacillus sakei* produces hydrogen peroxide when glucose and oxygen are no longer available. Although greening is usually associated with high-pH meat, it may also occur in normal pH meat (Borch *et al*., 1996).

B. FOOD BORNE DISEASES

Food borne diseases consume a substantial amount of health care resources and these diseases cause considerable mortality throughout the world. In 2000, Panisello and co-workers published data on food borne disease outbreaks in England and Wales for the period 1992 to 1996 with red meat being responsible for 18.7% of the outbreaks. Poultry (18.5%), seafood (15.7%) and desserts (15.7%) were also some of the more regular causes of food poisoning. In the case of red meat, *Clostridium perfringens* (44.9%), *Salmonella* spp. (38.8%), *Staphylococcus aureus* (4.1%), *Campylobacter* spp. (1.0%) and *Escherichia coli* 0157:H7 (5.1%) were the main organisms associated with food poisoning. Factors that contributed to outbreaks of food poisoning in red meat included improper heating and reheating, inadequate storage and thawing, and preparation long before consumption. Food handling and cross contamination also played a major role, while insufficient hygiene and inadequate facilities played less important roles (Panisello *et al.*, 2000).

There has been a rapid increase in food poisoning associated with bacteria such as *Escherichia coli* 0157:H7 and *Listeria monocytogenes* (Abee *et al*., 1995). *Escherichia coli* 0157:H7 presents a serious risk because of its low infectious level and the fact that it is highly adapted to acidic conditions (Työppönen *et al*., 2003). *Escherichia coli* 0157:H7 also produces a toxin during growth and reproduction in the human gastrointestinal tract. The majority of outbreaks are associated with the consumption of ground meat (Abee *et al*., 1995).

Listeria monocytogenes is a psychrotrophic food borne pathogen. It grows rapidly at refrigeration temperatures and is normally associated with dairy, poultry and meat products (Abee *et al*., 1995). *Listeria monocytogenes* is an invasive Gram-positive

and non-sporulating food pathogen. It is especially dangerous to young children, pregnant woman, immuno-compromised individuals and the elderly (Työppönen *et al*., 2003).

One of the most common food borne illnesses is caused by *Staphylococcus aureus*. It rarely causes death but the symptoms are severe and are usually associated with dairy and meat products. Food poisoning results from a toxin (0.2 to 1.0 μg) present in contaminated food. *Staphylococcus aureus* must be present at about 1×10^6 per g or higher levels to be able to produce this amount of toxin (Kennedy *et al*., 2000).

To minimize the health risk of potential pathogens and spoilage micoorganisms, it is currently recommended that the HACCP (Hazard Analysis Critical Control Point) principal is incorporated during the production of foods (Kennedy *et al*., 2000). Along with the improvement of the microbiological quality of food, good hygiene practice (GHP), good manufacturing practice (GMP) and HACCP, it is also important to prevent recontamination of food (Panisello *et al.*, 2000). The implementation of HACCP is unfortunately not enough for the extension of the shelf-life of products. The National Food Processor Association (NFPA) in the USA recommended the incorporation of a suitable food preservative into the product (Kennedy *et al*., 2000). For a compound to be considered a successful preservative, it must be effective in small quantities and have a broad spectrum of antimicrobial activity, but it should not lower the quality of the food and it should be safe to the consumer (Kennedy *et al*., 2000).

C. PRESERVATIVES

As a consequence of market globalisation, manufacturers of meat products are facing new daily challenges. Food service customers demand high quality and convenient meat products, with natural flavours as well as a relatively long shelf-life, but prefer the appearance of minimally processed food. To accommodate the demands of the consumer without compromising the safety of the meat product, new preservation technologies in the meat and food industry are needed (Hugas *et al*., 2002; Ross *et al*., 2002).

According to the Foodstuffs, Cosmetics and Disinfectants Act and Regulations 54/1972 in the Republic of South Africa, a preservative is any substance which inhibits, retards of arrests fermentation, acidification or other decomposition of foodstuffs, but does not include preservatives such as common salt (NaCl), sugar (sucrose), lactic acid, vinegar, alcohol or portable spirits, herbs, hop extract, spices and essential oils (Anon,

1972). Artificial chemical preservatives are currently employed to limit the number of micro-organisms capable of growing in foods (Abee *et al*., 1995). Existing preservatives include sulphites, sulphur dioxide, sodium chloride, phosphates, hydrogen peroxide, nitrates (NO_3), nitrites (NO_2), Na-diacetate, β-propiolactone, benzoic acid and benzoates, sorbic acid and sorbates, acetic acid and acetate salts, lactic acid, propionic acid, fumaric acid, citric acid, parabens and therapeutic antibiotics (Magnuson, 1997; Kennedy *et al*., 2000). The use of some of these chemical preservatives is being questioned with regard to their effect on human health (Kennedy *et al*., 2000).

The Foodstuffs, Cosmetics and Disinfectants Act and Regulations 54/1972, states that pimaricin may be used at 6 $mg.kg^{-1}$ or $mg.l^{-1}$; potassium and sodium nitrate at maximum 200 $mg.kg^{-1}$ or $mg.l^{-1}$; sorbic acid at 400 to 2000 $mg.kg^{-1}$ or $mg.l^{-1}$ depending on the product; benzoic acid at 750 $mg.kg^{-1}$ or $mg.l^{-1}$; and sulphur dioxide at 450 $mg.kg^{-1}$ or $mg.l^{-1}$ in meat products, including biltong; canned chopped meat; canned corned meat; cold, smoked, manufactured sausages; cooked, cured hams; cooked cured luncheon meat; cooked cured pork shoulder; frozen meat pie fillings; meat pastries, frozen, raw; manufactured meat products; processed meat products; and sausages and sausage meat (Anon, 1972).

The growth of many food spoilage bacteria and potential pathogens on meat are inhibited, or at least delayed, by the addition of salt, as it decreases water activity. Nitrous acid (HNO_2), the undissociated form of nitrite (NO_2), is able to pass through the bacterial cell membrane, which acts as an ion barrier. The presence of the HNO_2 disturbs the function of the bacterial enzymes and therefore also bacterial growth (Työppönen *et al*., 2003). Nitrite is also used as a colour enhancer in cured meat, poultry and fish products. Nitrates react with amines, ever-present in nature (food and biological systems) and substituted amides to form nitrosamines and nitrosamides, which are carcinogenic. Nitrites are still used as a food additive as the health effects from a food illness such as botulism (caused by *Clostridium botulinum*) are a far greater risk than the development of cancer from the small amounts of nitrites allowed in food. In some cases antioxidants (sodium ascorbate or sodium erythorbate) are added to inhibit the formation of nitrosamines and nitrosamides (Magnuson, 1997).

During the last century, several alternative or complementary preservation technologies to classical processing were developed. For example, gamma irradiation has been employed to improve the safety of fresh meat by reducing or eliminating food borne pathogens. The shelf-life of the meat at refrigeration temperatures is thus extended without detrimental effects on quality (Murano, 1995). A dosage of between 1.5 and 4.5 kGy is recommended for the irradiation of red meat in the U.S. (Food and Drug Administration, 1994). In a study done by Lebepe *et al*. (1990), the shelf-life of

vacuum packed pork loins were extended from 41 d at refrigeration temperatures to 90 d, after an irradiation dosage of 3 kGy (Hugas *et al*., 2002). There are several other examples of these mild preservation techniques that have good potential in the meat industry and include high pressure processing (HPP), controlled instantaneous decompression (DIC), oscillating magnetic fields (ohmic heating, dielectric heating, microwaves), high intensity pulsed light, X-rays and electron beams. However, the consumer acceptability for this kind of preservation method is low (Hugas *et al*., 2002).

Food suppliers also need to consider the use of more natural preservative alternatives such as "green technologies" and bio-preservation. Researchers have examined naturally occurring metabolites produced by lactic acid bacteria to inhibit the growth of undesirable micro-organisms (Abee *et al*., 1995; Kennedy *et al*., 2000; Ross *et al*., 2002). These "natural" preservatives can be used in a wide variety of foods (Abee *et al.*, 1995). The use of lactic acid bacteria and/or their metabolites may provide the suitable answer to this problem as the "natural" and "health-promoting" compounds are more acceptable to consumers (Montville & Winkowski, 1997). The bio-preservatives can either be used directly in the food in its purified and concentrated form as a food additive on its own or in combination with other preservatives (Abee *et al*., 1995; Ross *et al*., 2002). Certain strains that produce antimicrobial metabolites can even be incorporated into the starter culture for fermented foods or as protective cultures in non-fermented food (Ross *et al*., 2002).

Lactic acid bacteria produce a range of metabolic inhibitors, including organic acids, diacetyl, CO_2, hydrogen peroxide and even antibiotics. These inhibitors suppress the growth and survival of undesirable food spoilage and pathogenic micro-organisms in the foodstuffs. In addition to these antimicrobial compounds, these organisms are able to produce a wide range of antimicrobial peptides or bacteriocins (Abee *et al*., 1995; Ross *et al*., 2002; Magnusson *et al*., 2003).

D. BACTERIOCINS

Bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial proteins, peptides or peptide complexes that vary in activity spectrum, mode of action, molecular weight, genetic origin and biochemical properties (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002). These bio-active peptides are extracellularly released and are inhibitory or lethal to other genetically related bacteria, but also bacteria confined within the same ecological niche (Earnshaw, 1992; De Vuyst & Vandamme,

1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002). Some Gram-negative bacteria also become bacteriocin sensitive when subjected to chelating agents, hydrostatic pressure or other forms of cell injury (Montville & Winkowski, 1997). The producer strain is usually immune to its own bacteriocin (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002).

Most bacteriocins are small (3 to 10 kDa), with a high iso-electric point as well as hydrophobic and hydrophilic domains, but with different spectra of activity, biochemical characteristics and genetic determinants (Montville *et al.*, 1995; Montville & Winkowski, 1997; Cleveland *et al*., 2001). Bacteriocins are able to inhibit spoilage and pathogenic bacteria without changing the physico-chemical nature of the food, as observed by acidification, protein denaturation etc. (Montville & Winkowski, 1997).

The first bacteriocin-like substance was described in 1925 when Gratia noticed between inhibition two strains of *E. coli* (De Vuyst & Vandamme, 1994a; Frédericq, 1948). The antimicrobial substances produced by *E. coli* were named colicins (De Vuyst & Vandamme, 1994a; Frédericq, 1948). Colicins are a diverse group of antibacterial proteins, which kill closely related bacteria by inhibition of cell wall synthesis, permeabilising the target cell membrane or by inhibiting RNase or DNase activity (Cleveland *et al*., 2001; De Vuyst & Vandamme, 1994a). Later, in 1928, Rogers and Whittier resorted that Gram-positive bacteria also produce these 'colicin-like' substances. They observed the inhibitory effect that some lactococcal strains had on the growth of other lactic acid bacteria and proposed the name 'bacteriocins' (Cleveland *et al*., 2001; De Vuyst & Vandamme, 1994a; Rodgers & Whittier, 1928). Similar inhibition of cheese starter cultures was observed and the compounds were isolated and identified by Whitehead (1933), who found that the active antimicrobial was proteinaceous in nature and named the bacteriocin nisin (group N inhibitory substance) to indicate that it is produced by lactic streptococci of the serological group N (Mattick & Hirsch, 1947; Ross *et al*., 2002).

Bacteriocins produced by lactic acid bacteria, in particular, are attracting increasing attention as preservatives in the food processing industry to control undesirable spoilage organisms and food borne pathogens (Abee *et al*., 1995; Montville & Winkowski, 1997). Before bacteriocins can be used as a food preservative, it is important to know its origin, mode of action and genetics behind its preservative action.

Classification

Bacteriocins produced by lactic acid bacteria can be divided into three (De Vuyst & Vandamme, 1994b) or four groups (Klaenhammer, 1993). Class I bacteriocins, or lantibiotics, are small (< 5 kDa) heat-stable peptides (De Vuyst & Vandamme, 1994b; Cleveland *et al*., 2001) that are post-translocationally modified and have a broad host range (O'Keeffe & Hill, 1999). These bacteriocins typically have 19 to more than 50 amino acids and are characterized by the presence of unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. The Class I bacteriocins are further subdivided into Class Ia and Class Ib. Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid Class Ib. Class Ib bacteriocins, which include mersacidin, are globular peptides that have no net charge (Cleveland *et al*., 2001).

Class II bacteriocins are small (<15 kDa), heat-stable, unmodified peptides, which can be subdivided into Class IIa, IIb, IIc (Klaenhammer, 1993; De Vuyst & Vandamme, 1994b; O'Keeffe & Hill, 1999; Cleveland *et al*., 2001). A class IIa bacteriocin is synthesized in a form of a precursor that is processed after two glycine residues (active against *Listeria*) have a consensus of Tyr-Gly-Asn-Gly-Val-C in the N-terminal. Class IIa bacteriocins have two cysteines forming an S-S bond in the N terminal half of the peptide. This class includes pediocin-like *Listeria* active peptides, such as pediocin PA-1, sakacins A and P, leucocin A and carnobacteriocins (Klaenhammer, 1993; Cleveland *et al*., 2001).

The Class IIb bacteriocins are composed of two different peptides. Both of the peptides are necessary to form an active poration complex. The primary amino acid sequences of the peptides are different. Only one immunity gene is needed, though each is encoded by their own adjacent genes. Class IIb bacteriocins include lactococcins G and F, lactacin F and plantaricins EF and JK (Klaenhammer, 1993; Cleveland *et al*., 2001). Class IIc includes thiol-activated peptides that require reduced cysteine residues for activity (Klaenhammer, 1993).

Class III bacteriocins are large (>15 kDa), heat-labile peptides (Klaenhammer, 1993; De Vuyst & Vandamme, 1994a & b; O'Keeffe & Hill, 1999; Cleveland *et al*., 2001). This class includes helviticins J and V-1829, acidophilucin A, lactocin A and B and caseicin 80 (Klaenhammer, 1993; O'Keeffe & Hill, 1999; Cleveland *et al*., 2001).

Klaenhammer (1993) also reported the presence of a fourth class which includes complex bacteriocins, composed of protein and one or more chemical moieties such as lipids and carbohydrates. Class IV includes the bacteriocins plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1 (Klaenhammer, 1993).

Mode of Action

The potential application of bacteriocins produced by lactic acid bacteria as food preservatives requires a detailed knowledge of their bactericidal mode of action with the cell membrane as main target (Montville & Winkowski, 1997). Most of the bacteriocins produced by lactic acid bacteria appear to have the same mechanism of action, namely depleting the proton motive force (PMF) in the target cells by the formation of pores in the phospholipids bilayer of the cell membrane (Abee, 1995; O'Keeffe & Hill, 1999). These pores alter the membrane permeability, thus disturbing membrane transport and resulting in the uncontrolled efflux of ATP, amino acids and essential ions (Mg^{++} and K^{+}) (De Vuyst & Vandamme, 1994 b; Abee, 1995; O'Keeffe & Hill, 1999). This uncontrolled flow of substances in and out of the cell subsequently inhibits the energy production and biosynthesis of proteins or nucleic acids (De Vuyst & Vandamme, 1994 b). Some bacteriocins, like the colicins produced by Gram-negative *E. coli*, not only target the cell membrane, but also inhibit protein synthesis, degrade RNA or have other biological functions (Montville & Winkowski, 1997).

The mechanism through which pore formation, membrane destabilisation and ultimately, cell death is achieved appears to differ as shown by ultra structural studies done by Jack and co-workers (1995) on treated cells. In 1976, Tagg and co-workers proposed that bacteriocins adsorb to specific or non-specific receptors on the cell surface subsequently resulting in cell death.

Bacteriocins produced by lactic acid bacteria have a bactericidal effect on sensitive cells, but some have been reported to act bacteriostatically. This is mainly dependent on the number of arbitrary units, the buffer or broth used, the purity of the bacteriocin, the indicator species as well as the cell concentration used (De Vuyst & Vandamme, 1994b). Bacteriocins do not act equally against all target species. The phospholipid composition in the cell membranes of the target cells as well as the environmental pH has an influence on the minimal inhibitory concentration (MIC) required (Cleveland *et al.*, 2001).

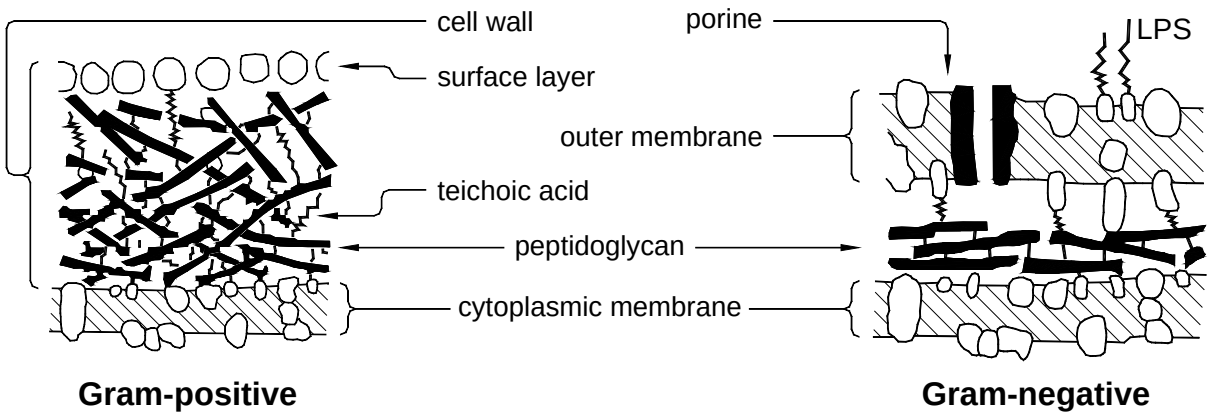

Figure 1. Schematic representation of the cell membrane of Gram-positive and Gram-negative bacteria (adapted from Abee *et al*., 1995).
LPS - lipopolysaccharides.

In both Gram-positive and Gram-negative bacteria, the cytoplasmic membrane forms a border between the cytoplasm and the external environment and is surrounded by a layer of peptidoglycan. In Gram-negative bacteria the peptidoglycan layer is significantly thinner than in Gram-positive bacteria, but the Gram-negative bacteria possesses an additional layer that is called the outer membrane, as illustrated in Fig 1 by Abee and co-workers (1995). The latter layer is composed of phospholipids, proteins and lipopolysaccharides (LPS). The outer membrane is impermeable to most molecules, but free diffusion of small molecules (< 600 Da) takes place through the pores. The smallest bacteriocins produced by lactic acid bacteria (3 kDa) are thus too large to pass through the outer membrane and reach the cytoplasmic membrane, their primary target (Abee *et al.,* 1995).

Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, associates with non-energized liposomes. According to Abee and co-workers (1995) the initial association of this positively charged peptide is charge dependent as the greatest interaction is with the negatively charged phospholipids. The association of the bacteriocins with the liposomes cause the formation of ion-permeable channels in the cytoplasmic membrane of sensitive cells, which increases the membrane permeability (De Vuyst & Vandamme, 1994b; Abee *et al.,* 1995). The type of pore formed by nisin is debatable. The “barrel-stave” or the “wedge” models for pore formation are mainly accepted (Cleveland *et al.*, 2001). An ion channel, that spans the membrane, is formed

as each nisin molecule orientates itself upright to the citoplasmic membrane of susceptible cells in the “barrel-stave” model (Ojcius & Young, 1991). In the “wedge” model, a critical number of nisin molecules associate with the membrane, insert simultaneously and form a wedge (Driessen *et al.*, 1995). A third model implies what appears to be a “docking molecule” on the target membrane that facilitates the interaction with the bacteriocin, thus increasing the effectiveness of the bacteriocin as demonstrated for nisin and mersacidin (Brotz *et al.*, 1998a & b; Breukink *et al.*, 1999).

Nisin A forms transient multistate pores of about 0.2 to 1.2 nm in diameter, which allows the passage of hydrophilic solutes with molecular masses up to 0.5 kDa. The membrane potential dissipates causing an efflux of ATP, amino acids and essential ions (potassium and magnesium). Finally, cell death is caused by the inhibition of energy production and biosynthesis of macromolecules (DNA, RNA, protein and polysaccharides). Nisin is dependent on the phospholipids composition of the membrane and does not require a membrane receptor but an energised membrane for its activity (De Vuyst & Vandamme, 1994b; Abee *et al.,* 1995).

Pep5, the lantibiotic produced by *Staphylococcus epidermidis* 5 (Sahl & Brandis, 1981), like nisin, also has a concentration-dependent mode of action which is affected by physiological conditions such as ionic strength, temperature, pH and growth phase of the target organism (Jack *et al.*, 1995). Cell death is caused by the inhibition of energy production and biosynthesis of macromolecules, similar to the case of nisin. Parallel findings have been reported for other lantibiotics, including SA-FF22 produced by *Streptococcus pyogenes* FF22 (Tagg & Wannamaker, 1978; Jack *et al.*, 1995) and epidermin produced by *Staphylococcus epidermidis* Tü3298 (Augustin *et al*., 1992; Jack *et al*., 1995).

Pediocin PA-1, produced by *Pediococcus pentosaceus*, consists of 44 amino acids, is highly hydrophobic and positively charged (Abee *et al.,* 1995). It acts on the cytoplasmic membrane and dissipates the ion gradients, thus inhibiting the transport of amino acids in the sensitive cell. The liposomes of the cell are not affected. The two disulphide bonds in positions 24 and 44 are essential for activity (Abee *et al.,* 1995). Pediocin PA-1 forms hydrophilic pores in the cytoplasmic membrane in a protein receptor mediated, voltage-independent manner, similar to the action of lactococcin A (Van Belkum *et al.*, 1991; Abee *et al.,* 1995).

Lactococcin A is produced by *Lactococcus lactis* and is a small 54 amino acid hydrophobic peptide that inhibits the growth of other *L. lactis* subspp. specifically. Lactococcin A recognises a *Lactococcus*-specific membrane receptor protein, which may be involved in the formation of pores (Van Belkum *et al.*, 1991). Bacteriocins like

lactococcin G, which consists of two peptides, do not affect the pH gradient over the cell membrane, but cause the dissipation of monovalent cations (Moll *et al.*, 1998).

Genetic organisation

The genetic information encoding the production of bacteriocins (*Bac*$^+$) and immunity is located on chromosomes, plasmids or both (Montville & Winkowski, 1997). Phenotypic and physical evidence, as well as genetic confirmation is needed to indicate if bacteriocin production is plasmid mediated (Montville & Winkowski, 1997). Lactococcins are an example of plasmid mediated bacteriocins. Some of the *Bac*$^+$ lactococcal strains, easily loose their ability to produce bacteriocins and become sensitive to their own bacteriocin (Montville & Winkowski, 1997). This indicates instability in the *Bac*$^+$ phenotype. Some of the *Bac*$^+$ strains are able to transfer the *Bac*$^+$ trait to a plasmid-free (*Bac*$^-$) recipient cell (Neve *et al.*, 1984), which includes potential pathogens and food spoilage bacteria. This may render immunity to the bacteriocin. In addition to the danger of transferring the *Bac*$^+$ characteristic to a potential pathogen, the *Bac*$^+$ trait may be "lost" (Montville & Winkowski, 1997). This may hamper the production of bacteriocins. Pediocin A, pediocin PA-1, sakacin A, lactocin S and carnobacteriocins A and B are plasmid-encoded (Montville & Winkowski, 1997). In many cases, bacteriocin production is correlated with the presence of a plasmid, but genes encoding several class IIa bacteriocins are located on the chromosome (Ennahar *et al.*, 2000).

Nisin was initially reported to be plasmid mediated, but there was no phenotypic and physical evidence or genetic confirmation. Nucleic acid hybridisation techniques indicated that the nisin structural gene was located on the chromosome of *Lactococcus lactis* (Montville & Winkowski, 1997). The nisin-producing trait is thus relatively stable and is less likely to be transferred to another microbe. The nisin gene resides within a 70-kb conjugative transposon and is genetically linked to the genes encoding sucrose metabolism. Helviticin J and lactacin B are also examples of chromosomally encoded lactic acid bacteria bacteriocins (Montville & Winkowski, 1997).

According to Montville & Winkowski (1997) the 'structural genes' for many bacteriocins seem to be located in an operon-like structure. A prepeptide is usually coded for by the structural gene (Montville & Winkowski, 1997). The prepeptide comprises of the precursor of the mature bacteriocin and is preceded by an N-terminal extension ("leader sequence"). The secondary structure of the N-terminal is α-helical. During maturation or export, this structure is cleaved. The role of the N-terminal extension is still unknown, but it may be involved in neutralising the bacteriocin activity within the cell to protect the producer strain (Montville & Winkowski, 1997).

The 'immunity gene' renders immunity of *Bac*+ cells to their own bacteriocins. Immunity seems to be co-ordinated with bacteriocin production and is rather specific. The immunity gene, for most non-lantibiotics, codes for a single polypeptide, which is located in the vicinity of, and in the same operon as the structural bacteriocin gene. The proteins involved in immunity ranges from 52 to 254 amino acids in size and are cationic (Nes & Holo, 2000). The 'processing and export genes' are responsible for the formation of a mature bacteriocin and its export from the cytoplasm. The 'regulatory genes' encode proteins that are homologues to the proteins of the two-component regulatory system. Histidine kinase (located in the membrane) senses an external signal and transduces it to the cell's interior by the phosphorylation of a second cytoplasmic protein (response regulator). This activates the biosynthesis of bacteriocins (Montville & Winkowski, 1997).

E. BACTERIOCINS AS FOOD PRESERVATIVES

A basic requirement for the development of a stable urban society is the ability to preserve food in a state that is both appetising and nutritious. It is both the food processors and retailer's responsibility to supply safe food to the customer (Kennedy *et al*., 2000).

Bacteriocins are considered natural bio-preservatives since they are proteins, readily degraded by proteases in the human gastrointestinal tract (Aymerich, 2000). Most bacteriocin-producing lactic acid bacteria have GRAS (generally regarded as safe) status (Vandenbergh, 1993; Abee *et al*., 1995; Schillinger *et al*., 2001).

Most of the bacteriocins produced by food-associated lactic acid bacteria have been explored and isolated, but it does not mean that they are effective in all food systems (Cleveland *et al*., 2001). Several bacteriocins have the potential to be applied as food preservatives when used under correct conditions, but it is recommended that they should rather be used as part of a multiple hurdle preservation system (Cleveland *et al*., 2001). The antimicrobial activity range and potency of bacteriocins can be increased dramatically when used in combination with stress factors, including pH, temperature and other preservatives (Stevens *et al*., 1991; Zhang & Mustapha, 1999; Nilsson *et al*., 2000). Helander and Mattila-Sandholm (2000) suggested the use of food grade permeabilisers such as lactic or citric acid in combination with bacteriocins as part of the hurdle concept in inhibiting Gram-negative food spoilage and pathogenic bacteria (Työppönen *et al*., 2003).

There are several applications for the use of bacteriocins in foodstuffs. Food spoilage and pathogenic organisms can be inhibited by directly adding the bacteriocin to food. Currently, products such as Danisco Nisaplin® Natural Antimicrobial (Anon, 2004a), Microgard (skim milk fermented using *Propionibacterium freudenreichii* subsp. *shermanii*) (Daeschel, 1989; Faye *et al*., 2000) and Microgard 200 (dextrose cultured with food grade dairy cultures) (Anon, 2004b) are available for this purpose. Bacteriocinogenic cultures are also used to control spoilage or pathogenic organisms. These bacteriocinogenic cultures can be added to food as a protective culture or used as a starter culture in fermented foods. The use of defined bacteriocin-producing strains as starter cultures has several advantages over indigenous strains as the quality and consistency of the fermented product is improved (Stevens *et al*., 1991; Zhang & Mustapha, 1999; Nilsson *et al*., 2000). When bacteriocinogenic cultures are used as starter cultures in food production, it is important that the amount of bacteriocin formed by the starter or protective culture is enough to ensure the desired preserving effect (Gänzle *et al.*, 1999; Cleveland *et al*., 2001). The bacteriocins produced may bind to the fat and/or protein present in the food and the food additives, and there are natural proteases or other inhibitors that will possibly inactivate them (Leroy & De Vuyst, 1999; Työppönen *et al*., 2003).

In many food products the concentration of *in situ* production of bacteriocins by lactic acid bacteria may be affected by the food composition, the storage temperature, the salt contents or the pH or a combination of these factors (Työppönen *et al*., 2003). In food matrices, the bacteriocin activity may be affected by the changes in solubility and the charge of the bacteriocin, the binding of the bacteriocin to the food components, the inactivation of the bacteriocin by proteases and changes in the cell envelope of the target organisms as a response to the environmental factors. The chemical composition as well as the physical conditions of food can have a significant influence on the bacteriocidic activity of the bacteriocins (Gänzle *et al.*, 1999; Cleveland *et al*., 2001). Although the bacteriocins are effective in inhibiting the target organisms in broth systems, it is important to confirm the effectiveness with applied studies in food systems.

There are a number of patented applications of bacteriocins in foodstuffs. Blackburn *et al.* (1998) patented the use of a combination of nisin, a chelating agent and a surfactant, as a food preservative, to inhibit both Gram-positive and Gram-negative micro-organisms in meat, eggs, cheese and fish. Wilhoit (1996) used *Streptococcus*-derived and *Pediococcus*-derived bacteriocins in combination with a chelating agent to protect food against *Listeria*. The number of *Listeria monocytogenes* in Manchengo cheese inoculated with a bacteriocin-producing strain of *Enterococcus faecalis* decreased by six log-cycles in only 7 d. The survival of *L. monocytogenes* in cheese

made with the commercial starter cultures were not affected (Nuñez *et al.*, 1997). When the bacteriocin producer *Lb. plantarum* was inoculated into a naturally contaminated salami sausage, the number of surviving *Listeria monocytogenes* decreased (Campanini *et al.*, 1993). In 1995, Vedamuthu patented a yoghurt product with increased shelf-life containing a bacteriocin derived from *Pediococcus acidilactici*. The plasmid-encoding pediocin expressed in *Lactococcus lactis,* was used as a starter culture for the production of cheddar cheese. This was done to aid the preservation of cheese and to ensure the microbial quality of the fermentation process (Buyong *et al.*, 1998). Pediocin PA-1 was also expressed in "*Streptococcus thermophilus"*, which is an important starter culture of dairy products (Coderre & Somkuti, 1999).

In meat

When high hydrostatic pressure (HHP) (600 MPa for 10 min at 30 C) is combined with antimicrobials, like bacteriocins, the death rate of the spoilage or pathogenic microbe can be increased because of sub lethal injuries to living cells. This treatment is able to extend the shelf-life of the marinated beef loin by controlling the growth of both spoilage and pathogenic bacteria, including *Salmonella* spp. and *Listeria monocytogenes*. HHP is a non-thermal process for meat products to avoid post-processing contamination. Both the physico-chemical and microbiological characteristics of cooked ham, dry cured ham and marinated beef loin, vacuum-packed and high pressure treated was substantially equivalent to the same untreated products (Hugas *et al*., 2002).

In a study done by Roller *et al*. (2002), carnocin (produced by *Carnobacterium piscicola*) was used in combination with chitosan and sulphite to preserve pork sausages. Carnocin did not protect the sausage from spoilage, but reduced the number of *Listeria innocua* by 2 log cfu.g^{-1} within the first five days of chill-storage (Roller *et al*., 2002).

The incorporation of bacteriocins into edible films and other forms of packaging is a very interesting and promising field. Sebti and Coma (2002) incorporated nisin into an edible hydroxy propyl methyl cellulose (HPMC) film instead of applying it to the product by spraying. This HPMC antimicrobial film was effective in controlling the growth of *Listeria monocytogenes* (*L. innocua*) and *Staphylococcus aureus*, but the water vapour barrier properties of the antimicrobial film were unsatisfactory because of the hydrophilic nature of cellulose. The water vapour barrier properties were improved by adding stearic acid, but this caused a decrease in the antimicrobial properties, because of the electrostatic interaction between stearic acid and nisin. When calcium ions were included, the antimicrobial properties improved (Sebti & Coma, 2002).

Some bacteriocin-producing strains of lactic acid bacteria have been applied as protective cultures in a variety of food products without a major change in physical and sensory properties, as in the case of fermented products. Especially in meat, where the modification of the product is undesirable, the use of homofermentative, mildly acidifying bacteriocinogenic lactic acid bacteria is ideal for bio-preservation. In some cases, relatively high numbers of these bacteriocin producers are needed to inhibit pathogens. The bacteriocin producers should be selected in such a way that it would not affect the products' taste and appearance negatively. The incorporation of purified bacteriocins can overcome this problem. The incorporation of other inhibiting factors at low levels can assist the bacteriocins in preventing the growth of bacteriocin-resistant pathogens (Abee *et al*., 1995).

Specific Bacteriocins

Microgard

Microgard is an antimicrobial agent produced from the fermentation of skim milk with *Propionibacterium freudenreichii* subsp. *shermanii* (Daeschel, 1989; Faye *et al*., 2000). Microgard 200 (cultured dextrose) is manufactured by growing food grade dairy cultures on dextrose. These cultures are then pasteurised, dried and converted into powder with maltodextrin as carrier. This antimicrobial agent is active against Gram-negative bacteria, as well as some yeasts and moulds and can be used at average levels ranging from 0.5% to 1.0% depending on the amount of water and the pH of the product. Microgard 200 is used to preserve refrigerated salad dressings, dips, sauces, salsas, fresh soups, fruit juices and pastas (Anon, 2004b).

Nisin

Nisin, probably the best-studied bacteriocin, is produced by *Lactococcus lactis* and was first marketed in England in 1953. It has since been approved for use as a food preservative in over 48 countries (Cleveland *et al*., 2001; Ross *et al*., 2002). In 1968, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives assessed nisin to be biologically safe (Ross *et al*., 2002; Ryan *et al*., 2002). Nisin was accepted as a food additive in processed cheese (12.5 mg pure nisin per kilogram product) by the FAO/WHO Codex Committee on milk and milk products. The EEC (Eastern European Community) (1983) added this bio-preservative to the food additive list (number E234). Although nisin is currently still restricted for use to prevent clostridial growth in processed cheese, cheese spreads and dairy desserts, its potential use in the food and bio-medical industry continues to grow (Ross *et al*., 2002; Ryan *et al*., 2002). Nisin was the first bacteriocin that received GRAS status (FDA, 1988;

Ross *et al*., 2002; Van Reenen *et al.*, 2002) and although it is currently the only bacteriocin legally approved for use in foodstuffs in the United States, many bacteriocins produced by other lactic acid bacteria have potential application as food preservatives (Gänzle *et al.*, 1999; Cleveland *et al*., 2001).

Nisaplin® Natural Antimicrobial (Danisco) contains approximately 2.5% nisin (Anon, 2004a). The primary applications for Nisaplin® include dairy products (processed cheese and cheese spreads, direct acidified cheeses, pasteurised dairy desserts and fresh and recombined milk), liquid egg, dressings and sauces, high moisture/reduced fat foods (baby foods), canned foods, crumpets and the processing of fermentation products (Montville & Winkowski, 1997; Anon, 2004a). Nisaplin® is manufactured by a controlled fermentation of *Lactococcus lactis* which produces nisin. The nisin is concentrated, separated and spray-dried, and then milled into fine particles and standardised by the addition of NaCl to a typical composition of 2.5% nisin, 77.5% NaCl, 12% protein, 6% carbohydrate and 2% moisture (Anon, 2004a).

Nisaplin® inhibits a broad range of Gram-positive bacteria, including clostridia, *Bacillus*, *Listeria* and lactic acid bacteria (Anon, 2004a). Although nisin has been used to inhibit listerial growth and biofilm formation when absorbed onto surfaces, *Clostridium botulinum* is considered as one of the main targets. Nisin causes botulinal spores to be more sensitive to heat treatment. About 1 000 $IU.ml^{-1}$ nisin is needed to reduce botulinal spores by 6 logs (Montville & Winkowski, 1997). The vegetative cells of *Listeria monocytogenes* are much more sensitive to nisin and ca. 200 $IU.ml^{-1}$ is needed to cause a 6-log reduction in vegetative cells (Montville & Winkowski, 1997).

The use of nisin in meat is probably one of the best-studied applications for bacteriocins in food. Generally nitrates are used to prevent the growth of *Clostridium botulinum* in cured meat, but the presence of nitrites, formed from the nitrates, raises concern in terms of meat safety. This encouraged the food industry to look at alternative methods of preservation (Cleveland *et al*., 2001; O'Keeffe & Hill, 1999). Abee *et al*. (1995) used Nisin A in the development of a nitrite-free meat curing system, as nitrite is eventually converted into carcinogenic N-nitrosamines (Abee *et al*., 1995). High levels of nisin are required when used on its own (Cleveland *et al*., 2001; O'Keeffe & Hill, 1999) and the bacteriocin alone is not successful, but the use of 100 to 250 $mg.kg^{-1}$ combined with 120 $mg.kg^{-1}$ nitrite was more effective than 156 $mg.kg^{-1}$ nitrite, used under normal conditions (Abee *et al*., 1995). This may be because it binds to the meat particles, is distributed unevenly, is poorly soluble in meat systems or because of interference by phospholipids. Nisin in combination with lower levels of nitrate is effective in preventing the growth of *Clostridium* (Cleveland *et al*., 2001; O'Keeffe & Hill, 1999). In most applications, nisin serves as part of a multiple barrier inhibitory system, such as

combining it with modified atmospheric storage, thermal stress and pH to increase its preserving effectiveness (Montville & Winkowski, 1997).

Abee *et al.* (1995) also transformed the transposon encoding nisin production and immunity into a commercial *Lactococcus lactis* starter culture, used for the production of Gouda cheese. Wessels *et al.* (1998) added bacteriocins such as nisin directly to cheese to prevent the growth of *Clostridium* and *Listeria*. Nisin inhibited the germination of *Clostridium botulinum* spores in cheese spreads (Abee *et al*., 1995).

Pediocin

Pediocin is produced by *Pediococcus pentosaceus* and is mostly inactive against spores, but has been shown to inhibit *Listeria monocytogenes* (Montville & Winkowski, 1997). In Europe, pediocin is used in the form of a dried powder or in a culture liquid to extend the shelf-life of salads and salad dressings, and to serve as an anti-listerial agent in products such as cream, cottage cheese and meats. In a meat system, and in some cases even in dairy products, pediocin is more effective than nisin (Montville & Winkowski, 1997).

Pediocin as purified bacteriocin, as well as viable cells of *Pediococcus*, can function as a bio-preservative to eliminate Gram-positive bacteria in fully cooked, cured meats like Wiener sausage and ground beef (Montville & Winkowski, 1997). Pediocin AcH is more effective against *L. monocytogenes* at 4ºC than at 25ºC. The effectiveness of pediocin is also improved when used together with emulsifiers such as Tween 80 (Montville & Winkowski, 1997).

Sakacin

Lactobacillus sake Lb 674, isolated from meat at levels of 10^5 to 10^6 cells.g^{-1} produce detectable amounts of a bacteriocin in vacuum-packed sliced Bologna-type sausage stored at 7ºC. The bacteriocin delayed or completely inhibited the growth of *Listeria monocytogenes*. The application of sakacin 674 as a purified additive had a marked initial effect against *L. monocytogenes* and reduced listerial growth during storage (data not shown) (Abee *et al*., 1995).

F. BACTERIOCINOGENIC ACTION AGAINST MOULDS AND YEASTS

Moulds and yeasts cause huge economic losses to the food industry. Moulds produce allergenic spores and in some cases myco-toxins, making them serious health hazards. There is thus a great interest in developing efficient and safe strategies to reduce mould and yeast growth in both the food and animal feed industry. Bio-preservation received a

lot of attention in this context. Lactic acid bacteria produce a variety of different antimicrobial compounds and play an important role in the bio-preservation of food and feed (Magnusson *et al.*, 2003). Most of the preserving effect is against bacteria, but there have been a few reports of the antifungal effect of bacteriocin-like substances and other low molecular mass compounds produced by lactic acid bacteria (Niku-paavola *et al*., 1999; Okkers *et al*., 1999).

G. CONCLUSIONS

Bacteriocins are often confused with antibiotics, which would limit their use in food applications from a legal point of view. In some countries, it is very important to draw the distinction between bacteriocins and antibiotics. Antibiotics are synthesized as a secondary metabolite and its varying spectrum of activity are mainly clinically applied. Bacteriocins, on the other hand, are ribosomally synthesized and have a narrow spectrum of activity. Their main mode of action is pore formation in the cytoplasmic membrane, but in some cases the biosynthesis of cell walls are also under attack. In the case of antibiotics, the target for attack is the cell membrane or intracellular organelles. Currently there are no known side effects regarding the use of bacteriocins, but in the case of antibiotics, there are many reported cases of side effects and toxicity (Cleveland *et al*., 2001). Bacteriocins are clearly distinguishable from clinical antibiotics and should be safe and effective to control the growth of target pathogens in foods (Cleveland *et al*., 2001).

Bio-preservation is defined as the use of lactic acid bacteria, their metabolic products, or both, to improve the safety and quality of foods that are not fermented (Montville & Winkowski, 1997). Bacteriocins have been consumed unknowingly for centuries. They can be isolated from food, which normally contains lactic acid bacteria such as meat and dairy products as well as a wide rage of fermented products (Cleveland *et al*., 2001). The use of bacteriocins produced by probiotic bacteria are used by the marketing professionals in the food industry to turn the “negative” need to preserve food into a “positive” way of preservation that might improve the consumers health (Montville & Winkowski, 1997).

A niche for natural food preservatives has been created as there is an increasing demand for high-quality, safe, natural and minimally processed food. It is therefore expected that bacteriocins and bacteriocin-producing lactic acid bacteria (as starter cultures or protective cultures) will be used in both fermented and non-fermented foods to improve its quality and safety (O’Keeffe & Hill, 1999). Before the application of

bacteriocins to food, the cytolitic abilities, physical and chemical properties, mode of action and structure-function relationship must be studied carefully (Abee *et al*., 1995).

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CHAPTER 3

IMPACT OF SIX BACTERIOCINS ON MEAT SPOILAGE MICROBES

ABSTRACT

Red meat is perishable at refrigerated temperatures due to the proliferation of spoilage and potential pathogenic micro-organisms. Different isolation media was used to isolate micro-organisms from beef, lamb and pork, obtained from four commercial retailers. The number of viable cells per gram (cfu.g^{-1}) ranged from 80 cfu.g^{-1} to 1.4 × 10^{8} cfu.g^{-1} three days after the sell-by date at 4ºC. About 53% of the meat isolates were Gram-negative, 35% were Gram-positive and 12% were identified as yeast. The microbial population of the meat was not influenced by the type of meat (beef, lamb or pork), but rather by the origin, i.e. the retailer. Bacteriocins produced by *Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lactobacillus plantarum* 423, *Lactobacillus casei* LHS, *Lactobacillus salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 were screened for activity against the meat isolates. Between 16% and 21% of the isolates, identified as members of *Klebsiella*, *Shigella*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus* and *Bacillus*, as well as some yeast, were sensitive to the bacteriocins, suggesting that they may be used as preservatives.

INTRODUCTION

Red meat does not have a long shelf-life at refrigerated temperatures (0º - 4ºC). Beef has a shelf-life of approximately 10 to 14 d, lamb between 7 and 10 d and pork about 4 d. When packaged in an airtight and moisture proof container at -18ºC, the shelf-life of beef may be extended to about 10 months, lamb to about eight months and pork to between four and six months (Anon., 2002).

Meat spoilage generally occurs as a result of the proliferation of microbes at near freezing point (Merck, undated). A viable aerobic cell count of 1 × 10^{6} cfu.cm^{-2} (Merck, undated) or 1 × 10^{7} cfu.g^{-1} is an indication of spoilage (ICMSF, 1986).

Most of the micro-organisms present on the surface of meat are acquired during the dressing process (Merck, undated). The implementation of systems like HACCP (Hazard Analysis Critical Control Points) to minimize the risk of potential pathogens and

spoilage micro-organisms is not always sufficient to extend the shelf-life of fresh meat (Kennedy *et al*., 2000; Panisello *et al.*, 2000). In 2000, the NFPA (National Food Processor Association) in the USA recommended the incorporation of a suitable food preservative into meat products (Kennedy *et al*., 2000). For a compound to be considered a successful preservative, it must be effective in small quantities and have a broad spectrum of antimicrobial activity, but it should not alter the quality of the food and has to be safe for consumption (Kennedy *et al*., 2000).

Bacteriocins produced by lactic acid bacteria are attracting increasing attention as food preservatives to control undesirable spoilage organisms and food borne pathogens (Abee *et al*., 1995; Montville & Winkowski, 1997). Bacteriocins are antimicrobial proteins or peptides that inhibit bacteria closely related to the producer strain and bacteria confined within the same ecological niche (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; Van Reenen *et al*., 2002). Bacteriocins inhibit the growth of spoilage and pathogenic bacteria without changing the physico-chemical nature of the food, as found during acidification and protein denaturation (Montville & Winkowski, 1997).

Bacteriocins may be used as part of a multiple hurdle preservation system (Cleveland *et al*., 2001) or bacteriocinogenic cultures may be used to control spoilage organisms or the proliferation of pathogens. Bacteriocins may be added to non-fermented foods or ‘bacteriocin-producing’ bacteria may be used as starter cultures in fermented foods (Stevens *et al*., 1991; Zhang & Mustapha, 1999; Nilsson *et al*., 2000).

The application of a number of bacteriocins to foodstuffs has been patented. The applications include their addition to meat, eggs, cheese and fish (Blackburn *et al.*, 1998), salami (Campanini *et al.*, 1993), nitrite-free meat curing systems (Abee *et al*., 1995) and fully cooked, cured meats like Wiener sausage, as well as ground beef (Montville & Winkowski, 1997). Bacteriocin-producing starter cultures have also been used to ferment salami produced from ostrich meat (Böhme *et al.*, 1996; Dicks *et al*., 2004).

The aim of this study was to evaluate the sensitivity of bacteria, isolated from red meat obtained from four different commercial retailers, against bacteriocins produced by *Enterococcus feacalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lactobacillus plantarum* 423, *Lactobacillus casei* LHS, *Lactobacillus salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacteriocin-producing strains of lactic acid bacteria (LAB), their origin and growth conditions are listed in Table 1.

Isolation and quantification of micro-organisms from red meat

Samples of meat (loin chops of beef, lamb and pork) were collected from four different food chain stores in and around Stellenbosch. The samples were randomly selected and collected on non-specific times and dates. The meat was kept at 4ºC for three days past the sell-by date. Ten grams of each sample was then homogenised with 90 ml of sterile dilution liquid (0.1%, w/v, peptone; 0.85%, w/v, NaCl) and serially diluted. The dilutions were plated out and incubated on different isolation media (Table 2) and the number of viable cells determined. Pure cultures of all isolates were obtained by streaking on the isolation media. Cultures were grown in the respective media that they were isolated from and stored at -80ºC in 40% (v/v) glycerol.

Initial identification of red meat isolates

The meat isolates were grouped according to their Gram-reaction, morphology, catalase reaction and mobility. Gram staining (Bartholomew & Mittwer, 1950) was used to verify the Gram-reaction and the morphology of the micro-organisms. Motility was determined on wet mount slides. Hydrogen peroxide (10% v/v) was used in catalase tests (Harrigan & McCance, 1976).

Sensitivity of isolates to bacteriocins produced by LAB

The bacteriocin-producing strains were inoculated into the growth medium and incubated as described in Table 1. Cell-free supernatants were prepared by centrifugation (14 000 × *g*, 4ºC, 1 h). The pH of the cell-free supernatants was adjusted to 6.0 - 7.0 with sterile 1 N NaOH to eliminate the inhibitory effect of lactic acid. Proteolytic enzymes were inactivated by treating the cell-free supernatant at 80ºC for 10 min.

Table 1. Growth conditions of bacteriocin-producing lactic acid bacteria.

Species	Source	Growth conditions	Reference
Enterococcus faecalis BFE 1071	Faeces of Göttingen minipigs (W.H. Holzapfel, Bundesforschungsanstalt für Ernáhrung, Institut für Hygiene und Toxikologie, Karlsruhe, Germany)	BHI, 37ºC, 24 h	Balla *et al*. (2000)
Lactobacillus curvatus DF 38	Italian Salami (F. Dellaglio, Instituto Policattedra, Università delgi Studi di Verona, Italy)	MRS, 30ºC, 24 h	Böhme *et al*. (1996); Dicks *et al*. (2004)
Lactobacillus plantarum 423	Sorghum Beer (own culture collection)	MRS, 30ºC, 24 h	Van Reenen *et al*. (1998)
Lactobacillus casei LHS	Wine (own culture collection)	MRS, 30ºC, 24 h	Van Jaarsveld (1991)
Lactobacillus salivarius 241	Ileum of piglets (own culture collection)	MRS, 30ºC, 24 h	Maré (2004)
Pediococcus pentosaceus ATCC 43201	Fermenting cucumbers (North Carolina, USA)	MRS, 30ºC, 24 h	Daeschel & Klaenhammer (1985)

ATCC - American Type Culture Collection

BHI - Brain Heart Infusion (Biolab, Merck Laboratories, Milnerton, South Africa)

MRS - De Man-Rogosa-Sharpe medium (Biolab)

Table 2. Selective and differential growth media.

Medium	Organisms	Incubation conditions	Colony characteristics
MRS Agar, pH 5.6	Lactic acid bacteria	30ºC, 72 h to 5 d, anaerobic *	Catalase-negative colonies
Plate Count Agar	Aerobes	26ºC, 48 h	All colonies
m-Enterococcus Agar	Enterococci	37ºC, 48 h	Catalase-negative; pink to red colonies
VRBD Agar	*Enterobacteriaceae*	30ºC, 48 h, anaerobic*	Colonies > 1 mm
CASO Agar	*Pseudomonas*	26ºC, 72 h	Oxidase-positive cells
DRCM Agar	Mesophilic clostridia	37ºC, 72 h, anaerobic*	Black colonies
Yeast Extract Glucose Agar	Yeast and moulds	26ºC, 72 h to 5 d	Yeast and moulds
SS Agar	*Salmonella* and *Shigella* spp.	26ºC, 24 h and 48 h	Colourless, pink to red and grey to black colonies
KRANEP Agar	Micrococci and staphylococci	37ºC 48 h	Catalase-positive cells; cocci

* Anaerobic conditions were created using a Gas Generating Kit, Anaerobic System BR 038B, Oxoid, Wade Road, Basingstoke, Hampshire, RG24 8PW, England.

The antimicrobial activity of the crude bacteriocins was then tested against all strains isolated from meat. An aliquot of 10 μl of the cell-free supernatant was spotted on BHI agar (2% w/v agar), left to diffuse into the medium and were then covered with soft agar (1% w/v), seeded with the test organism (ca. 1×10^6 cfu.ml^{-1}). The plates were incubated overnight at the optimal growth temperature of the specific test organism. An inhibition zone of at least 2 mm in diameter was taken as positive for bacteriocin sensitivity.

Identification of sensitive bacteria isolated from red meat

The meat isolates that were found to be sensitive to the bacteriocins were further identified to genus level. The presence of endospores, growth at aerobic and anaerobic conditions and the presence of oxidase was determined as described by Harrigan and McCance (1976).

The Hugh and Leifson's medium (Hugh & Leifson, 1953) was used to determine whether the isolates were able to ferment glucose to acid and/or gas (Harrigan & McCance (1976). The results were noted as a colour change in the medium caused by a change in pH. If a colour-change occurred at the surface of the medium, the organism was taken as oxidase positive and if the change was at the bottom of the tube, the organism was considered to be fermentative. A colour change throughout the medium indicated that the organism was both oxidative and fermentative.

RESULTS AND DISCUSSION

As a result of the large volume of data compiled during this study, and to simplify the results and discussion section, the data is listed in an Appendix at the end of this chapter.

Isolation and quantification of micro-organisms from red meat

The number of bacteria and yeasts isolated from the various red meat samples are listed in Tables 3 to 5.

Beef – The data summarised in Table 3 show that the beef loin chops from Retailer 1 reached a viable aerobic count of 3.8×10^7 cfu.g^{-1} within three days after the sell-by date when stored at 4ºC. From the counts on the different isolation media (Table 3), it was concluded that members of the *Enterobacteriaceae*, *Pseudomonas* spp., yeasts and moulds were the most dominant micro-organisms to contribute to the

spoilage. Three days after the sell-by date, the meat from Retailers 2, 3 and 4 showed a viable aerobic count between 1.2×10^6 and 6.5×10^6 cfu.g^{-1}, which may be considered to be just below the spoilage limit (1×10^7 cfu.g^{-1}, ICMSF, 1986). Growth on the isolation media indicated that members of the *Enterobacteriaceae*, *Pseudomonas*, yeasts, moulds, *Salmonella* and *Shigella* were probably the main contributors to the viable aerobic counts of the meat from Retailers 2 and 3. In addition to these contaminants, meat from Retailer 4 contained enterococci and mesophilic clostridia.

Lamb - The lamb loin chops from Retailers 1, 2 and 3 reached viable cell numbers of approximately 1×10^7 cfu.g^{-1} within 3 d after the sell-by date (Table 4). Meat from Retailer 4 yielded a viable aerobic count of 2.3×10^6 cfu.g^{-1}, which is just below the spoilage limit, as suggested by the ICMSF (1986). The microbial population on lamb chops from Retailers 1, 2 and 3, mainly consisted of members of the *Enterobacteriaceae*, *Pseudomonas* spp., yeasts and moulds according to growth on the different isolation media. In addition to the mentioned contaminants, meat from Retailer 4 showed microbial growth on media selective for lactic acid bacteria, enterococci, mesophilic clostridia, *Salmonella* and *Shigella*.

Pork - Viable aerobic counts of pork loin chops (Table 5) showed that the meat from Retailers 1 and 4 were below the 1×10^6 cfu.g^{-1} level, even 3 d past the sell-by date when stored at refrigeration temperature. The viable microbial count of meat from Retailers 2 and 3 were above the spoilage limit. Meat from Retailers 1, 2 and 3 had a microbial population containing members of *Enterobacteriaceae*, *Pseudomonas*, yeasts and moulds as indicated by the growth on the different isolation media. Lactic acid bacteria, enterococci, mesophilic clostridia, *Salmonella* and *Shigella* spp. were isolated from meat collected from Retailer 4, in addition to *Enterobacteriaceae*, *Pseudomonas*, yeasts and moulds.

The microbial populations of meat from Retailers 1, 2 and 3 were consistent throughout the study. Meat from Retailer 4 had a broader microbial population that would probably contribute to spoilage in comparison with that of Retailers 1, 2 and 3, according to the viable cell counts on the different selective media. The type of red meat (beef, lamb or pork) did not have such an influence on the microbial population as was the case with the origin of the meat, i.e. the Retailer.

Table 3. Viable cell numbers (cfu.g^{-1}) recorded three days after the sell-by date for beef loin chops obtained from four retailers.

	MRS	PCA	m-Ent	VRBD	CASO	DRCM	YE	SS	KRANEP
Retailer 1	7.8×10^3	3.8×10^7	2.6×10^3	1.6×10^7	2.6×10^7	3.0×10^4	2.3×10^7	2.1×10^4	nd
Retailer 2	6.0×10^3	6.5×10^6	8.0×10^1	9.7×10^5	1.4×10^8	1.9×10^3	4.3×10^6	1.6×10^6	nd
Retailer 3	2.5×10^4	2.0×10^6	nd	1.6×10^5	7.2×10^5	nd	4.6×10^5	4.9×10^3	nd
Retailer 4	1.0×10^6	1.2×10^6	6.3×10^5	2.5×10^3	1.1×10^6	1.6×10^6	1.1×10^6	1.2×10^4	nd

nd – not detected

MRS - De Man-Rogosa-Sharpe medium (Biolab, Merck Laboratories, Milnerton, South Africa)

PCA – Plate Count Agar (Biolab)

m-Ent – m-Enterococcus agar (Difco, Merck Laboratories, Milnerton, South Africa)

VRBD – Violet Red Bile Dextrose Agar (Biolab)

CASO – Casein-peptone Soy meal-peptone Agar (Biolab)

DRCM – Differential Reinforced Clostridial Broth (Biolab)

YE – Yeast Extract Agar (Biolab)

SS – Salmonella Shigella Agar (Biolab)

KRANEP – Potassium thiocyanate Actidion Sodium azide Egg-yolk Pyruvate Agar Basis (Fluka, Merck Laboratories, Milnerton, South Africa)

Table 4. Viable cell numbers (cfu.g^{-1}) recorded three days after the sell-by date for lamb loin chops obtained from four retailers.

	MRS	PCA	m-Ent	VRBD	CASO	DRCM	YE	SS	KRANEP
Retailer 1	1.1×10^3	1.1×10^7	8.5×10^1	1.5×10^6	3.8×10^6	4.0×10^2	1.8×10^6	3.7×10^3	nd
Retailer 2	1.2×10^3	1.2×10^7	1.0×10^1	1.0×10^7	1.4×10^7	nd	6.6×10^6	5.2×10^3	nd
Retailer 3	3.5×10^2	2.5×10^7	nd	1.2×10^6	6.0×10^6	nd	1.6×10^5	1.6×10^4	nd
Retailer 4	1.3×10^6	2.3×10^6	7.0×10^5	1.2×10^6	1.8×10^6	8.7×10^5	1.9×10^6	9.0×10^5	nd

Abbreviations as in the footnote of Table 3.

Table 5. Viable cell numbers (cfu.g^{-1}) recorded three days after the sell-by date for pork loin chops obtained from four retailers.

	MRS	PCA	m-Ent	VRBD	CASO	DRCM	YE	SS	KRANEP
Retailer 1	1.3×10^3	1.3×10^6	nd	1.7×10^5	2.0×10^6	1.2×10^3	1.2×10^6	4.0×10^3	nd
Retailer 2	5.0×10^3	1.3×10^7	nd	1.3×10^7	2.1×10^7	2.8×10^2	1.6×10^7	4.6×10^2	nd
Retailer 3	3.5×10^2	2.5×10^7	nd	1.2×10^6	6.8×10^6	nd	1.6×10^5	1.6×10^4	nd
Retailer 4	1.0×10^5	3.2×10^6	3.1×10^3	7.6×10^5	2.3×10^6	1.4×10^5	2.2×10^6	1.3×10^5	nd

Abbreviations as in the footnote of Table 3.

Presumptive microbial and source identification of red meat isolates

The results for the initial identification of the isolates from the three different meat species from the four retailers are shown in Tables A1 to A12 as given in the Appendix. The ratio of yeast, Gram-positive and Gram-negative bacteria isolated from the red meat obtained from the four retailers is shown in Fig 1. Approximately 12% of the total microbes isolated from the meat were identified as yeast, 35% as Gram-positive and 53% was Gram-negative bacteria.

Gram-positive - The percentage and origin of the meat isolates are listed in Table 6. Nineteen percent of the total Gram-positive bacteria isolated from the meat obtained from Retailer 1. Four, three and 13%, respectively, of the total isolated Gram-positive bacteria from Retailer 1 were isolated from the beef, lamb and pork. The beef (6%), lamb (6%) and pork (7%) from Retailer 2 contributed to 19% of the total isolated Gram-positive bacteria. The percentage of isolated Gram-positive bacteria from the meat obtained from Retailer 3 was 21% of which 6% were isolated from beef, 8% from lamb and 7% from pork. Forty-one percent of the total isolated Gram-positive bacteria were isolated from the meat obtained from Retailer 4 of which 13%, 15% and 14%, respectively, were isolated from beef, lamb and pork.

Gram-negative - The meat from Retailer 1 contributed to 17% of the total isolated Gram-negative bacteria of which 6% was from the beef, 3% from the lamb and 17% from the pork. Four, eight and 4%, respectively, of the total Gram-negative bacteria were isolated from beef, lamb and pork obtained from Retailer 2. The percentage of isolated Gram-negative bacteria from the meat obtained from Retailer 3 was 17% of which 5% was isolated from beef, 5% from lamb and 17% from pork. The beef (11%), lamb (14%) and pork (25%) from Retailer 4 contributed to 51% of the total isolated Gram-negative bacteria.

Yeasts - Five percent of the total yeasts isolated came from the pork of Retailer 1. Five percent and 14%, respectively, of the total isolated yeasts was isolated from beef and lamb obtained from Retailer 2, which contributed to 19% of the total isolated yeasts. The percentage of isolated yeasts from the meat obtained from Retailer 3 was 67% of which 24% was isolated from beef, 10% from lamb and 33% from pork. Ten percent of the total isolated yeasts were isolated from the meat obtained from Retailer 4, 5% from beef and 5% from pork. No moulds were detected.

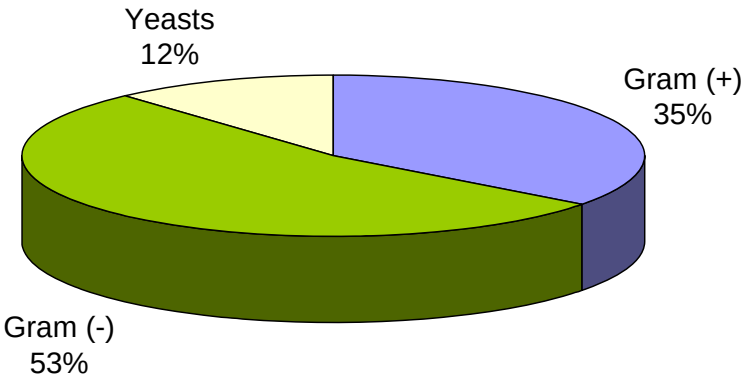

Figure 1. Isolation percentages of yeast and Gram-positive and Gram-negative bacteria isolated from red meat samples from four retailers.

Table 6. Percentage of yeasts, Gram-positive and Gram-negative bacteria isolated from the beef, lamb and pork obtained from the four different retailers.

		Retailer 1	Retailer 2	Retailer 3	Retailer 4	Total
Beef	Gram-positive	4	6	6	13	29
	Gram-negative	6	4	5	11	26
	Yeasts	0	5	24	4	33
Lamb	Gram-positive	3	6	7	15	31
	Gram-negative	3	8	5	14	30
	Yeasts	0	14	10	0	24
Pork	Gram-positive	12	7	7	14	40
	Gram-negative	8	4	8	24	44
	Yeasts	5	0	33	5	43
Total	Gram-positive	19	19	20	42	35
	Gram-negative	17	16	18	49	53
	Yeasts	5	19	67	9	12

Sensitivity of isolates to the lactic acid bacteria bacteriocins

The sensitive meat isolates are shown in Table A13 to A16 in the Appendix. The percentage sensitive isolates, calculated by dividing the number of sensitive micro-organisms by the total number of micro-organisms isolated from the specific retailer, is shown in Table 7. Enterocin had the lowest activity (15%) against the isolated organisms. Pediocin was more active at 16%. Approximately 21% of the organisms isolated from red meat were sensitive to curvacin, plantaricin, caseicin and salivaricin.

In Table 7, the sensitive isolates are expressed as a percentage of total isolated yeasts, Gram-positive and negative bacteria. In terms of this study, the Gram-negative bacteria and the yeasts were found to be more sensitive to the bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS and *Lb. salivarius* 241. The Gram-positive bacteria were more sensitive to the bacteriocin produced by *Pediococcus pentosaceus* ATCC 43201. Overall, the bacteriocins produced by *Enterococcus feacalis* BFE 1071 and *Pediococcus pentosaceus* ATCC 43201, were not as effective against the meat isolates as those produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS and *Lb. salivarius* 241. The activity of bacteriocins against meat isolates is important when selecting them as preservatives. The bacteriocin(s) with the widest spectrum of activity will be more effective in preventing the outgrowth of microbes on the meat surface and thus the most effective in preventing meat spoilage.

Identification of sensitive isolates from red meat

The micro-organisms sensitive to the lactic acid bacteria bacteriocins were further identified. The results are shown in Table A13 to A16 in the Appendix. In Table 8, the isolated microbes that are sensitive to the bacteriocins were grouped into different genera. As expected, most of the microbes that were sensitive to the bacteriocins, were Gram-positive. Approximately 7% of the total sensitive organisms were Gram-positive, catalase positive cocci and could not be identified by the methods used. Six percent of the total sensitive organisms were Gram-positive, catalase positive cocci and identified as *Staphylococcus*. The Gram-positive, catalase negative cocci represented 3.6% of the total sensitive organisms and were identified as lactococci and could possibly belong to the genus *Streptococcus, Lactococcus, Enterococcus, Leuconostoc* or *Pediococcus. Lactobacillus* (Gram-positive, catalase negative rods) represented 20.5% and *Bacillus* (Gram-positive, catalase positive rods) represented 1.2% of the total sensitive

organisms. Thirty-six percent of the total sensitive organisms were Gram-positive, catalase positive rods and could not be identified by the methods used.

Approximately 8.4% of the total sensitive organisms were Gram-negative rods of which 1.2% were catalase negative and could not be identified. About 7.2% were catalase positive of which 2.4% could not be identified and 4.8% were identified as microbes belonging to the genus *Klebsiella* or *Shigella*. Seventeen percent of the total sensitive organisms were identified as yeasts.

Table 7. The percentage of Gram-positive and Gram-negative organisms and yeasts from the respective retailers sensitive to bacteriocins produced by lactic acid bacteria.

		Sensitivity to Bacteriocins produced by					
		E. faecalis **BFE 1071**	***Lb. curvatus*** **DF 38**	***Lb. plantarum*** **423**	***Lb. casei*** **LHS**	***Lb. salivarius*** **241**	***P. pentosaceus*** **ATCC 43201**
Retailer 1	Gram-pos	7	20	20	27	27	27
	Gram-neg	7	23	23	23	23	20
	Yeast	83	83	83	83	83	17
	Total	16	29	29	31	31	22
Retailer 2	Gram-pos	6	6	6	6	6	28
	Gram-neg	8	31	31	31	31	19
	Yeast	29	29	29	29	29	29
	Total	10	22	22	22	22	24
Retailer 3	Gram-pos	13	17	17	17	17	4
	Gram-neg	4	16	20	16	16	4
	Yeast	6	19	19	13	19	6
	Total	8	17	19	16	17	5
Retailer 4	Gram-pos	33	29	26	29	26	29
	Gram-neg	11	13	13	15	15	10
	Yeast	67	33	33	33	33	0
	Total	21	20	19	21	20	17
Total	Gram-pos	19	20	19	21	20	22
	Gram-neg	8	19	20	20	20	13
	Yeast	31	34	34	31	34	13
	Total	15	21	21	22	22	16

Table 8. Grouping of 'bacteriocin-sensitive' microbial isolates from red meat samples.

Gram Reaction	Morphology	Catalase	Genus	% of total sensitive organisms
-	Rod	-	Unidentified	1.2
-	Rod	+	Unidentified	2.4
-	Rod	+	*Klebsiella/Shigella*	4.8
+	Cocci	+	Unidentified	7.2
+	Cocci	+	*Staphylococcus*	6.0
+	Cocci	-	*Streptococcus/Lactococcus/Enterococcus/ Leuconostoc/Pediococcus*	3.6
+	Rod	-	*Lactobacillus*	20.5
+	Rod	+	*Bacillus*	1.2
+	Rod	+	Unidentified	36.2
.	Yeasts	+	Unidentified	16.9

CONCLUSIONS

The bacteriocins produced by *Enterococcus feacalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 were screened for activity against meat isolates to determine whether these bacteriocins have potential in the meat industry as preservatives. Microbes were enumerated and isolated from beef, lamb and pork obtained from four retailers 3 d after the sell-by date. The viable aerobic counts of the red meat from the various retailers reached values near and beyond the spoilage limit of 1×10^7 $cfu.g^{-1}$ (ICMSF, 1986). From the growth on the isolation media, similarities were noted between the beef, lamb and pork obtained from the same retailer. From this observation it may be concluded that the source of the meat may be the main contributor to contamination with meat spoilage and pathogenic bacteria. This observation is confirmed by Merck (undated), who states that most of the microbes on the meat are acquired during the dressing process. There is a need for the application of systems like HACCP (Kennedy *et al*., 2000; Panisello *et al*., 2000) to lower the chance of contamination of the meat before packaging. To further increase the shelf-life and the possible outgrowth of the microbes in the consumers' refrigerator, a preservative of some kind is required, as also suggested by Kennedy *et al*. (2000).

The initial identification of the meat isolates indicated that 12% were yeasts, 35% Gram-positive and 53% Gram-negative bacteria. The bacteriocins produced by *Enterococcus feacalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 were active against approximately 16% to 21% of the total meat isolates. On average, the bacteriocins from *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS were more active against the meat isolates. The sensitivity of the meat isolates indicates that there is potential for bacteriocins as a preservative for red meat to extend the shelf-life. The stability and the efficiency of the bacteriocins in various conditions, especially those present on red meat at refrigerated temperatures, should be studied further to ensure their effectiveness as a preservative.

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APPENDIX

To Chapter Three

Tables A1 - A16 are given in this Appendix. The data recorded was placed in a separate appendix to simplify the discussion section of this chapter.

Table A1. Initial characterisation of isolates from pork (Retailer 1) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
29	MRS	+	Cocci	-	-	+	+	+	+	+	-
45	DRCM	+	Cocci	+	-	-	-	-	-	-	-
47	DRCM	+	Rod	+	-	-	-	-	-	-	-
48	DRCM	+	Rod	+	-	-	+	+	+	+	-
49	DRCM	+	Cocci	+	-	-	+	+	+	+	-
78	VRBD	+	Cocci	+	-	-	-	-	-	-	+
114	PCA	+	Cocci	+	-	-	-	-	+	+	+
140	CASO	+	Cocci	+	-	-	-	-	-	-	-
143	YE	+	Rod	+	-	-	-	-	-	-	-
25	MRS	×	Yeast	+	-	-	-	-	-	-	-
26	MRS	×	Yeast	+	-	+	+	+	+	+	-
27	MRS	×	Yeast	+	-	+	+	+	+	+	-
28	MRS	×	Yeast	+	-	+	+	+	+	+	-
30	MRS	×	Yeast	+	-	+	+	+	+	+	-
115	PCA	×	Yeast	+	-	+	+	+	+	+	+
46	DRCM	-	Rod	+	-	-	-	-	-	-	-
50	DRCM	-	Rod	+	-	-	+	+	+	+	-
62	SS	-	Rod	+	-	-	-	-	-	-	-
113	PCA	-	Rod	+	-	-	-	-	-	-	+
116	PCA	-	Rod	+	-	-	-	-	-	-	+
117	PCA	-	Rod	+	-	-	-	-	-	-	+
130	CASO	-	Rod	-	-	-	+	+	+	+	-
138	CASO	-	Rod	+	-	-	-	-	-	-	-
139	CASO	-	Rod	+	-	-	-	-	-	-	-
141	CASO	-	Rod	+	-	-	-	-	-	-	-
142	YE	-	Rod	+	-	-	-	-	-	-	-
144	YE	-	Rod	+	-	-	-	-	-	-	-
145	YE	-	Rod	+	-	-	-	-	-	-	-
146	YE	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A2. Initial characterisation of isolates from pork (Retailer 2) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
66	SS	+	Rod	+	-	-	-	-	-	-	+
67	SS	+	Rod	+	+	-	-	-	-	-	+
86	VRBD	+	Rod	+	-	-	-	-	-	-	-
87	VRBD	+	Rod	+	-	-	-	-	-	-	-
89	VRBD	+	Rod	+	-	-	-	-	-	-	-
151	YE	+	Rod	-	-	-	-	-	-	-	-
301	VRBD	+	Rod	-	-	+	+	+	+	+	+
9	PCA	-	Rod	+	-	-	+	+	+	+	-
10	PCA	-	Rod	+	-	-	+	+	+	+	+
14	PCA	-	Rod	+	-	+	+	+	+	+	-
63	SS	-	Rod	+	-	-	-	-	-	-	+
64	SS	-	Rod	+	-	-	-	-	-	-	-
65	SS	-	Rod	+	-	-	-	-	-	-	-
112	mEnt	-	Rod	+	-	-	-	-	-	-	+

Table A3. Initial characterisation of isolates from pork (Retailer 3) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
36	DRCM	+	Rod	-	-	-	-	-	-	-	-
38	DRCM	+	Rod	-	-	+	+	+	+	+	-
39	DRCM	+	Rod	-	-	+	+	+	+	+	-
58	SS	+	Rod	+	+	-	-	-	-	-	+
105	VRBD	+	Rod	+	-	+	-	-	-	-	-
107	VRBD	+	Rod	+	-	-	-	-	-	-	-
109	VRBD	+	Cocci	+	-	-	-	-	-	-	-
136	CASO	+	Cocci	+	-	-	-	-	-	-	-
140	CASO	+	Cocci	+	-	-	-	-	-	-	-
2	MRS	×	Yeast	+	-	-	-	-	-	-	-
98	VRBD	×	Yeast	+	-	-	-	-	-	-	-
99	VRBD	×	Yeast	+	-	-	-	-	-	-	-
100	VRBD	×	Yeast	+	-	-	-	-	-	-	-
108	VRBD	×	Yeast	+	-	-	-	-	-	-	-
110	VRBD	×	Yeast	+	-	-	-	-	-	-	-
111	VRBD	×	Yeast	+	-	-	-	-	-	-	-
15	PCA	-	Rod	+	-	+	+	+	+	+	-
57	SS	-	Rod	+	-	-	-	-	-	-	-
59	SS	-	Rod	+	-	-	-	-	-	-	-
60	SS	-	Rod	+	-	-	-	-	-	-	-
61	SS	-	Rod	+	-	-	-	-	-	-	-
106	VRBD	-	Rod	+	-	-	-	-	-	-	-
135	CASO	-	Rod	+	-	-	-	-	-	-	-
138	CASO	-	Rod	+	-	-	-	-	-	-	-
139	CASO	-	Rod	+	-	-	-	-	-	-	-
159	YE	-	Rod	+	-	-	-	-	-	-	-
160	YE	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A4. Initial characterisation of isolates from pork (Retailer 4) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
179	VRBD	+	Rod	+	-	-	-	-	-	-	-
215	YE	+	Rod	+	-	-	-	-	-	-	-
238	CASO	+	Cocci	+	-	-	-	-	-	-	-
248	mEnt	+	Rod	-	-	-	-	-	-	-	-
276	DRCM	+	Rod	-	-	+	+	+	+	+	+
282	DRCM	+	Rod	-	-	+	+	+	-	-	-
288	MRS	+	Rod	-	-	+	+	++	+	+	+
289	MRS	+	Cocci	-	-	+	+	+	-	-	+
305	SS	+	Rod	+	-	-	-	-	-	-	-
303	SS	?	Rod	+	-	-	-	-	-	-	-
216	YE	×	Yeast	+	-	+	-	-	-	-	-
180	VRBD	-	Rod	+	-	-	-	-	-	-	-
181	VRBD	-	Rod	+	?	-	-	-	-	-	-
182	VRBD	-	Rod	+	-	-	-	-	-	-	-
183	VRBD	-	Rod	+	-	-	-	-	-	-	-
200	PCA	-	Rod	+	-	-	-	-	-	-	-
201	PCA	-	Rod	+	-	-	-	-	-	-	-
202	PCA	-	Rod	+	-	-	-	-	-	-	-
219	YE	-	Rod	+	-	-	-	-	-	-	-
220	YE	-	Rod	+	-	-	-	-	-	-	-
221	YE	-	Rod	+	-	-	-	-	-	-	-
236	CASO	-	Rod	+	-	-	-	-	-	-	-
237	CASO	-	Rod	+	-	-	-	-	-	-	-
239	CASO	-	Rod	+	-	-	-	-	-	-	-
249	mEnt	-	Rod	-	-	-	-	-	-	-	-
269	DRCM	-	Rod	+	-	-	-	-	-	-	-
270	DRCM	-	Rod	+	-	-	-	-	+	-	-
275	DRCM	-	Rod	+	-	+	+	+	+	+	+
277	DRCM	-	Rod	+	-	-	-	-	-	-	-
278	DRCM	-	Rod	+	-	-	-	-	-	-	-
279	DRCM	-	Rod	+	-	-	-	-	-	-	-
280	DRCM	-	Rod	+	-	-	-	-	-	-	-
281	DRCM	-	Rod	+	-	-	-	-	-	-	-
283	DRCM	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A5. Initial characterisation of isolates from beef (Retailer 1) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
75	SS	+	Rod	+	-	-	-	-	-	-	+
78	VRBD	+	Cocci	+	-	-	-	-	-	-	+
118	PCA	+	Cocci	+	-	-	-	-	-	-	-
302	VRBD	×	Yeast	+	-	-	-	-	-	-	-
74	SS	-	Rod	+	-	-	-	-	-	-	-
76	SS	-	Rod	+	-	-	-	-	-	-	+
119	PCA	-	Rod	+	-	-	-	-	-	-	+
132	CASO	-	Rod	+	-	-	+	+	+	+	-
133	CASO	-	Rod	+	-	-	-	-	-	-	-
137	CASO	-	Rod	+	-	-	+	+	+	+	-
152	YE	-	Rod	+	+	-	-	-	-	-	-
153	YE	-	Rod	+	-	-	-	-	-	-	-
198	VRBD	-	Rod	+	-	-	-	-	-	-	-
199	VRBD	-	Rod	+	-	-	-	-	-	-	-
300	VRBD	-	Rod	+	-	+	+	+	+	+	+

× - Gram-stained to determine morphology

Table A6. Initial characterisation of isolates from beef (Retailer 2) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
16	MRS	+	Rod	-	-	-	-	-	-	-	-
90	VRBD	+	Rod	+	-	-	-	-	-	-	-
91	VRBD	+	Cocci	+	-	-	-	-	-	-	-
92	VRBD	+	Cocci	+	-	-	-	-	-	-	-
93	VRBD	×	Yeast	+	-	-	-	-	-	-	+
94	VRBD	+	Rod	+	-	-	-	-	-	-	-
95	VRBD	+	Rod	+	-	-	-	-	-	-	-
96	VRBD	+	Cocci	+	-	-	-	-	-	-	+
17	MRS	.	Yeast	+	-	+	+	+	+	+	-
18	MRS	.	Yeast	+	-	+	+	+	+	+	-
19	MRS	.	Yeast	+	-	-	-	-	-	-	-
120	PCA	-	Rod	+	-	-	-	-	-	-	+
121	PCA	-	Rod	-	+	-	-	-	-	-	-
122	PCA	-	Rod	+	-	-	-	-	-	-	-
123	PCA	-	Rod	-	-	-	+	+	+	+	-

× - Gram-stained to determine morphology

Table A7. Initial characterisation of isolates from beef (Retailer 3) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
51	SS	+	Rod	-	+	-	-	-	-	-	-
53	SS	+	Rod	+	-	-	-	-	-	-	-
55	SS	+	Rod	+	-	-	-	-	-	-	-
97	VRBD	+	Cocci	+	-	-	-	-	-	-	-
126	PCA	+	Cocci	+	-	-	+	+	+	+	-
163	YE	+	Rod	+	-	-	-	-	-	-	-
1	MRS	×	Yeast	+	-	-	-	-	-	-	-
35	DRCM	×	Yeast	+	-	+	+	+	+	+	-
98	VRBD	×	Yeast	+	-	-	-	-	-	-	-
99	VRBD	×	Yeast	+	-	-	-	-	-	-	-
124	PCA	×	Yeast	+	-	-	+	+	-	+	-
125	PCA	×	Yeast	+	-	-	+	+	+	+	+
165	YE	×	Yeast	+	-	-	-	-	-	-	-
52	SS	-	Rod	+	-	-	-	-	-	-	-
54	SS	-	Rod	+	-	-	-	-	-	-	-
137	CASO	-	Rod	+	-	-	+	+	+	+	-
141	CASO	-	Rod	+	-	-	-	-	-	-	-
161	YE	-	Rod	+	+	-	-	-	-	-	-
162	YE	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A8. Initial characterisation of isolates from beef (Retailer 4) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
203	PCA	+	Cocci	+	-	+	+	+	+	+	+
204	PCA	+	Cocci	+	-	+	+	+	+	+	+
205	PCA	+	Cocci	Slow gas release	-	+	-	-	-	-	-
207	PCA	+	Cocci	-	-	-	-	-	-	-	-
240	CASO	+	Cocci	-	-	-	-	-	-	-	-
244	CASO	+	Cocci	+	-	-	-	-	-	-	-
245	CASO	+	Cocci	+	-	-	-	-	-	-	-
265	DRCM	+	Cocci	+	-	+	+	+	+	+	+
268	DRCM	+	Cocci	-	?	+	+	+	-	+	+
272	DRCM	+	Rod	-	-	-	-	-	+	-	-
273	DRCM	+	Rod	+	-	+	+	-	+	+	+
285	MRS	+	Rod	-	-	-	-	-	-	-	-
286	MRS	+	Rod	-	-	-	-	-	+	-	+
287	MRS	+	Cocci	-	-	+	+	+	+	+	+
306	SS	+	Rod	+	-	-	-	-	-	-	-
304	SS	?	Rod	+	-	-	-	-	-	-	-
226	YE	×	Yeast	+	-	-	-	-	-	-	-
185	VRBD	-	Rod	+	-	-	-	-	-	-	-
186	VRBD	-	Rod	+	-	-	-	-	-	-	-
187	VRBD	-	Rod	-	-	-	-	-	-	-	-
188	VRBD	-	Rod	+	-	-	-	-	-	-	-
189	VRBD	-	Rod	+	-	-	-	-	-	-	-
190	VRBD	-	Rod	+	-	-	-	-	-	-	-
191	VRBD	-	Rod	+	-	-	-	-	-	-	-
192	VRBD	-	Rod	+	-	-	-	-	-	-	-
193	VRBD	-	Rod	+	-	-	-	-	-	-	-
206	PCA	-	Rod	-	-	+	-	-	-	-	-
241	CASO	-	Rod	+	+	-	-	-	-	-	-
242	CASO	-	Rod	+	-	-	-	-	-	-	-
243	CASO	-	Rod	+	-	-	-	-	-	-	-
246	CASO	-	Rod	+	-	-	-	-	-	-	-
251	mEnt	-	Rod	-	-	-	-	-	-	-	-
266	DRCM	-	Rod	+	-	-	-	-	+	-	-
267	DRCM	-	Rod	-	-	+	+	+	-	+	+
274	DRCM	-	Rod	-	-	+	+	+	-	+	-

× - Gram-stained to determine morphology

Table A9. Initial characterisation of isolates from lamb (Retailer 1) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
78	VRBD	+	Cocci	+	-	-	-	-	-	-	+
131	CASO	+	Cocci	+	-	-	-	-	-	-	-
140	CASO	+	Cocci	+	-	-	-	-	-	-	-
9	PCA	-	Rod	+	-	-	+	+	+	+	-
14	PCA	-	Rod	+	-	+	+	+	+	+	-
77	VRBD	-	Rod	+	-	-	-	-	-	-	-
138	CASO	-	Rod	+	-	-	-	-	-	-	-
139	CASO	-	Rod	+	-	-	-	-	-	-	-

Table A10. Initial characterisation of isolates from lamb (Retailer 2) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
68	SS	+	Rod	+	-	-	-	-	-	-	-
82	VRBD	+	Rod	+	-	-	-	-	-	-	+
83	VRBD	+	Rod	+	-	-	-	-	-	-	-
148	YE	+	Rod	+	+	-	-	-	-	-	-
84	VRBD	×	Yeast	+	-	-	-	-	-	-	-
85	VRBD	×	Yeast	+	-	-	-	-	-	-	+
147	YE	×	Yeast	+	-	-	-	-	-	-	-
7	PCA	-	Rod	+	-	-	+	+	+	+	-
9	PCA	-	Rod	+	-	-	+	+	+	+	-
15	PCA	-	Rod	+	-	+	+	+	+	+	-
69	SS	-	Rod	+	-	-	-	-	-	-	+
70	SS	-	Rod	+	-	-	-	-	-	-	-
129	CASO	-	Rod	+	-	-	-	-	-	-	-
137	CASO	-	Rod	+	-	-	+	+	+	+	-
138	CASO	-	Rod	+	-	-	-	-	-	-	-
139	CASO	-	Rod	+	-	-	-	-	-	-	-
149	YE	-	Rod	+	-	-	-	-	-	-	-
194	VRBD	-	Rod	+	?	-	-	-	-	-	-
195	VRBD	-	Rod	+	-	-	-	-	-	-	-
196	VRBD	-	Rod	+	-	-	-	-	-	-	-
197	VRBD	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A11. Initial characterisation of isolates from lamb (Retailer 3) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
41	DRCM	+	Cocci	+	-	-	-	-	-	-	-
42	DRCM	+	Cocci	+	-	-	-	-	-	-	-
43	DRCM	+	Rod	+	-	-	-	-	-	-	-
56	SS	+	Rod	+	-	-	-	-	-	-	-
103	VRBD	+	Rod	+	-	-	-	-	-	-	-
104	VRBD	+	Cocci	+	-	-	-	-	-	-	-
140	CASO	+	Cocci	+	-	-	-	-	-	-	-
9A	PCA	+	Cocci	+	-	-	+	+	+	+	-
2	MRS	×	Yeast	+	-	-	-	-	-	-	-
100	VRBD	×	Yeast	+	-	-	-	-	-	-	-
12	PCA	-	Rod	+	-	-	+	+	+	+	-
13	PCA	-	Rod	+	-	-	+	+	+	+	+
40	DRCM	-	Rod	+	-	-	-	-	-	-	-
101	VRBD	-	Rod	+	-	-	-	+	-	-	-
102	VRBD	-	Rod	+	-	-	-	-	-	-	-
134	CASO	-	Rod	+	-	-	-	-	-	-	-
156	YE	-	Rod	-	+	-	-	-	-	-	-
157	YE	-	Rod	-	-	-	-	-	-	-	-
158	YE	-	Rod	+	-	-	-	-	-	-	-

× - Gram-stained to determine morphology

Table A12. Initial characterisation of isolates from lamb (Retailer 4) and their sensitivity to six different bacteriocins.

						Sensitivity to Bacteriocins produced by					
Isolate Number	**Media**	**Gram Stain**	**Cell Morphology**	**Catalase Reaction**	**Motility**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**
176	VRBD	+	Rod	+	-	-	-	-	-	-	-
177	VRBD	+	Rod	+	-	-	-	-	-	-	-
209	PCA	+	Rod	+	-	-	-	-	-	-	-
211	PCA	+	Rod	-	-	-	-	-	-	-	-
214	PCA	+	Rod	+	-	-	-	-	-	-	-
218	YE	+	Cocci	+	-	+	-	-	-	-	-
224	YE	+	Rod	-	-	+	-	-	+	+	+
225	YE	+	Rod	+	-	-	-	-	-	-	-
228	CASO	+	Cocci	-	-	-	-	-	-	-	-
232	CASO	+	Cocci	-	--	-	-	-	-	-	-
233	CASO	+	Rod	+	-	-	-	-	-	-	-
234	CASO	+	Rod	+	-	-	-	-	-	-	-
250	mEnt	+	Rod	-	-	-	-	-	-	-	-
255	DRCM	+	Cocci	+	-	-	-	-	-	-	-
256	DRCM	+	Cocci	+	-	+	+	+	+	+	+
257	DRCM	+	Rod	-	-	-	-	-	-	-	-
262	DRCM	+	Cocci	+	-	+	+	+	+	+	-
229	CASO	?	Rod	-	-	-	-	-	-	-	-
235	CASO	?	Cocci	+	-	-	-	-	-	-	-
210	PCA	×	Yeast	+	-	+	+	+	+	+	-
173	VRBD	-	Rod	+	-	-	-	-	-	-	-
174	VRBD	-	Rod	+	-	-	-	-	-	-	-
175	VRBD	-	Rod	+	-	-	-	-	-	-	-
178	VRBD	-	Rod	+	-	-	-	-	-	-	-
184	VRBD	-	Rod	+	+	-	-	-	-	-	-
208	PCA	-	Rod	+	-	-	-	-	-	-	-
212	PCA	-	Rod	+	-	-	-	-	-	-	-
213	PCA	-	Rod	+	?	+	+	+	+	+	+
217	YE	-	Rod	+	-	-	+	+	+	+	-
222	YE	-	Rod	+	-	-	-	-	-	-	-
223	YE	-	Rod	+	-	-	-	-	-	-	-
227	CASO	-	Rod	+	-	-	-	-	-	-	-
230	CASO	-	Rod	+	-	-	-	-	-	-	-
231	CASO	-	Rod	+	-	-	-	-	-	-	-
252	DRCM	-	Rod	+	-	-	-	-	-	-	-
253	DRCM	-	Rod	+	-	-	-	-	-	-	-
254	DRCM	-	Rod	+	-	-	-	-	+	+	+
258	DRCM	-	Rod	+	-						
259	DRCM	-	Rod	+	?	+	+	+	+	+	+
260	DRCM	-	Rod	+	-	-	-	-	+	-	-
263	DRCM	-	Rod	+	-	+	+	+	+	+	-
264	DRCM	-	Rod	+	-	+	+	+	-	+	+

× - Gram-stained to determine morphology

Table A13. Further identification of bacteria isolated from red meat of Retailer 1, sensitive to the bacteriocins produced by lactic acid bacteria.

													Sensitivity to Bacteriocins produced by						
Isolate	**Meat Type**	**Medium**	**Gram**	**Morphology**	**Aerobic growth**	**Anaerobic Growth**	**Endospores**	**Motility**	**Catalase**	**Oxidase**	**Glucose fermentation**	**Glucose O/F Medium**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**	**Identification**
75	Beef	SS	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
76	Beef	SS	-	Rod	+	+	-	-	+	+	+	O/F	-	-	-	-	-	+	Unidentified
78	Beef	VRBD	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
119	Beef	PCA	+	Rod	+	+	-	-	+	+	+	O/F	-	-	-	-	-	+	Unidentified
132	Beef	CASO	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
137	Beef	CASO	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
300	Beef	VRBD	-	Rod	+	+	-	-	+	+	+	O/F	+	+	+	+	+	+	Unidentified
78	Lamb	VRBD	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
27	Pork	MRS	+	Rod	slow	slow	-	-	-	-	homo	O/F	+	+	+	+	+	-	LAB*
28	Pork	MRS	+	Rod	+	+	-	-	-	-	homo	O/F	+	+	+	+	+	-	LAB*
29	Pork	MRS	+	Cocci	+	+	-	-	+	+	+	O	+	+	+	+	+	-	Unidentified
30	Pork	MRS	+	Rod	+	+	-	-	-	-	homo	O/F	+	+	+	+	+	-	LAB*
48	Pork	DRCM	+	Rod	+	+	-	-	+	-	+	O	-	+	+	+	+	-	*Staphylococcus*
49	Pork	DRCM	+	Cocci	+	slow	-	-	+	-	+	O	-	+	+	+	+	-	*Staphylococcus*
50	Pork	DRCM	-	Rod	+	+	-	-	+	-	+	F	-	+	+	+	+	-	*Klebsiella* or *Shigella*
78	Pork	VRBD	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
113	Pork	PCA	+	Rod	+	+	-	-	+	+	+	O/F	-	-	-	-	-	+	Unidentified
115	Pork	PCA	+	Cocci	+	+	-	-	+	+	+	O/F	+	+	+	+	+	+	Unidentified
116	Pork	PCA	+	Rod	+	+	-	+	+	-	+	O/F	-	-	-	-	-	+	Unidentified
117	Pork	PCA	+	Rod	+	+	-	-	+	+	+	O/F	-	-	-	-	-	+	Unidentified

*LAB - Lactic acid bacteria including *Lactobacillus* (rods) and *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc* or *Pediococcus* (cocci).

Table A14. Further identification of bacteria isolated from red meat of Retailer 2, sensitive to the bacteriocins produced by lactic acid bacteria.

													Sensitivity to Bacteriocins produced by					
Isolate	**Meat Type**	**Medium**	**Gram**	**Morphology**	**Aerobic growth**	**Anaerobic Growth**	**Endospores**	**Motility**	**Catalase**	**Oxidase**	**Glucose fermentation**	**Glucose O/F Medium**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**	**Identification**
17	Beef	MRS	+	Rod	+	+	-	-	-	-	homo	F	+	+	+	+	+	-	LAB*
93	Beef	VRBD	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
120	Beef	PCA	-	Rod	+	+	-	-	+	-	+	F	-	-	-	-	-	+	*Klebsiella* or *Shigella*
123	Beef	PCA	-	Rod	+	+	-	-	-	-	+	O/F	-	+	+	+	+	-	Unidentified
7	Lamb	PCA	+	Rod	+	+	-	-	-	-	homo	F	-	+	+	+	+	-	LAB*
15	Lamb	PCA	+	Rod	+	slow	-	-	-	-	homo	F	+	+	+	+	+	-	LAB*
68	Lamb	SS	+	Rod	+	+	-	-	+	-	+	O	-	-	-	-	-	-	*Staphylococcus*
69	Lamb	SS	+	Rod	+	+	-	-	+	+	+	O/F	-	-	-	-	-	+	Unidentified
82	Lamb	VRBD	+	Rod	+	slow	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
137	Lamb	CASO	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
10	Pork	PCA	+	Rod	+	+	-	-	-	-	homo	F	-	+	+	+	+	+	LAB*
66	Pork	SS	+	Rod	+	+	-	-	+	+	+	O	-	-	-	-	-	+	Unidentified
67	Pork	SS	+	Rod	+	+	+	-	+	+	+	O	-	-	-	-	-	+	*Bacillus*
112	Pork	mEnt	-	Rod	+	+	-	-	+	-	+	F	-	-	-	-	-	+	*Klebsiella* or *Shigella*
301	Pork	VRBD	+	Rod	+	+	-	-	+	+	+	F	+	+	+	+	+	+	Unidentified

*LAB - Lactic acid bacteria including *Lactobacillus* (rods) and *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc* or *Pediococcus* (cocci).

Table A15. Further identification of bacteria isolated from red meat of Retailer 3, sensitive to the bacteriocins produced by lactic acid bacteria.

													Sensitivity to Bacteriocins produced by					
Isolate	**Meat Type**	**Medium**	**Gram**	**Morphology**	**Aerobic growth**	**Anaerobic Growth**	**Endospores**	**Motility**	**Catalase**	**Oxidase**	**Glucose fermentation**	**Glucose O/F Medium**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**	**Identification**
124	Beef	PCA	+	Rod	+	+	-	-	+	-	+	O/F	-	+	+	-	+	-	*Staphylococcus*
125	Beef	PCA	+	Cocci	+	+	-	-	+	+	+	O/F	-	+	+	+	+	+	Unidentified
126	Beef	PCA	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
137	Beef	CASO	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
12	Lamb	PCA	+	Rod	+	+	-	-	-	-	homo	F	-	+	+	+	+	-	LAB*
13	Lamb	PCA	+	Rod	+	+	-	-	-	-	hetero	F	-	+	+	+	+	+	LAB*
9A	Lamb	PCA	+	Cocci	+	+	-	-	-	-	homo	F	-	+	+	+	+	-	LAB*
15	Pork	PCA	+	Rod	+	slow	-	-	-	-	homo	F	+	+	+	+	+	-	LAB*
38	Pork	DRCM	+	Cocci	+	+	-	-	+	-	+	O	+	+	+	+	+	-	*Staphylococcus*
39	Pork	DRCM	+	Rod	+	+	-	-	-	-	homo	F	+	+	+	+	+	-	LAB*
58	Pork	SS	+	Rod	+	+	-	-	+	-	+	O	-	-	-	-	-	+	*Staphylococcus*

*LAB - Lactic acid bacteria including *Lactobacillus* (rods) and *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc* or *Pediococcus* (cocci).

Table A16. Further identification of bacteria isolated from red meat of Retailer 4, sensitive to the bacteriocins produced by lactic acid bacteria.

													Sensitivity to Bacteriocins produced by						
Isolate	**Meat Type**	**Medium**	**Gram**	**Morphology**	**Aerobic growth**	**Anaerobic Growth**	**Endospores**	**Motility**	**Catalase**	**Oxidase**	**Glucose fermentation**	**Glucose O/F Medium**	***E. faecalis* BFE 1071**	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	***Lb. casei* LHS**	***Lb. salivarius* 241**	***P. pentosaceus* ATCC 43201**	**Identification**
204	Beef	PCA	+	Rod	+	+	-	-	-	-	homo	F	+	+	+	+	+	+	LAB*
205	Beef	PCA	+	Cocci	+	+	-	-	+	-	+	F	+	-	-	-	-	-	*Staphylococcus*
265	Beef	DRCM	+	Rod	+	+	-	-	+	-	+	O/F	+	+	+	+	+	+	*Staphylococcus*
267	Beef	DRCM	+	Rod	+	+	-	-	-	-	homo	O/F	+	+	+	-	+	+	LAB*
268	Beef	DRCM	+	Rod	+	+	-	-	-	-	homo	F	+	+	+	-	+	+	LAB*
272	Beef	DRCM	+	Rod	+	+	-	-	+	+	+	F	-	-	-	+	-	-	Unidentified
273	Beef	DRCM	+	Rod	+	+	-	-	+	-	+	F	+	+	-	+	+	+	*Staphylococcus*
274	Beef	DRCM	+	Rod	+	+	-	-	-	-	homo	O/F	+	+	+	-	+	-	LAB*
287	Beef	MRS	+	Rod	+	+	-	-	+	-	+	F	+	+	+	+	+	+	*Staphylococcus*
210	Lamb	PCA	+	Cocci	+	+	-	-	+	+	+	O/F	+	+	+	+	+	-	Unidentified
213	Lamb	PCA	+	Cocci	+	+	-	-	-	-	hetero	O/F	+	+	+	+	+	+	LAB*
217	Lamb	YE	+	Rod	+	+	-	-	+	+	+	O/F	-	+	+	+	+	-	Unidentified
218	Lamb	YE	+	Cocci	+	+	-	-	+	+	+	F	+	-	-	-	-	-	Unidentified
224	Lamb	YE	+	Cocci	+	+	-	-	+	-	+	F	+	-	-	+	+	+	*Staphylococcus*
254	Lamb	DRCM	-	Rod	+	+	-	-	+	-	+	O/F	-	-	-	+	+	+	*Klebsiella* or *Shigella*
259	Lamb	DRCM	+	Rod	+	+	-	+	+	-	+	O/F	+	+	+	+	+	+	Unidentified
263	Lamb	DRCM	+	Cocci	+	+	-	-	+	+	+	O/F	+	+	+	+	+	-	Unidentified
264	Lamb	DRCM	+	Rod	+	+	-	-	-	-	+	O/F	+	+	+	-	+	+	LAB*
276	Pork	DRCM	+	Cocci	+	+	-	-	+	-	+	F	+	+	+	+	+	+	*Staphylococcus*
282	Pork	DRCM	+	Cocci	+	+	-	-	-	-	+	F	+	+	+	-	-	-	LAB*
288	Pork	MRS	+	Rod	+	+	-	-	+	+	+	O	+	+	++	+	+	+	Unidentified
289	Pork	MRS	+	Rod	+	+	-	-	+	-	+	F	+	+	+	-	-	+	*Staphylococcus*

*LAB - Lactic acid bacteria including *Lactobacillus* (rods) and *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc* or *Pediococcus* (cocci).

CHAPTER 4

PRELIMINARY CHARACTERISATION OF BACTERIOCINS PRODUCED BY *Lactobacillus curvatus* DF 38, *Lactobacillus plantarum* 423 AND *Lactobacillus casei* LHS

ABSTRACT

Lactobacillus curvatus DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS were screened for the production of bacteriocins. All three strains produced antimicrobial peptides of between 2.35 and 3.4 kDa in size, according to tricine-SDS-PAGE. They were active against some species of *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Listeria*, *Bacillus*, *Clostridium* and *Propionibacterium*. The bacteriocins remained stable at 121ºC for 20 min, in buffers with a pH ranging from 2 to 10 and in salt concentrations of between 0.1 and 10%. Like most peptides, they were sensitive to proteolytic enzymes. Curvacin was sensitive to amylase, suggesting that this bacteriocin might be glycosylated. The bacteriocins were partially purified using ammonium sulphate precipitation and a Sep Pak C_{18} cartridge.

INTRODUCTION

During fermentation lactic acid bacteria produce a range of metabolites with antimicrobial activity, including organic acids (lactic, acetic and formic), diacetyl, carbon dioxide, hydrogen peroxide, aldehydes and antibiotics (Abee *et al*., 1995; Ross *et al*., 2002; Magnusson *et al*., 2003). In addition to these antimicrobial compounds, many species of lactic acid bacteria produce antimicrobial peptides or bacteriocins. These antimicrobial compounds suppress the growth and survival of undesirable food spoilage and pathogenic micro-organisms in fermented food (Abee *et al*., 1995; Ross *et al*., 2002; Magnusson *et al*., 2003).

Bacteriocins are a heterogeneous group of small (3 to 10 kDa) ribosomally synthesized antimicrobial proteins or peptides (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002). They may vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Bacteriocins can be bacteriostatic or bactericidal to other bacteria, especially those closely related or confined to the same

ecological niche. Producer strains are usually immune to their own bacteriocins (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002). Bacteriocins produced by lactic acid bacteria can be divided into four groups (Klaenhammer, 1993). The four classes and their subclasses are listed in Table 1.

Antimicrobial peptides or bacteriocins produced by food-associated micro-organisms such as lactic acid bacteria are attracting increasing attention as food preservatives (Abee *et al*., 1995; Montville & Winkowski, 1997). Several of these bacteriocins have potential in the food industry when used at correct conditions (Cleveland *et al*., 2001). It is important that applied studies be done to confirm the effectiveness of bacteriocins in food, as they are not effective in all food systems (Gänzle *et al.*, 1999; Cleveland *et al*., 2001).

There are a number of patented applications of bacteriocins in foodstuffs. Blackburn *et al.* (1998) patented the use of a combination of nisin, a chelating agent and a surfactant, as a food preservative, to inhibit both Gram-positive and Gram-negative micro-organisms in meat, eggs, cheese and fish. Wilhoit (1996) used *Streptococcus* and *Pediococcus*-derived bacteriocins in combination with a chelating agent to protect food against *Listeria*. The number of *Listeria monocytogenes* in Manchengo cheese inoculated with a bacteriocin-producing strain of *Enterococcus faecalis* decreased by six log cycles in only 7 days. The survival of *L. monocytogenes* in cheese made with the commercial starter cultures were not affected (Nuñez *et al.*, 1997). Campanini *et al.* (1993) found that when the bacteriocin producer *Lb. plantarum* was inoculated into a naturally contaminated salami sausage, the number of surviving *Listeria monocytogenes* decreased. In 1995, Vedamuthu patented a yoghurt product with increased shelf-life containing a bacteriocin derived from *Pediococcus acidilactici*. The plasmid-encoding pediocin expressed in *Lc. lactis,* was used as a starter culture for the production of cheddar cheese. This was done to aid the preservation of the cheese and to ensure the microbial quality of the fermentation process (Buyong *et al.*, 1998). Pediocin PA-1 was also expressed in "*Streptococcus thermophilus"* and is an important organism in the dairy fermentation industry (Coderre & Somkuti, 1999).

Bacteriocins are also considered as natural bio-preservatives because they are readily degraded by the protease-enzyme in the human gastrointestinal tract. Most bacteriocin-producing lactic acid bacteria have GRAS (generally regarded as safe) status (Vandenbergh, 1993; Abee *et al*., 1995; Schillinger *et al.*, 1996; Aymerich *et al.*, 2000). The aim of this study was to fundamentally characterise the bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS for the use as preservatives on fresh meat stored at 4ºC.

Table 1. Classification of bacteriocins produced by lactic acid bacteria.

Primary Structure	Molecular Mass	Sensitivity to Heat	Bacteriocin (as example)	Producing Organism
Class I				
Lantibiotics	< 5 kDa	Heat stable (30 min at 100ºC to 15 min at 121ºC)	Nisin	*Lactococcus lactis* subsp. *lactis* ATCC 11454
			Carnocin	*Carnobacterium piscicola* UI49
Class II				
Small non-lantibiotics	< 15 kDa	Heat stable (30 min at 100ºC to 15 min at 121ºC)	Pediocin PA-1	*Pediococcus acidilactici* PAC-1·0
			Curvacin A	*Lactobacillus curvatus* LTH 1174
			Enterocin 1146	*Enterococcus faecium* DPC 1146
Class III				
Large non-lantibiotics	> 15 kDa	Heat labile	Helviticin J	*Lactobacillus helveticus* 481
			Caseicin 80	*Lactobacillus casei* B80
Class IV				
Complex bacteriocins	Protein or peptide plus 1 or 2 moieties (lipids or carbohydrates)		Plantaricin S	*Lactobacillus plantarum*
			Leuconocin S	*Leuconostoc* spp.

(Klaenhammer, 1993; De Vuyst & Vandamme, 1994a; Cleveland *et al*., 2001)

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus curvatus DF 38, isolated from Italian salami, was obtained from F. Dellaglio, (Instituto Policattedra, Università delgi Studi di Verona, Verona, Italy) (Böhme *et al*., 1996; Dicks *et al*., 2003). *Lactobacillus plantarum* 423 was isolated from sorghum beer (Van Reenen *et al.*, 1998) and *Lb. casei* LHS from fortified wine (Van Jaarsveld, 1991).

Lactobacillus curvatus DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS were cultured separately in De Man-Rogosa-Sharpe (MRS) medium (Biolab, Merck Laboratories, Milnerton, South Africa) at 30ºC. The indicator strains and their growth conditions are listed in Table 2.

Screening for activity against indicator strains

The bacteriocin-producing strains were inoculated into the respective growth medium and incubated at 30ºC for 24 h. The cells were harvested (8 000 × *g*, 4ºC, 30 min) and the pH of the cell-free supernatant was adjusted to 6.0 with 1 N NaOH. Proteolytic enzymes were inactivated by heating the supernatants for 10 min at 80ºC. An aliquot of 10 μl of each of the cell-free supernatants was spotted onto solid medium (1% w/v agar), seeded with active growing cells (ca. 10^6 cfu.ml^{-1}) of the test organism and incubated for 12 to 24 h at the respective optimal growth conditions (Table 2). An inhibition zone of at least 2 mm in diameter was recorded as positive sensitivity to the bacteriocins.

Sensitivity to heat, pH, NaCl and proteolytic enzymes

Cell-free supernatants at pH 6.0 and heat-treated were used in all these tests, with *Lactobacillus sakei* DSM 20017 as indicator strain. The cell-free supernatants were heat-treated at 40, 60, 80 and 100ºC for 10, 30, 60 and 120 min, respectively, and 121ºC for 20 min. The samples were then screened for antimicrobial activity as described previously.

The pH of the cell-free supernatants was adjusted from 2 to 12, with intervals of two units, with sterile NaOH or HCl and incubated at 37ºC for 30 min and 2 h, respectively. After incubation, the samples were neutralised to pH 7 and screened for antimicrobial activity as before.

Sensitivity to salt were determined by adding 0.1%, 0.5%, 1.0%, 3.0%, 5.0%, 7.0% and 10.0% NaCl (w/v), respectively, to each of the cell-free supernatants. As control, the same concentrations of NaCl were added to MRS broth. The samples were incubated for 2 h at 37ºC and then screened, as described previously.

Resistance of the bacteriocins to proteolytic enzymes was determined by treating the cell-free supernatants with Proteinase K, pepsin and trypsin (final concentration = 1 μg.ml^{-1}). After 2 h at 37ºC, the enzymes were inactivated (100ºC for 10 min) and the samples tested for antimicrobial activity, as described previously.

Partial purification of bacteriocins

Strains *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS were cultured in 2 *l* MRS broth (Biolab) for 24 h at 30ºC. Cell-free supernatants were obtained by centrifugation (14 000 × *g*, 4ºC, 1 h). The pH of each of the supernatants was adjusted and protease enzymes inactivated as described previously. Ammonium sulphate was added gradually to a saturation of 60% (Sambrook *et al.*, 1989) and the proteins precipitated overnight at 4ºC. The supernatants were centrifuged at 20 000 × *g* for 1 h at 4ºC, the pellets resuspended in 25 mM ammonium acetate buffer (pH 6.5) and then loaded onto separate Sep Pak C_{18} cartridges (Waters, Millipore). The cartridges were activated with 80% *i*-propanol in 25 mM ammonium acetate buffer (pH 6.5) and then washed with 25 mM ammonium acetate buffer (pH 6.5). Bacteriocins were eluted with 60% *i*-propanol in 25 mM ammonium acetate buffer (pH 6.5). The fractions collected were dried under reduced pressure (Speed-Vac; Savant) and kept at -20ºC.

Determination of the specific activity of the bacteriocins

The protein concentration at each stage of purification was determined spectrophotometrically, using the Bradford method (Ausubel *et al*., 1998). Bacteriocin activity was expressed in arbitrary units per millilitre (AU.ml^{-1}). One AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of inhibition against *Lactobacillus sakei* DSM 20017.

Size determination

The partially purified bacteriocins of *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS were separated on a tricine-SDS-PAGE (Schägger & Von Jagow, 1987). A low molecular weight marker with sizes ranging from 2.35 to 46 kDa (Amersham International, UK) was used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa) (Van Reenen *et al*., 1998). The position of the active bacteriocin was determined by overlaying the other half of the gel (not stained and extensively pre-washed with sterile distilled water) with viable cells of *Lb. sakei* DSM 20017 (approximately 1 × 10^6 cfu.ml^{-1}), embedded in De Man-Rogosa-Sharpe (MRS) agar (1.0% agar, w/v). The overlaid gel was incubated for 24h at 30°C.

Table 2. Indicator bacteria and their growth conditions.

Strain	Species	Incubation Temperature (ºC)	Growth Medium	Incubation
DSM 20017	*Lactobacillus sakei*	30	MRS	Aerobic
LMG 13550	*Lactobacillus acidophilus*	37	MRS	Anaerobic
LMG 13551	*Lactobacillus bulgaricus*	42	MRS	Anaerobic
LMG 13552	*Lactobacillus casei*	37	MRS	Anaerobic
LMG 13553	*Lactobacillus curvatus*	30	MRS	Anaerobic
LMG 13554	*Lactobacillus fermentum*	37	MRS	Anaerobic
LMG 13555	*Lactobacillus helveticus*	42	MRS	Anaerobic
LMG 13556	*Lactobacillus plantarum*	37	MRS	Anaerobic
LMG 13557	*Lactobacillus reuteri*	37	MRS	Anaerobic
LMG 13558	*Lactobacillus sakei*	30	MRS	Anaerobic
LMG 13560	*Pediococcus pentosaceus*	30	MRS	Anaerobic
LMG 13562	*Leuconostoc cremoris*	25	MRS	Anaerobic
LMG 13564	*Streptococcus thermophilus*	42	MRS	Anaerobic
LMG 13566	*Enterococcus faecalis*	37	BHI	Aerobic
LMG 13567	*Staphylococcus carnosus*	37	BHI	Aerobic
LMG 13568	*Listeria innocua*	30	BHI	Aerobic
LMG 13569	*Bacillus cereus*	37	BHI	Aerobic
LMG 13570	*Clostridium sporogenes*	37	RCM	Anaerobic
LMG 13571	*Clostridium tyrobutyricum*	30	RCM	Anaerobic
LMG 13572	*Propionibacterium acidipropionici*	32	GYP	Anaerobic
LMG 13573	*Propionibacterium* sp.	32	GYP	Anaerobic
LMG 13574	*Propionibacterium* sp.	32	GYP	Anaerobic

MRS - De Man-Rogosa-Sharpe broth (Biolab, Merck Laboratories, Milnerton, South Africa)

BHI - Brain Heart Infusion (Biolab)

RCM - Reinforced Clostridial Medium (Biolab)

GYP - glucose 5 $g.l^{-1}$, yeast extract 3 $g.l^{-1}$, peptone 10 $g.l^{-1}$, meat extract 10 $g.l^{-1}$, NaCl 5 $g.l^{-1}$.

RESULTS AND DISCUSSION

Inhibitory activity

The bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS were active against *Lb. sakei* DSM 20017 (Table 3). Curvacin DF 38, plantaricin 423 and caseicin LHS showed no activity against *Lb. bulgaricus*, *Lb. casei*, *Lb. helveticus*, *Leuconostoc cremoris*, *Streptococcus thermophilus*, *Staphylococcus carnosus* and *Clostridium sporogenes*. Bacteriocins from *Lactobacillus plantarum* 423 and *Lb. casei* LHS gave mild activity against *Lb. acidophilus*, *Lb. curvatus*, *Lb. fermentum*, *Lb. plantarum*, *Enterococcus faecalis*, *Bacillus cereus* and *Clostridium tyrobutyricum*, while the bacteriocin produced by *Lb. curvatus* DF 38 showed no activity. *Lactobacillus sakei*, *Pediococcus pentosaceus*, *Listeria innocua* and *Propionibacterium acidipropionici* were even more sensitive to bacteriocins produced by *Lactobacillus plantarum* 423 and *Lb. casei* LHS. *Lactobacillus reuteri* was sensitive to the bacteriocin produced by *Lb. curvatus* DF 38. The *Propionibacterium* species were sensitive to all three bacteriocins.

Sensitivity to heat, pH, NaCl and proteolytic enzymes

Curvacin DF 38 (produced by *Lactobacillus curvatus* DF 38), plantaricin 423 (produced by *Lb. plantarum* 423) and caseicin LHS (produced by *Lb. casei* LHS) were found to be resistant to heat treatments of 40, 60, 80 and 100ºC for 10, 30 and 60 min, respectively. Treatment for 120 min, however, resulted in a slight decrease (*ca*. 20%) in activity. Approximately 30% of the antimicrobial activity was lost during autoclaving (121ºC for 20 min).

Incubation of the various bacteriocins in buffers ranging from pH 2 to 10 had no effect on their antimicrobial activity. However, a decrease (ca. 80%) in activity was noted at pH 12. The bacteriocins produced by *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS remained active against *Lb. sakei* DSM 20017 at NaCl concentrations between 0.1% and 10% (w/v).

All three bacteriocins were sensitive to Proteinase K, pepsin and trypsin. Amylase had no effect on plantaricin 423 and caseicin LHS, but destroyed the activity of curvacin DF 38, suggesting that the bacteriocin might by glycosylated.

Table 3. Spectrum of activity of bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS.

Micro-organism	Bacteriocins produced by		
	***Lb. curvatus* DF 38**	***Lb. plantarum* 423**	**Lb. casei LHS**
Lactobacillus sakei	++	++	++
Lactobacillus acidophilus	-	+	+
Lactobacillus bulgaricus	-	-	-
Lactobacillus casei	-	-	-
Lactobacillus curvatus	-	+	+
Lactobacillus fermentum	-	+	+
Lactobacillus helveticus	-	-	-
Lactobacillus plantarum	-	+	+
Lactobacillus reuteri	++	-	-
Lactobacillus sakei	-	++	++
Pediococcus pentosaceus	-	++	++
Leuconostoc cremoris	-	-	-
Streptococcus thermophilus	-	-	-
Enterococcus faecalis	-	+	+
Staphylococcus carnosus	-	-	-
Listeria innocua	-	++	++
Bacillus cereus	-	+	+
Clostridium sporogenes	-	-	-
Clostridium tyrobutyricum	-	+	+
Propionibacterium acidipropionici	-	++	++
Propionibacterium sp.	+	+++	+++
Propionibacterium sp.	+++	+++	+++

- no activity
+ mild activity (2 to 5 mm zone)
++ moderate activity (5 to 10 mm zone)
+++ extreme activity (≥ 10 mm zone)

Bacteriocin purification and determination of specific activity

The results of the bacteriocin purification and determination of specific activity are shown in Tables 4 to 6. During ammonium sulphate precipitation, all of the proteins (the bacteriocins and the proteins present in the growth media) are precipitated (Yang *et al*., 1992). As expected, the total activity as well as the total protein decreased after each purification step. The decrease in the protein concentration is a result of the elimination of the inactive proteins and peptides; while some activity is lost as the active proteins are damaged or inactivated during the purification steps.

Size Determination

Separation of the bacteriocin produced by *Lb. curvatus* DF 38 yielded an active band of between 2.35 and 3.4 kDa in size (Fig. 1). As previously mentioned, curvacin might be glycosylated. Although the bacteriocin was sensitive to amylase, it remained stable when subjected to tricine-SDS-PAGE.

Separation of the bacteriocins produced by *Lactobacillus plantarum* 423 and *Lb. casei* LHS yielded active peptide bands within the range of 2.35 to 3.4 kDa (Figs. 2 and 3).

CONCLUSIONS

According to their size and the heat stability, the bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS belong to the Class I lantibiotics, based on the classification by Klaenhammer (1993), De Vuyst & Vandamme (1994b) and Cleveland *et al.* (2001). Curvacin DF 38 may be glycosylated, which groups it into Class IV, i.e. complex bacteriocins with one or two moieties of carbohydrate or lipids. Further studies should be done to determine the exact composition of curvacin DF 38.

Table 4. Purification of the bacteriocin produced by *Lactobacillus curvatus* DF 38.

Sample	Activity ($AU.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Total Activity (AU)	Total Protein (mg)	Specific Activity ($AU.mg^{-1}$)	Yield (%)	Purification Factor
Supernatant (400 ml)	1.28×10^4	8.00×10^1	5.12×10^6	3.20×10^4	1.60×10^2	100	1
60% $(NH_4)_2SO_4$ precipitation (5 ml)	8.19×10^5	2.68×10^2	4.10×10^6	1.34×10^3	3.06×10^3	80	19.1
Sep Pak 60% *i*-propanol (5 ml)	4.10×10^5	9.38×10^1	2.05×10^6	4.69×10^2	4.37×10^3	40	27.3

Table 5. Purification of the bacteriocin produced by *Lactobacillus plantarum* 423.

Sample	Activity ($AU.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Total Activity (AU)	Total Protein (mg)	Specific Activity ($AU.mg^{-1}$)	Yield (%)	Purification Factor
Supernatant (400 ml)	2.56×10^4	1.74×10^2	1.02×10^7	6.96×10^4	1.47×10^2	100	1
60% $(NH_4)_2SO_4$ precipitation (5 ml)	1.64×10^6	3.60×10^2	8.19×10^6	1.80×10^3	4.55×10^3	80	30.9
Sep Pak 60% *i*-propanol (5 ml)	8.19×10^5	1.02×10^2	4.10×10^6	5.08×10^2	8.06×10^4	40	54.8

Table 6. Purification of the bacteriocin produced by *Lactobacillus casei* LHS.

Sample	Activity ($AU.ml^{-1}$)	Protein ($mg.ml^{-1}$)	Total Activity (AU)	Total Protein (mg)	Specific Activity ($AU.mg^{-1}$)	Yield (%)	Purification Factor
Supernatant (400 ml)	2.56×10^4	1.22×10^2	1.02×10^7	4.87×10^4	2.10×10^2	100	1
60% $(NH_4)_2SO_4$ precipitation (5 ml)	1.64×10^6	5.75×10^2	8.19×10^6	2.88×10^3	2.85×10^3	80	13.6
Sep Pak 60% *i*-propanol (5 ml)	8.10×10^5	8.30×10^1	4.10×10^6	4.15×10^2	9.87×10^3	40	46.9

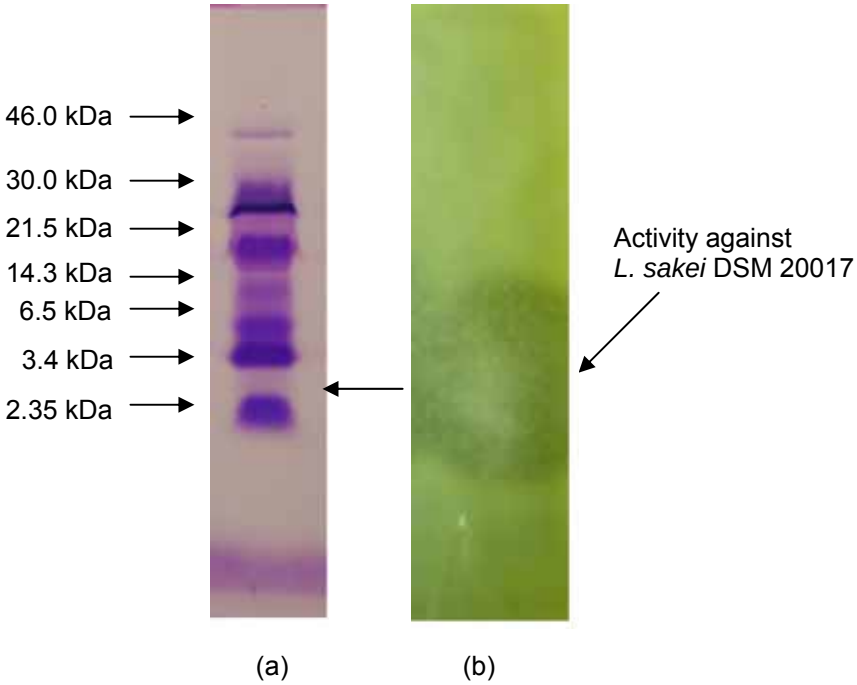

Figure 1. Tricine-SDS-PAGE profiles of partially purified *Lactobacillus curvatus* DF 38 bacteriocin. (a) Gel stained with Coomassie brilliant blue R250; (b) gel placed onto MRS agar surface and overlaid with *Lb. sakei* DSM 20017.

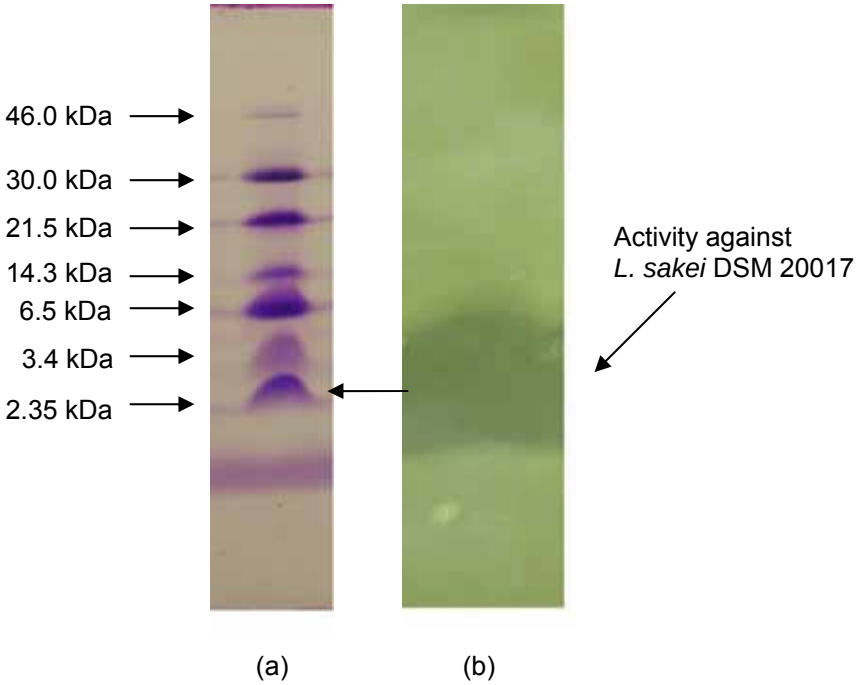

Figure 2. Tricine-SDS-PAGE profiles of partially purified *Lactobacillus plantarum* 423 bacteriocin. (a) Gel stained with Coomassie brilliant blue R250; (b) gel placed onto MRS agar surface and overlaid with *Lb. sakei* DSM 20017.

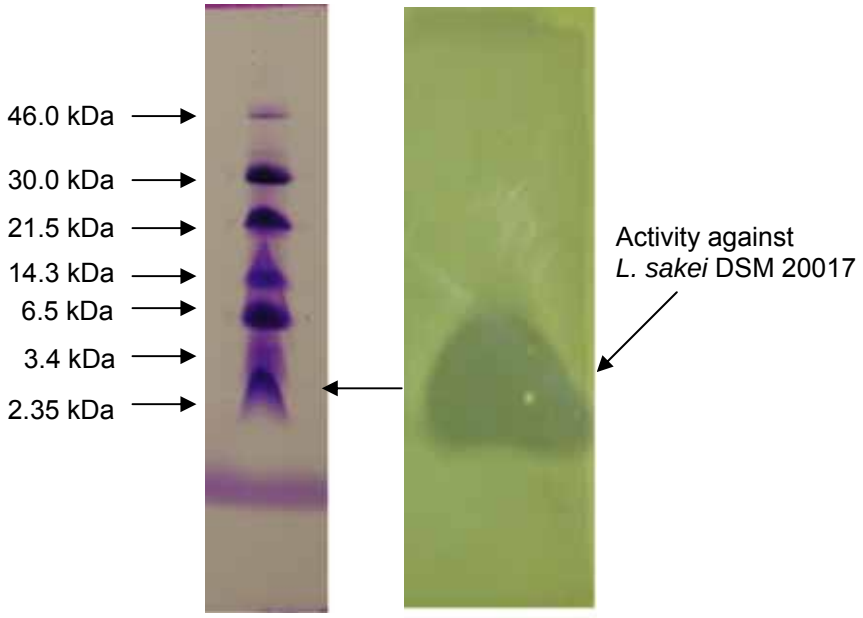

Figure 3. Tricine-SDS-PAGE profiles of partially purified *Lactobacillus casei* LHS bacteriocin. (a) Gel stained with Coomassie brilliant blue R250; (b) gel placed onto MRS agar surface and overlaid with *Lb. sakei* DSM 20017.

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CHAPTER 5

PRESERVATION OF PORK LOIN CHOPS WITH BACTERIOCINS PRODUCED BY *Lactobacillus curvatus* DF38, *Lactobacillus plantarum* 423 AND *Lactobacillus casei* LHS

ABSTRACT

Even though red meat is perishable at both refrigerated and frozen temperatures, consumers demand products that have a long shelf-life and are safe for consumption. Scientists are therefore seeking means to improve the shelf-life of meat and the use of bacteriocins produced by lactic acid bacteria is one of these methods. The bacteriocins produced by *Lactobacillus curvatus* DF38, *Lb. plantarum* 423 and *Lb. casei* LHS were partially purified and used to extend the microbiological shelf-life of pork loins. In a pilot study, the microbiological spoilage limit of untreated meat was reached after 6 d while that of the bacteriocin-treated sample was reached after 8 d. Meat colour evaluation was used as an indicator of the effect that the bacteriocin-treatment may have on the consumers' acceptance there of. The bacteriocin-treated pork sample was significantly ($P \leq 0.05$) darker than the control sample. The sensory attributes of the control pork samples, pork samples 2 d (48 h ± 2 h), and pork samples 4 d (96 h ± 2 h) after bacteriocin application, was compared. Descriptive sensory evaluation by a seven-member panel indicated that there was a significant difference ($P \leq 0.05$) regarding the aroma, sustained juiciness, first bite and metallic taste attributes of the control and the 4 day-treated samples. The control and 2 day-treated samples and the 2 day- and 4 day treated samples did not differ significantly for these attributes. Bacteriocins have the potential to be successful natural preservatives, to extend the shelf-life of pork and to improve the safety at refrigerated temperatures with little or no effect on the sensory attributes.

INTRODUCTION

A nutritious, well balanced diet consists of a daily intake of the correct portions of red meat and/or meat alternatives, milk and milk products, fruit, vegetables, and bread, rice, cereal and pasta (Anon., 2002a). Unfortunately, when stored at refrigerated temperatures, the shelf-life of red meat is limited. Beef has a shelf-life of 10 to 14 d, lamb 7 to 10 d and pork approximately 4 d at refrigerated temperatures. When

packaged in an airtight and moisture proof container and stored at -18ºC, the shelf-life of beef, lamb and pork is extended to 10, 8 and 4-6 months, respectively (Anon., 2002b).

The spoilage of chilled red meat at refrigeration temperatures is due to the proliferation of various bacteria, yeasts and moulds on the meat surface, mostly acquired during the dressing process (Jensen, 1954). These spoilage organisms not only cause bad odours and off-flavours, but also discolouration, slime and gas production as well as a decrease in pH (Borch *et al*., 1996).

The modern food processing industry is facing new challenges daily, as the consumer demands products that are both safe for consumption and have a long shelf-life (Ross *et al*., 2002). Artificial chemical preservatives are currently employed to limit the number of micro-organisms capable of growing in foods (Abee *et al*., 1995). Existing chemical food preservatives, including sulphites, sulphur dioxide, nitrates, nitrites, Na-diacetate, β-propiolactone, benzoic acid, sorbic acid, and therapeutic antibiotics, are increasingly being questioned with regard to their effects on human health (Kennedy *et al*., 2000). These concerns result in a trend towards minimally processed foods that are free of chemical preservatives (Ross *et al*., 2002). Food suppliers are thus forced to consider the use of more natural alternatives such as “green technologies” and bio-preservation to improve the shelf-life of red meat. Anti-microbial peptides or bacteriocins produced by food-associated micro-organisms such as lactic acid bacteria, in particular, are attracting increasing attention as food preservatives (Abee *et al*., 1995; Montville & Winkowski, 1997; Kennedy *et al*., 2000; Ross *et al*., 2002).

Bacteriocins have possible applications in a wide variety of foods (Abee *et al.*, 1995). These bio-preservatives can either be used directly in the food in the purified, concentrated form as a food additive, on their own or in combination with other preservatives (Abee *et al*., 1995; Ross *et al*., 2002). Certain strains of lactic acid bacteria that produce bacteriocins can also be incorporated into the starter culture of fermented foods or can be used as protective cultures (Ross *et al*., 2002). The *in situ* production of bacteriocins by the starter or protective cultures may be affected by the composition of the food, the temperature of storage, the salt contents or the pH (Työppönen *et al*., 2003). In addition, the bacteriocin activity may be affected by the changes in solubility and ionic charge of the bacteriocin, as well as the binding of the bacteriocin to food components, such as fat and/or protein, and the food additives present. The activity of the bacteriocins are also affected by the presence of natural proteases or other inhibitors present in the food as well as changes in the cell envelope of the target organisms as a response to environmental factors (Gänzle *et al.*, 1999; Leroy & De Vuyst, 1999; Cleveland *et al*., 2001; Työppönen *et al*., 2003).

In Chapters Three and Four of this thesis, the efficiency of bacteriocins in inhibiting microbes was investigated in broth systems. The first aim of this investigation was to apply bacteriocins produced by *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS on the surface of pork to confirm their effectiveness as food preservatives. The purchase and repurchase of a bacteriocin treated product by the average consumer will be greatly influence by the sensory attributes. The second aim was therefore to determine whether the bacteriocins cause negative sensory effects in terms of visual perception (colour), flavour, tenderness and juiciness of pork.

MATERIALS AND METHODS

Bacteriocin production and partial purification

The bacteriocin producing strains include *Lactobacillus curvatus* DF38, *Lb. plantarum* 423 and *Lb. casei* LHS. *Lactobacillus curvatus* DF 38, which was originally isolated from Italian salami, was obtained from Professor F. Dellaglio (Instituto Policattedra, Università delgi Studi di Verona, Verona, Italy) (Böhme *et al*., 1996; Dicks *et al*., 2004). *Lactobacillus plantarum* 423 was isolated from sorghum beer (Van Reenen *et al.,* 1998) and *Lb. casei* LHS from fortified wine (Van Jaarsveld, 1991).

Strains *Lb. curvatus* DF38 (producing curvacin DF38), *Lb. plantarum* 423 (producing plantaricin 423) and *Lb. casei* LHS (producing caseicin LHS) were cultured in 2 L MRS broth (Biolab, Merck Laboratories, Milnerton, South Africa) for 24 h at 30ºC. Cell-free supernatants were obtained by centrifugation (14 000 × g, 4ºC, 1 h). The pH of the cell-free supernatant was adjusted to 6.0 with NaOH and proteolytic enzymes were inactivated by heating the supernatants for 10 min at 80ºC. Ammonium sulphate was gradually added to give a final saturation of 60% (w/v) (Sambrook *et al*., 1989). The proteins precipitated overnight at 4ºC. The supernatants were then centrifuged at 20 000 × g for 1 h at 4ºC, where after the pellets were resuspended in 25 mM ammonium acetate buffer (pH = 6.5) and loaded separately on Sep Pack C_{18} cartridges (Waters, Millipore). The cartridges were activated using 80% *i*-propanol in 25 mM ammonium acetate buffer (pH = 6.5) and then washed with 25 mM ammonium acetate buffer (pH = 6.5). Bacteriocins were eluted with 60% *i*-propanol in 25 mM ammonium acetate buffer (pH = 6.5). The bacteriocin fractions were dried under reduced pressure (Speed-Vac; Savant) and stored at -20ºC.

Bacterial strains and growth conditions

Two bacterial strains were used to spike the pork samples in the shelf-life study. The first was a strain of *Leuconostoc mesenteroides* subsp. *mesenteroides*, which was isolated from pork obtained from a local supermarket in Stellenbosch, South Africa (as described in Chapter 3). It was cultured and maintained in MRS medium (Biolab). Strain 106 was isolated by Michèle de Kwaadsteniet (University of Stellenbosch, Stellenbosch, South Africa, 2003) from biofilms in steel pipes of the company Specialised Protein Products (SPP), Potchefstroom, South Africa and was identified as a *Bacillus* sp. It was cultured in Brain Heart Infusion broth (BHI) (Biolab).

Shelf-life study

Pork loin chops, *longissimus lumborum* (LL), were obtained from a local supermarket. Proximal chemical analyses were carried out on the raw LL, from which all visible fat had been removed. The muscle samples were cut into smaller portions and to ensure homogeneity, minced three times through a 2 mm sieve where after the samples were chemically analysed. The percentage of moisture and protein of the meat sample was determined according to AOAC methods (AOAC, 2002). The lipid content was determined by means of chloroform:methanol extraction (Lee *et al*., 1996). The protein content was determined by the Dumas combustion method (AOAC 968.06) on the defatted sample using a FP528 Nitrogen Analyser (AOAC, 2002). The moisture content was determined by drying 2 g of meat at 100ºC for 24 h as described by the AOAC 934.01 method.

In a preliminary study shelf-life investigation, samples of bacteriocin-treated and a control sample of pork were compared. Five-gram cubes (2.5 × 2.5 cm) of meat were placed in 10 sterile McCartney bottles, respectively. Five of the samples were treated with 500 µl (approximately 4 000 $AU.ml^{-1}$) of the mixture (1:1:1) of curvacin DF 38, plantaricin 423 and caseicin LHS (prepared as previously described) and the other 5 was used as a control (treated with 500 µl sterile distilled water). The samples were stored at 4ºC for 8 d and microbial enumeration was done on days 0 (directly after the bacteriocin application), 2, 4, 6 and 8.

In the main investigation the meat was spiked with either *Leuconostoc mesenteroides* subsp. *mesenteroides* or *Bacillus* sp. or a 1:1 mixture of the two and the effect of the various bacteriocins and a mixture of the three were investigated. Cubes (2.5 × 2.5 cm) of meat (approximately 5 g/bottle) were placed in 75 sterile McCartney bottles. The bacteriocins, prepared as previously described, were resuspended in sterile distilled water. Five hundred micro litres of bacteriocin (approximately 4 000 $AU.ml^{-1}$) were added to each sample and shaken carefully to ensure that the entire surface of the

meat was covered with the bacteriocin suspension. Fifteen samples were thus treated with curvacin DF 38, 15 samples with plantaricin 423, 15 with caseicin LHS and 15 samples were treated with a combination (1:1:1) of the three bacteriocins. Fifteen control samples were also prepared where the bacteriocin suspension was replaced with 500 µl of sterile distilled water.

Five samples of each of the groups of 15 (treated with the respective bacteriocins as well as the control) were subsequently inoculated with 500 µl (approximately 1×10^6 cfu.ml^{-1}) *Leuconostoc mesenteroides* subsp. *mesenteroides*, another 5 samples with the *Bacillus* sp. and 5 samples with a combination (approximately 1:1) of the two microbes. The bottle was again shaken to ensure the distribution of the cells over the surface of the meat sample.

The meat was stored for 8 d at 4ºC. The 15 samples for each microbial analyses (days 0, 2, 4, 6 and 8) therefore consisted of 3 samples treated with curvacin DF 38, 3 samples treated with plantaricin 423, another 3 with caseicin LHS, 3 samples treated with a combination (1:1:1) of the three bacteriocins and 3 control samples. One sample of each of the groups of three was inoculated with *Leuconostoc mesenteroides* subsp. *mesenteroides*, another sample with *Bacillus* sp. and the other sample with the mixture (1:1) of the micro-organisms.

On each target date, 5 ml of sterile peptone water (0.1% peptone, 0.85% NaCl) was added to each of the fifteen 5 g samples and mixed thoroughly for 1 min on a Vortex mixer. A serial dilution was made and plated out on BHI (Biolab) agar. The cfu.g^{-1} was determined after an incubation of 48 h at 37ºC. Aerobic cell counts were repeated on day 2, 4, 6 and 8 (1 d = 24 h ± 2 h). This experiment was repeated three times.

Meat colour evaluation

The sensory characteristics of red meat, including the visual appearance, strongly influence the purchase decision of the consumer (Bolte, 2002). Thus the effect of the bacteriocin-treatment on the colour of the meat was determined. Two pork loin slices were used for the colour evaluation. Five hundred microlitres of a 1:1:1 mixture of curvacin DF38, plantaricin 423 and caseicin LHS (4 000 AU.ml^{-1}) were applied to one of the loin slices. The bacteriocins were prepared as previously described. The meat was packed separately in styrofoam containers and covered with oxygen permeable cling film (O_2 transmission, 12 000 cm^3.m^{-2}.24h^{-1}; CO_2 transmission 76 000 cm^3.m^{-2}.24h^{-1}) in a similar manner to packaging used by supermarkets. The colour of the raw treated and untreated *longissimus lumborum* muscle was recorded on day 0 (directly after application) and on day 1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and 14 (1 d = 24 h ± 2 h) using a Colour-guide 45°/0° colorimeter (Cat no: 6805; BYK-Gardner, USA). The initial colour

measurements for the control (C) were taken in triplicate and five measurements were taken in the case of the bacteriocin-treated (B) meat at randomly selected positions, thereafter; consequent measurements were taken at the same position. Colour was expressed by the coordinates L*, a*, b* of the CIE Lab colorimetric space (Honikel, 1998). The L*-, a*- and b*-values are an indication of the lightness (black-white axis), redness (red-green spectrum) and yellowness (blue-yellow spectrum) of colour respectively. bL*, ba* and bb* indicates the same colour co-ordinates taken on the bacteriocin-treated samples and cL*, ca* and cb* indicates the same colour co-ordinates taken on the untreated or control samples. The hue-angle and the a* and b* chroma are psychometric correlates of perceived hue and chroma (Honikel, 1998) and were determined using the following equation (Honikel, 1998):

Chroma: $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

Hue angle: $h_{ab} = \tan^{-1}\left\{\frac{b^*}{a^*}\right\}$

Descriptive sensory analysis

If the bacteriocin-treated product is purchased, the repurchasing of the product will depend on whether the product is acceptable to the consumer, with specific reference to the flavour, and thus taste. Samples of pork loin (*longissimus lumborum)*, from which all visible fat was removed, were obtained from a local retailer. The loins were cut in 1.5 cm slices and divided randomly into three groups of which two were frozen at – 18ºC. Group 1 (sample B_4) was treated with a mixture (1:1:1) of the three bacteriocins, prepared as described previously, by distributing an aqueous solution containing approximately 4 000 $AU.ml^{-1}$ evenly onto the surface of the meat. The meat was then stored at 4ºC for 4 d (96 h ± 2 h). The second group of meat (sample B_2) was thawed overnight at 4ºC (on day 1) and treated on day 2 with the bacteriocins as described. The meat was then stored at 4ºC for 2 d (48 h ± 2 h). The last sample was used as a control (C), thawed overnight at 4ºC (on day 3) and left untreated. On day 4 the samples were wrapped in cooking bags and placed on a rack of an open roasting pan. A thermocouple (for internal temperature control) was inserted into each of the samples, which were prepared at 160ºC in two computerised electronic temperature controlled electric Defy 835 ovens (Viljoen *et al*., 2001). The meat was roasted to an internal temperature of 71ºC (American Meat Science Association, 1978).

After cooking, the meat was allowed to cool for 10 min. Six cubed (1.5 × 1.5 cm) samples were then taken from the middle of each slice. Immediately after which, each of the cubed samples were wrapped individually in aluminium foil and marked with a

random three-digit code and placed in glass ramekins in a preheated oven at 100ºC. The samples were evaluated within 10 min by the sensory panel. A seven-member panel was selected and trained according to the American Meat Science Associations guidelines (AMSA, 1995). The score sheet (Fig. 1) was compiled and refined by the panel during the training session. The descriptive sensory evaluation performed on the meat included pork aroma, initial impression of juiciness, sustained juiciness, first bite, residue, pork flavour and metallic taste. The definitions of these attributes are listed in Table 1.

The meat was evaluated in seven sessions over a period of 3 d using an eight-point ordinal scale (Jeremiah & Phillips, 2000). The meat for each sensory evaluation session was prepared exactly the same ensuring that the meat was untreated (C), treated for two (B_2) and four days (B_4), respectively. The panel members were seated in individual booths in a light-controlled and temperature controlled room. Each panel member received three samples in a randomised order. Crackers and distilled water were used to cleanse the palate between samples (AMSA, 1995).

Statistical analysis

The shelf-life data was analysed using Proc GLM (General Linear Models) (SAS for Windows 2000 Version 8.2) (Little *et al.*, 1996). All the variations of the full model were fitted, including treatment, time and bacteria; treatment, time by bacteria; and treatment by time.

The data obtained from the meat colour evaluation was statistically analysed using Proc Mixed and Proc GLM (SAS) and two models were fitted during Proc GLM, one with a time*treatment interaction and one without this interaction. As there were no significant interactions, only the main effects were included in the final model.

In the case of the descriptive sensory analysis, the ordinal scores (non-parametric data) awarded by the panel members were used to rank the treatments for each attribute, respectively. These ranks were subjected to a two-way analysis of variance, similar to Friedman's two-way analysis by ranks for non-parametric data (Siegel, 1956). Tukey's LSD (least significant difference) was calculated at a 5% significance level to compare the treatment rank means. Scores were tabulated in contingency tables for treatments and chi-square tests for independence or similar patterns were performed (Snedecor & Cochran, 1967). The SAS program, (SAS, 1999) was used to statistically analyse the data.

SESSION: **DATE:**

PANEL MEMBER: **NAME:**

Evaluate the samples in the order that they are presented.
Rinse your mouth with water and crackers between samples and sets.

CHARACTERISTIC	SCORE			
PORK AROMA INTENSITY Take a few short sniffs as soon as you remove the foil	8 Extremely intense 7 Very intense 6 Moderately intense 5 Slightly intense 4 Slightly bland 3 Moderately bland 2 Very bland 1 Extremely bland			
INITIAL IMPRESSION OF JUICINESS The amount of fluid exuded on the cut surface when pressed between your thumb and forefinger	8 Extremely juicy 7 Very juicy 6 Moderately juicy 5 Slightly juicy 4 Slightly dry 3 Moderately dry 2 Very dry 1 Extremely dry			
SUSTAINED JUICINESS The impression that you form after the first two to three chews between the molar teeth	8 Extremely juicy 7 Very juicy 6 Moderately juicy 5 Slightly juicy 4 Slightly dry 3 Moderately dry 2 Very dry 1 Extremely dry			
FIRST BITE The impression of tenderness after the first two to three chews between the molar teeth	8 Extremely tender 7 Very tender 6 Moderately tender 5 Slightly tender 4 Slightly tough 3 Moderately tough 2 Very tough 1 Extremely tough			
RESIDUE The amount of residue left in the mouth after the first twenty to thirty chews.	8 None 7 Practically none 6 Traces 5 Slightly 4 Moderate 3 Excessive amount 2 Moderately abundant 1 Abundant			
OVERALL PORK FLAVOUR This is a combination of taste and swallowing	8 Extremely typical 7 Very typical 6 Moderately typical 5 Slightly typical 4 Slightly untypical 3 Moderately untypical 2 Very untypical 1 Extremely untypical			
METALLIC TASTE Metallic taste while chewing the sample/ taste associated with frozen pork	8 Extremely metallic taste 7 Very prominent metallic taste 6 Moderate metallic taste 5 Slight metallic taste 4 Very little metallic taste 3 Traces of metallic taste l 2 Practically no metallic taste 1 No metallic taste			

Figure 1. The score sheet used for the sensory evaluation of pork.

Table 1. Definition of attributes used for the descriptive sensory analysis

Attribute and Scale	Definition
Pork Aroma 1 = Extremely bland; 8 = Extremely intense	Characteristic aroma associated with the meat of the animal species
Initial Juiciness 1 = Extremely dry; 8 = Extremely juicy	Amount of fluid exuded on the cut surface when pressed between fingers
Sustained Juiciness 1 = Extremely dry; 8 = Extremely juicy	Amount of water perceived during mastication
First Bite 1 = Extremely tough; 8 = Extremely tender	Force needed to compress the meat sample between molar teeth on the first bite
Residue 1 = Abundant; 8 = None	Amount of connective tissue remaining after mastication
Pork Flavour 1 = Extremely typical 8 = Extremely untypical	Characteristic flavour associated with the meat of the animal species
Metallic Taste 1 = Extreme metallic taste 8 = No metallic taste	Metallic taste experienced while chewing the sample

RESULTS AND DISCUSSION

Shelf-life study

At refrigerated temperatures, pork has a shelf-life of approximately 4 d (Anon., 2002b). The legally set microbiological standard for meat is a general viable count of less than 1 $\times$ 10^7 cfu.g^{-1} (ICMSF, 1986). A microbial analysis was used to determine whether the bacteriocins were an effective preservative to increase the shelf-life of the pork. The pork loins analysed in the shelf-life study contained 73.9% moisture, 23.5% crude protein and 1.3% total fat (w/w). This chemical composition is typical of pork *longissimus lumborum* (Johansson *et al*., 2002; Lindahl *et al*., 2004).

In Fig. 2, the $\log_{10}$ cfu.g^{-1} of meat samples treated with a mixture of bacteriocins and untreated samples are compared. In this pilot study, the maximum acceptable microbial level for red meat (1 × 10^7 cfu.g^{-1}) (ICMSF, 1986) of the untreated sample was reached after just over 6 d, however, this level was reached after just under 8 d in the case of the bacteriocin treated sample. The bacteriocin treatment was thus effective against the microbes naturally present on the meat. This shelf-life expansion of approximately 2 d at 4ºC may have economical benefits for the retail industry. When a statistical model was fitted with both main effects of treatment and time, both of the main effects are significant, however, the contribution of the treatment is low in comparison with the time. When only the treatment is included in the model the r^2 = 0.06, which is very low and the treatment is then not significant. The variation in the data is therefore primarily attributed to time. The differences between the treatments are therefore statistically not significant.

The change in $\log_{10}$ cfu.g^{-1} over 8 d of control pork samples and pork samples treated with either curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins and spiked with *Leuconostoc mesenteroides* subsp. *mesenteroides* is represented in Fig. 3. Statistically (Proc GLM), there were no significant differences between the respective treatments (including the control), however, as expected, time played a significant role in the aerobic cell counts. The aerobic cell counts of day 0 differed significantly ($P \leq 0.05$) from days 2, 4, 6 and 8. The aerobic cell counts of day 2 also differed significantly ($P \leq 0.05$) from that of day 6 and 8. Similarly, the aerobic cell counts from day 4 differed significantly ($P \leq 0.05$) from day 6. The aerobic cell counts from day 2 and 4 did not differ significantly from each other.

In Fig. 4, the control pork samples and pork samples treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins were spiked with *Bacillus* sp. Again the respective treatments did not differ significantly and time was the major influence. There were significant differences ($P \leq 0.05$) between the aerobic cell

counts of day 0 and 8, however day 0 and days 2, 4 and 6 did not differ significantly. The aerobic cell counts from day 2 differed significantly ($P \leq 0.05$) from day 6 and 8, and 4 and 8. The aerobic cell counts from day 4 and 6 did not differ significantly; however, there was a significant ($P \leq 0.05$) difference between that of day 4 and 8. The difference between day 6 and 8 was not significant, however, there was sufficient evidence to indicate that there are a difference at a probability level of 90% ($P = 0.07$).

The microbial cell count of the control pork samples and pork samples treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins inoculated with *Leuconostoc mesenteroides* subsp. *mesenteroides* and the *Bacillus sp.* (1:1) are shown in Fig. 5. The respective treatments did not differ significantly. There were significant differences ($P \leq 0.05$) between the aerobic cell count of day 0 and days 6 and 8. The aerobic cell counts from day 2 differed significantly from that of day 6 and 8. There was also a significant difference ($P \leq 0.05$) between the aerobic cell count of day 4 and 8. The differences between day 4 and 6, and day 6 and 8 was not significant, however, it differs significantly at a probability level of 90% ($P = 0.06$).

From this study, it was clear none of the bacteriocin treatments had any influence on the growth of *Leuconostoc mesenteroides* subsp. *mesenteroides* and the *Bacillus* sp. However, it would be interesting to see whether these bacteriocins would be effective on more closely related bacteria when applied to the meat.

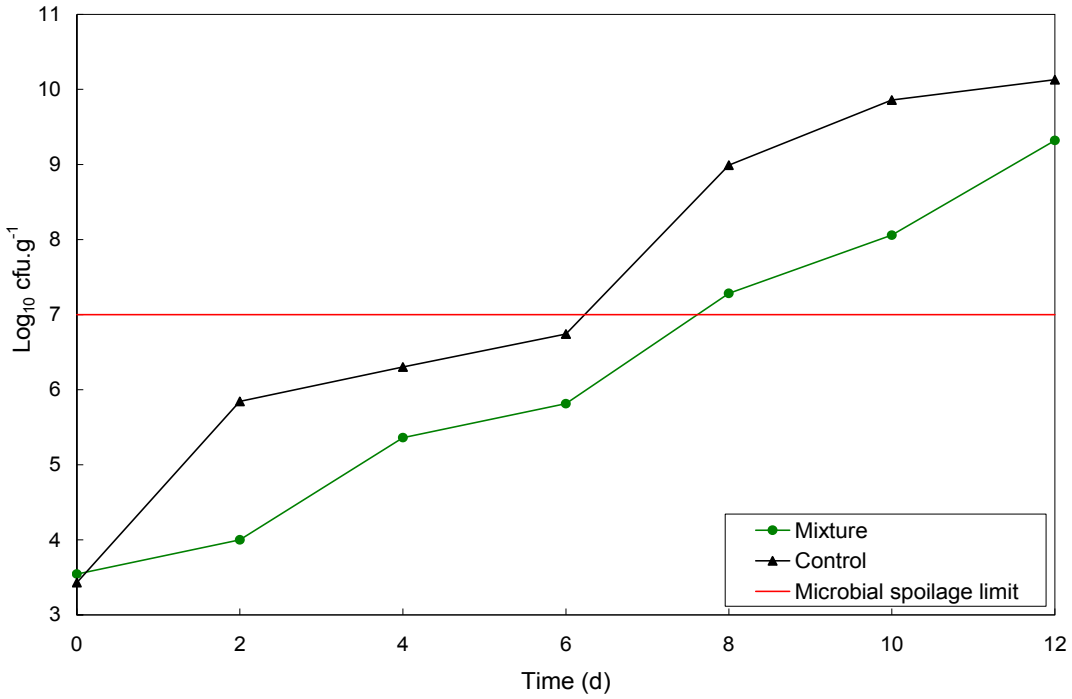


Figure 2. Colony forming units per gram of a pork sample treated with a mixture of curvacin DF 38, plantaricin 423 and caseicin LHS over a period of 12 d (raw data is shown in the Appendix to Chapter 5, Table A1).

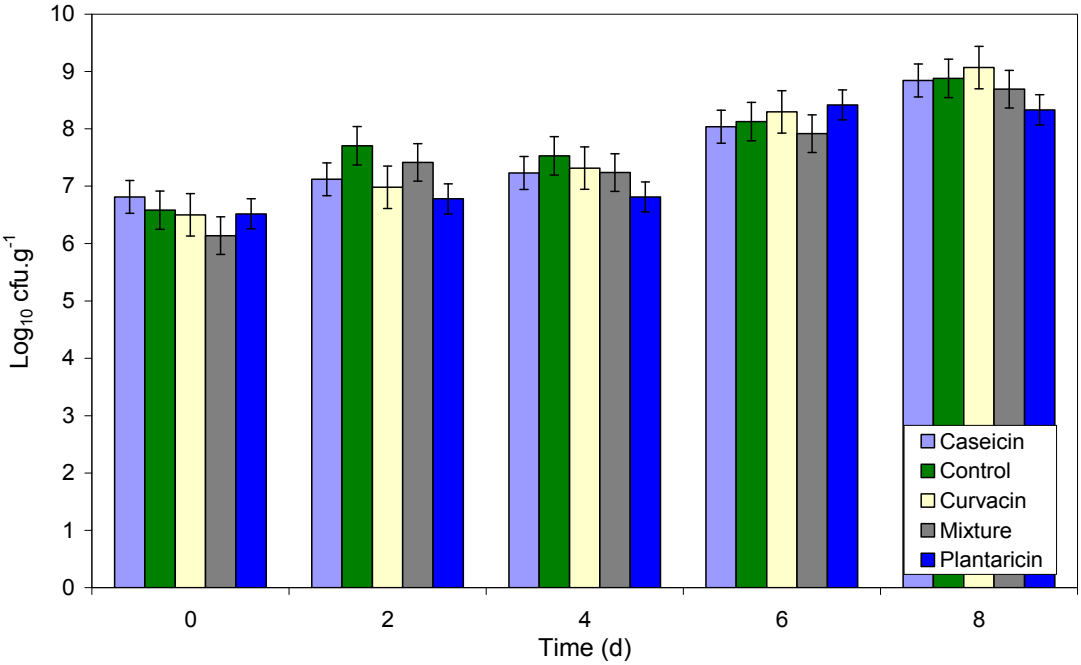


Figure 3. Total colony forming units per gram of pork sample spiked with *Leuconostoc mesenteroides* subsp. *mesenteroides* and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins (raw data is shown in the Appendix to Chapter 5, Table A2).

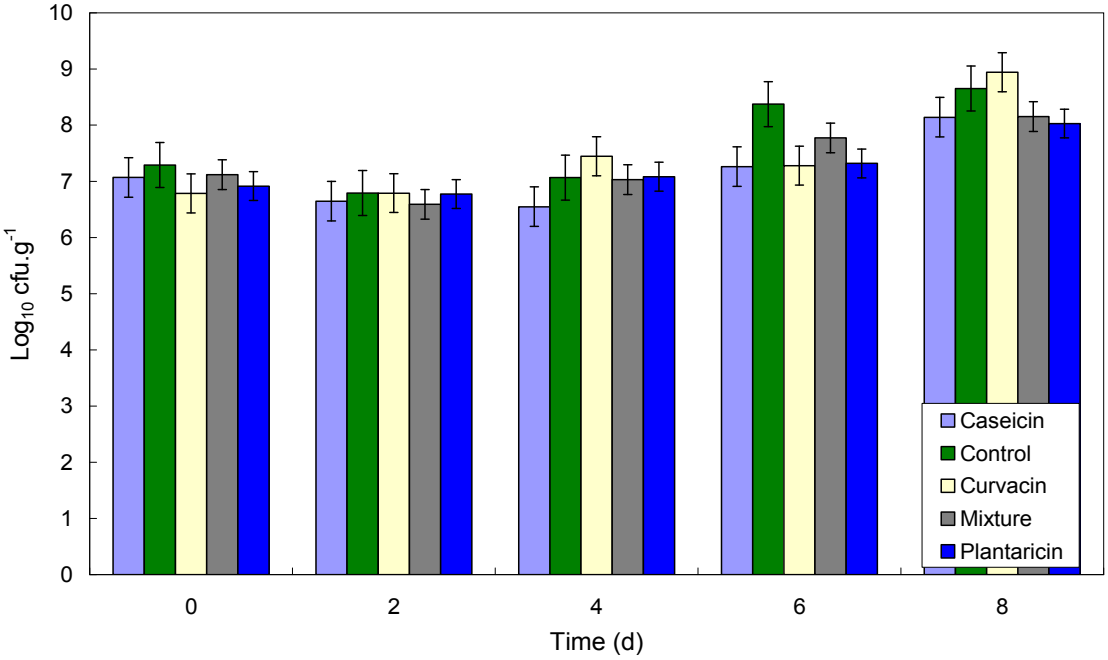


Figure 4. Total colony forming units per gram of pork sample spiked with *Bacillus* sp. and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins (raw data is shown in the Appendix to Chapter 5, Table A3).

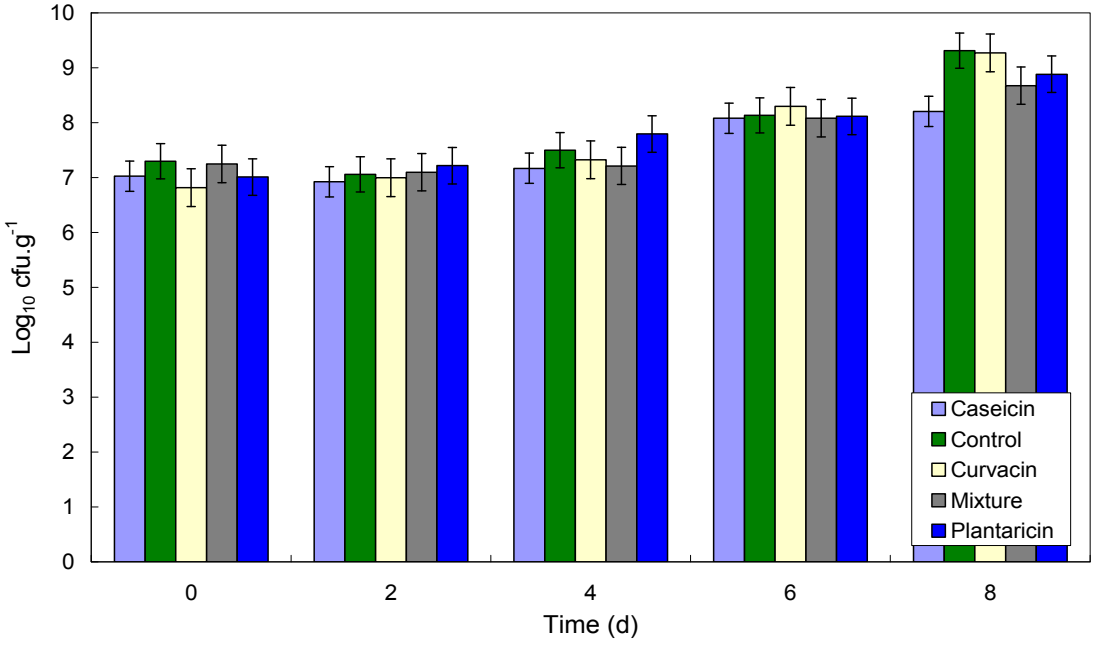


Figure 5. Total colony forming units per gram of pork sample spiked with a mixture of Leuconostoc mesenteroides subsp. Mesenteroides and Bacillus sp. and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins raw data is shown in the Appendix to Chapter 5, Table A4).

Meat colour evaluation

Colour is one of the most important sensory attributes, as colour will influence the purchase intent of the consumer (Borch *et al*., 1996). There were two treatments, including bacteriocin-treated (B) and a control (C), which was not treated, in this experiment. The measurements (L*-, a*-, b*-, h_{ab}- and C*-values) were taken over 14 d on day 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 11 and 14. The results were fitted to a full model (Proc GLM) for time, treatment and for the time-treatment interaction. There was no interaction, but the main effects of treatment and time were significant for L*-, a*-, b*-, h_{ab}- and C*-values.

The L*-value is an indication of the lightness of the meat. In Fig. 6, the change in bL* (L*-value of the bacteriocin treated pork sample) and cL* (L*-value of the control sample) is indicated over 14 d (1 d = 24 h ± 2 h). Although there were changes in bL* and cL*, the L*-values did not change significantly ($P > 0.05$) over time. However, there were significant differences ($P \leq 0.05$) between bL* and cL* for each time period of sampling (Table 2). bL* was initially (day 0) 7.8 units significantly darker than cL* and on day 14 bL* was still significantly darker than cL* ($P \leq 0.05$).

The a*-values indicate the ranking of the meat colour on a red-green scale. The change in ba* and ca* from day 0 to 14, is illustrated in Fig. 7. The bacteriocin treated sample was 3.2 units greener ($P = 0.0022$) than the control (Table 3) on day 0. The control changed by only 2.69 units in comparison with the 5.3 unit change in the control sample over the first 8 d. From day 8 a drop in the a*-values are noted. This could most probably be due to the outgrowth of micro-organisms as the microbial limit (1×10^7 cfu.g^{-1}, ICMSF, 1986) was reached as indicated in the pilot study (See Fig. 2), causing the meat and consequently the colour of the meat to deteriorate more rapidly. The LSM ± SD, and the p-values of the a*-values for the effect of treatment by time is shown in Table 3. The bacteriocin-treated sample (B) and the control (C) differed significantly from day 0 to 14, except on day 5, 8 and 9. The non-significant differences on day 8 and 9 may be explained by microbial deterioration.

The blue-yellow spectrum is indicated by the b*-values. Fig. 8 illustrates the change of the b*-value of the bacteriocin treated and control sample of pork over time. The b* value of the bacteriocin-treated sample was significantly (Table 4) more toward the blue end of the b*-spectrum than the control causing the meat to be perceived as darker (also note that bL* is lower than cL* in Table 2 and Fig. 6). The b*-value for both the control and bacteriocin treated sample decreased by 1.9 and 2.4 units, respectively, over 8 d where after it drastically decreased, once more most probably due to the outgrowth of micro-organisms. The difference in the b*-value between the bacteriocin-treated (B) and the control (C) samples was significant ($P \leq 0.05$) for the whole 14 d trial.

The hue angle (h_{ab}) values of both bh_{ab} and ch_{ab} increased by 7.11 and 13.69 units, respectively over the first 8 d (Fig. 9 and Table 5). However, the hue_{ab}-values of the bacteriocin-treated (B) and the control (C) samples did not differ significantly ($P > 0.05$) over the 14 d period, except on day 0 and day 14. After day 8, there was a drastic increase in both the bh_{ab} and ch_{ab} values, presumably due to the decrease in the ba* and ca*-values during the microbial deterioration.

Figure 10 illustrates the change in the chroma-values over time. There was a gradual decrease in both bC* and cC* over the first 9 d. The C*-values on day 0 for the bacteriocin treated and the control sample differed by 3.7 units, which was significant ($P \leq 0.05$) (Table 6) and on day 8 it differed by 3.1 units ($P \leq 0.05$). After day 8, probably because of microbial deterioration, there was a more rapid decrease in the C*-values. There were a significant difference ($P \leq 0.05$) between bC* and cC* over the 14 d period (Table 6).

Overall, the bacteriocin-treated sample was either darker or browner than the untreated sample. This difference in colour could have been due to the colour of the aqueous bacteriocin solution that was applied to the meat. The colour difference could be avoided by further purifying the bacteriocin solution. However, it must be remembered that microbial deterioration also plays an important role in the colour of the meat.

Table 2. The Least Square Means, Standard Deviation and p-values of L*-values of bacteriocin-treated (bL*) and control sample (cL*) for the effect of treatment by time over a 14 day period (raw data is shown in the Appendix to Chapter 5, Table A5).

Time (d)	[a]LSM ± [b]SD		p
	bL*	cL*	
0	50.39 ± 1.22	58.16 ± 2.15	0.0005
1	51.56 ± 1.48	58.56 ± 2.56	0.0024
2	51.49 ±1.03	58.82 ± 2.78	0.0014
3	51.75 ± 1.34	59.39 ± 2.39	0.0010
4	52.16 ± 1.25	59.17 ± 2.64	0.0019
5	52.50 ± 1.08	58.61 ± 2.38	0.0022
6	52.92 ± 1.41	59.61 ± 2.70	0.0032
7	53.26 ± 1.46	59.81 ± 1.79	0.0013
8	54.04 ± 0.93	59.42 ± 3.01	0.0081
9	53.33 ± 1.03	58.72 ± 3.48	0.0147
11	53.02 ± 1.14	58.76 ± 2.77	0.0053
14	53.33 ± 1.23	58.10 ± 2.88	0.0151

[a]LSM - Least Square Mean

[b]SD - Standard Deviation

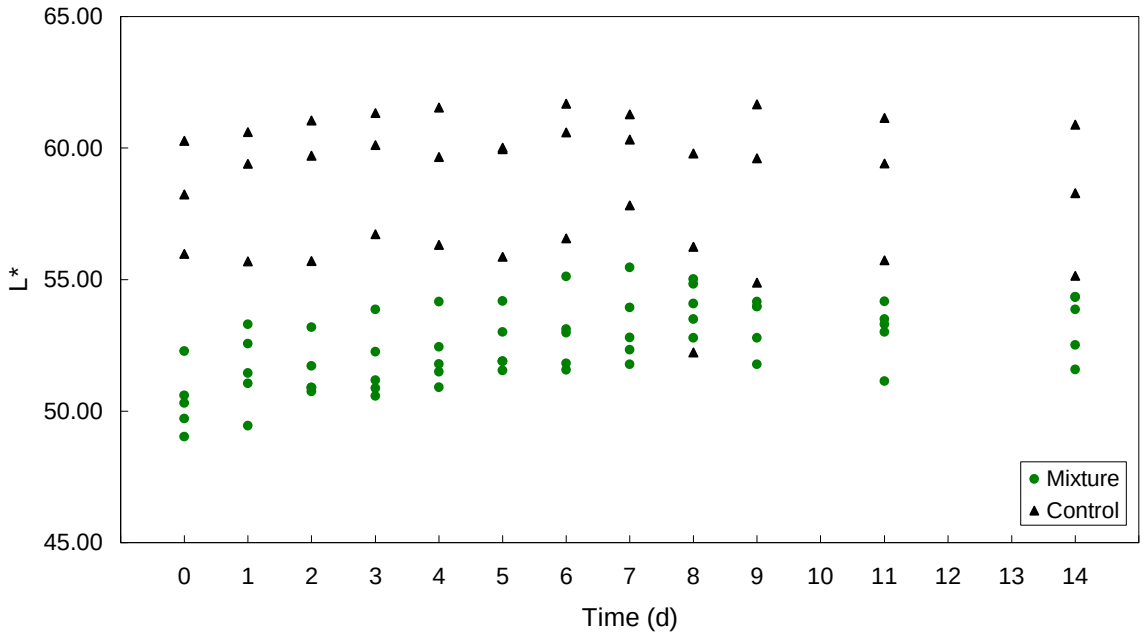


Figure 6. Change in L* over time of a sample treated with a mixture of curvacin, plantaricin and caseicin, and a control sample .

Table 3. The Least Square Means, Standard Deviation and p-values of a*-values of bacteriocin-treated (B) and control sample (C) for the effect of treatment by time over a 14 day period (raw data is shown in the Appendix to Chapter 5, Table A5).

Time (d)	[a]LSM ± [b]SD		p
	ba*	ca*	
0	6.13 ± 1.02	9.37 ± 0.44	0.0022
1	6.30 ± 0.93	8.61 ± 0.88	0.0134
2	6.23 ± 0.80	8.40 ± 1.03	0.0154
3	6.37 ± 0.78	7.89 ± 0.75	0.0356
4	6.33 ± 0.83	7.93 ± 0.83	0.0380
5	6.14 ± 0.71	7.14 ± 1.02	0.1473
6	5.49 ± 0.56	7.05 ± 0.94	0.0240
7	5.24 ± 0.56	6.81 ± 0.68	0.0118
8	3.44 ± 0.92	4.12 ± 0.95	0.3553
9	2.68 ± 0.54	2.67 ± 0.75	0.9650
11	2.30 ± 0.51	3.44 ± 0.44	0.0187
14	2.32 ± 0.53	4.58 ± 0.47	0.0009

[a]LSM - Least Square Mean

[b]SD - Standard Deviation

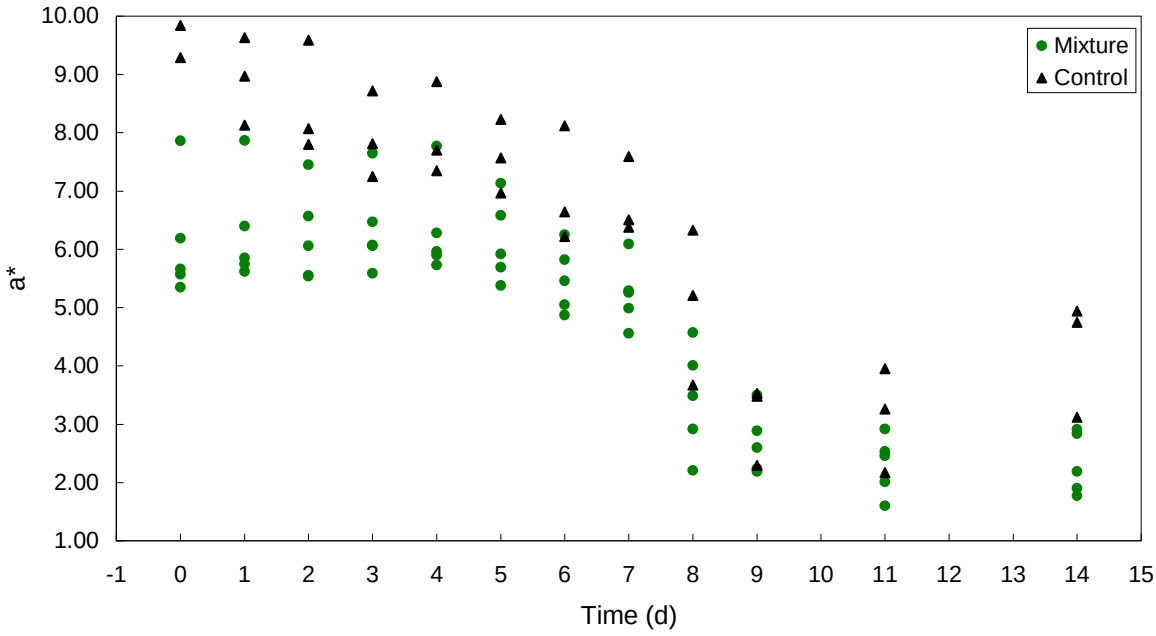


Figure 7. Change in a* over time of a sample treated with a mixture of curvacin, plantaricin and caseicin, and a control sample.

Table 4. The Least Square Means, Standard Deviation and p-values of b*-values of bacteriocin-treated (B) and control sample (C) for the effect of treatment by time over a 14 day period (raw data is shown in the Appendix to Chapter 5, Table A5).

Time (d)	[a]LSM ± [b]SD		p
	bb*	cb*	
0	14.25 ± 0.52	16.77 ± 0.29	0.0003
1	13.80 ± 1.10	16.26 ± 0.45	0.0115
2	13.67 ± 0.75	16.16 ± 0.17	0.0015
3	13.78 ± 0.65	15.98 ± 0.30	0.0017
4	13.67 ± 0.43	15.81 ± 0.42	0.0005
5	13.58 ± 0.56	16.14 ± 0.16	0.0003
6	13.44 ± 0.37	16.21 ± 0.39	< 0.0001
7	13.28 ± 0.62	15.97 ± 0.13	0.0004
8	11.82 ± 0.75	14.83 ± 0.08	0.0005
9	11.36 ± 0.57	14.32 ± 0.67	0.0006
11	11.40 ± 0.59	13.56 ± 0.63	0.0028
14	9.91 ± 0.50	12.43 ± 0.86	0.0017

[a]LSM - Least Square Mean

[b]SD - Standard Deviation

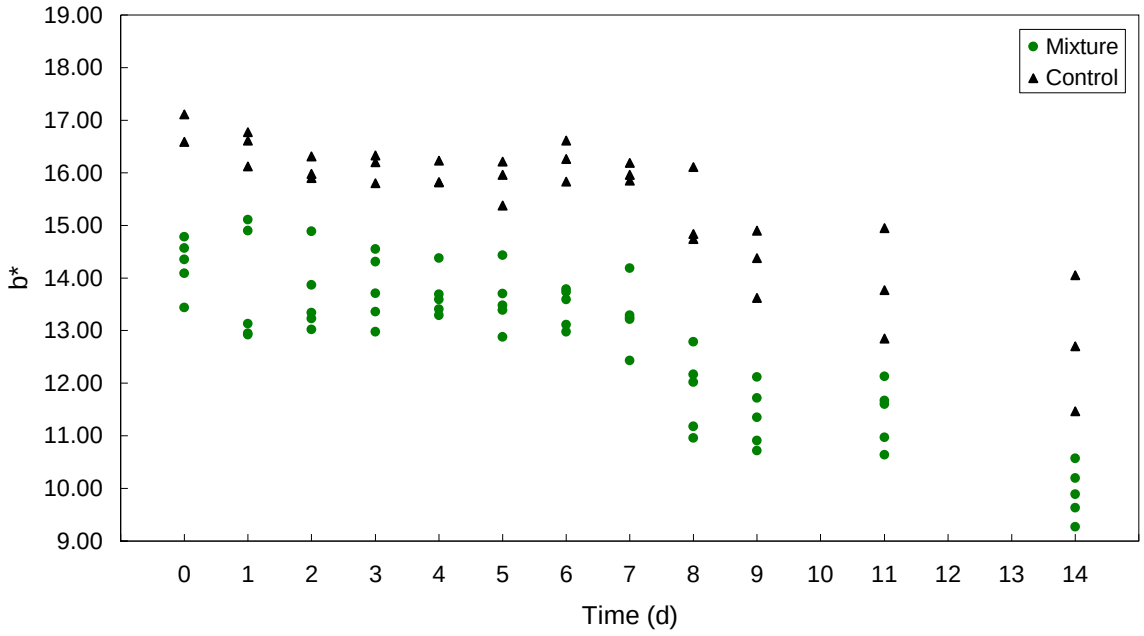


Figure 8. Change in b* over time of a sample treated with a mixture of curvacin, plantaricin and caseicin, and a control sample.

Table 5. The Least Square Means, Standard Deviation and p-values of h_{ab}-values of bacteriocin-treated (B) and control sample (C) for the effect of treatment by time over a 14 day period (raw data is shown in the Appendix to Chapter 5, Table A5).

Time (d)	[a]LSM ± [b]SD		p
	bh_{ab}	ch_{ab}	
0	66.80 ± 3.14	60.82 ± 1.29	0.0221
1	65.56 ± 1.74	62.12 ± 2.63	0.0649
2	65.50 ± 2.88	62.58 ± 3.07	0.2223
3	65.26 ± 2.06	63.74 ± 2.49	0.3839
4	65.21 ± 2.82	63.37 ± 2.50	0.3919
5	65.67 ± 2.83	66.19 ± 3.00	0.8146
6	67.79 ± 2.24	66.56 ±2.34	0.4891
7	68.41 ± 2.98	66.92 ± 2.21	0.4870
8	73.91 ± 3.67	74.51 ± 3.36	0.8260
9	76.71 ± 2.72	79.39 ± 3.42	0.2626
11	78.57 ± 2.66	75.69 ± 2.44	0.1792
14	76.82 ± 3.02	69.68 ± 3.08	0.0184

[a]LSM - Least Square Mean

[b]SD - Standard Deviation

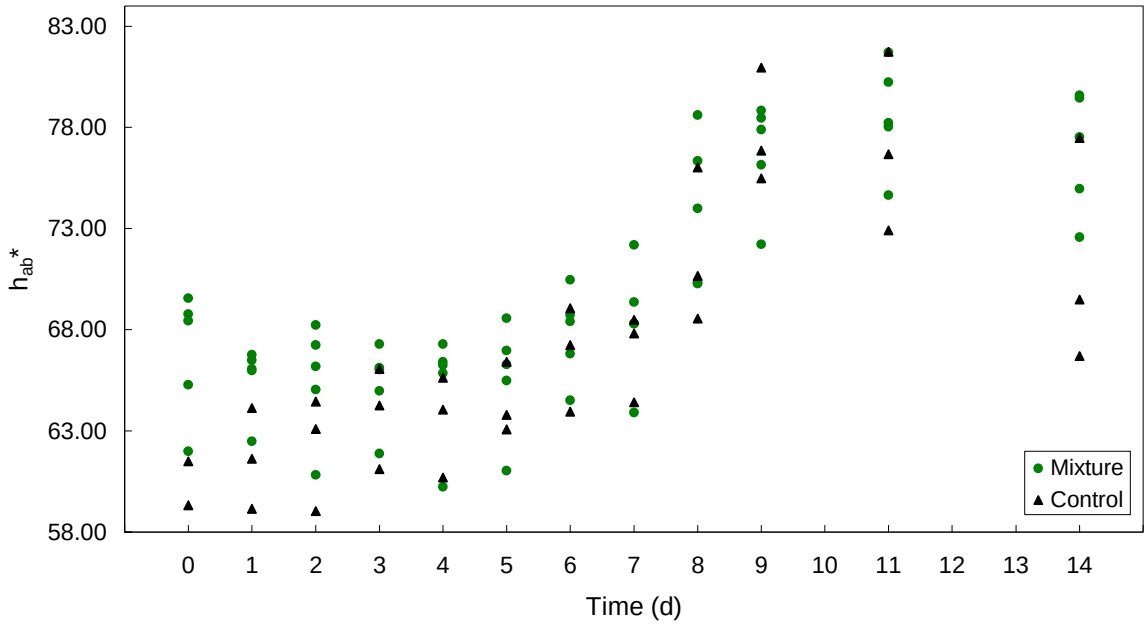


Figure 9. Change in h_{ab} over time of a sample treated with a mixture of curvacin, plantaricin and caseicin, and a control sample.

Table 6. The Least Square Means, Standard Deviation and p-values of C*-values of bacteriocin-treated (B) and control sample (C) for the effect of treatment by time over a 14 day period (raw data is shown in the Appendix to Chapter 5, Table A5).

Time (d)	[a]LSM ± [b]SD		p
	bC*	cC*	
0	15.53 ± 0.74	19.21 ± 0.30	0.0002
1	15.18 ± 1.36	18.41 ± 0.51	0.0083
2	15.04 ± 0.80	18.23 ± 0.35	0.0007
3	15.19 ± 0.84	17.84 ± 0.23	0.0022
4	15.08 ± 0.55	17.70 ± 0.51	0.0005
5	14.91 ± 0.53	17.67 ± 0.44	0.0003
6	14.53 ± 0.36	17.69 ± 0.71	0.0001
7	14.29 ± 0.39	17.37 ± 0.18	< 0.0001
8	12.33 ± 0.89	15.41 ± 0.28	0.0014
9	11.69 ± 0.56	14.58 ± 0.52	0.0004
11	11.64 ± 0.58	14.00 ± 0.49	0.0011
14	10.19 ± 0.50	13.26 ± 0.68	0.0003

[a]LSM - Least Square Mean

[b]SD - Standard Deviation

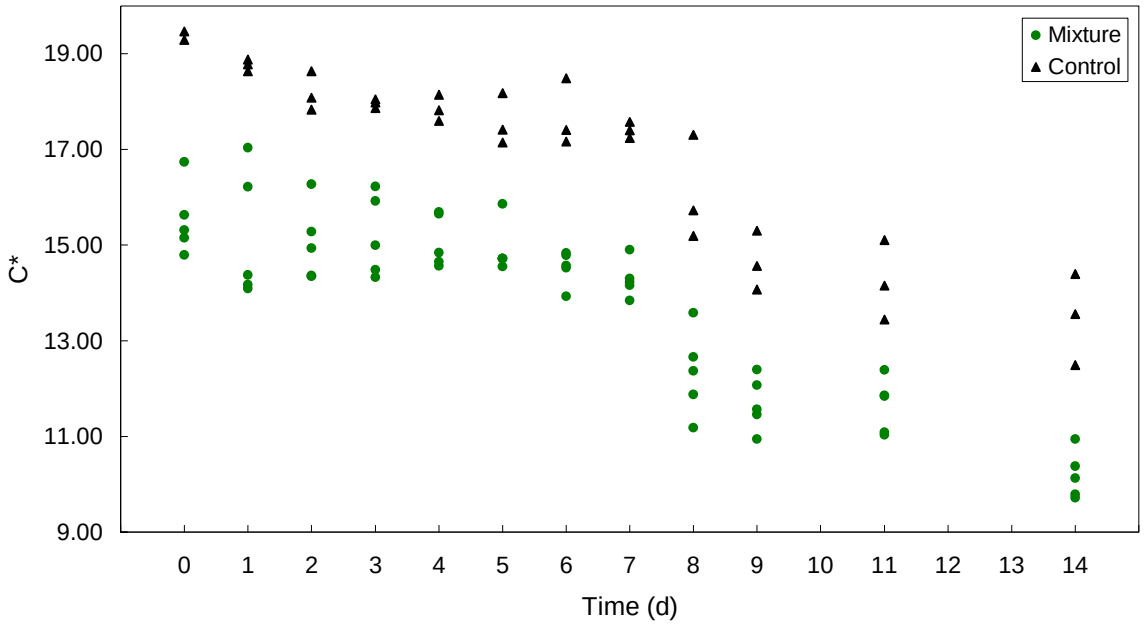


Figure 10. Change in C* over time of a sample treated with a mixture of curvacin, plantaricin and caseicin, and a control sample.

Descriptive sensory analysis

The results of the statistical analysis of the data obtained in the sensory analysis are shown in Table 7. The rank means of the aroma-attribute of the control (C) and the two d treated sample (B_2) and sample B_2 and the four d treated sample (B_4) did not differ significantly ($P \leq 0.05$). However, the control and sample B_4 differed significantly ($P \leq 0.05$). The aroma of the control sample was rated less intense than that of sample B_2 and B_4. This difference could be attributed to enzymatic action of proteolysis, which contributes to the development of meat flavour (Wood *et al.*, 1996), the latter being a combination of odour or aroma and taste. Flavour is also influenced by texture, pH and temperature (Moelich, 1999). Enzymes are more active at refrigerated temperatures than at frozen temperatures (Parson, 1993) and sample B_4 was kept at 4ºC for longer than the control sample, which was only defrosted the day before testing. The bacteriocins itself could also have an effect on the aroma, as they are peptides (Earnshaw, 1992; De Vuyst & Vandamme, 1994a & b; Abee *et al*., 1995; Montville & Winkowski, 1997; O'Keeffe & Hill, 1999; Van Reenen *et al*., 2002), which would denature during the cooking process, contributing to the aroma, and thus flavour (Parson, 1993).

Juiciness is the eating characteristic of meat, which is associated with the fat proportion (Moelich *et al.*, 2003). The initial juiciness of the untreated, two-day and four-day treated samples did not differ significantly ($P \leq 0.05$). In the case of the sustained juiciness, samples C and B_2 and samples B_2 and B_4 did not differ significantly, although sample C and B_4 did differ significantly ($P \leq 0.05$). None the less, for both the initial and the sustained juiciness, sample B_4 was perceived as having a slightly higher juiciness than sample B_2, which was in turn juicier than the control sample. Since the different treated samples came from the muscle of the same animal, it was not expected that the samples would differ.

In the case of the first bite-attribute, the control sample differed significantly ($P \leq 0.05$) from samples B_2 and B_4, which did not differ significantly from each other ($P > 0.05$). Post mortem storage at refrigerated conditions has a tenderising effect on *longissimus* as a result of the enzymatic degradation of myofibrillar and related proteins (Koohmaraie, 1996; Koohmaraie *et al.*, 2002). This may also be linked to the amount of residue left after 20 to 30 chews, however, the residue attribute between the three different samples did not differ significantly ($P > 0.05$), although less residue was recorded for sample B_4 than for sample B_2 and the control sample.

The pork flavour for the control sample was more typical than that of samples B_2 and B_4, however, there was no significant difference between the three samples ($P > 0.05$). In the case of the metallic taste-attribute, samples B_2 and B_4 and the control and sample B_2 did not differ significantly, however, the control sample and sample B_4 differed

significantly ($P \leq 0.05$). The metallic taste was more prominent for sample B_4 than B_2; however, the average rating was below 2, which was described as “practically no metallic taste”. The metallic taste is most probably as a result of the yeast extracts present in the MRS medium in which the bacteriocin-producing microbes were cultured. This potentially unacceptable taste may be avoided by the more extensive purification of the bacteriocins.

The frequency distributions of the scores for the different attributes by the seven panel members are shown in Fig. 11. The frequency distribution for aroma indicates a chi-square value of 7.51 with 8 degrees of freedom. The association between the treatments is not significant ($P > 0.05$). The panel used the range between 4 and 8 to describe aroma. The majority of judges awarded 6 points for the aroma attribute. A high score is perceived as more positive. The frequency distribution for initial juiciness shows that the association between the treatments was not significant (Fig. 11). The chi-square value with 6 degrees of freedom was 3.78. The judges used the 6 most frequently and the scores ranged between 4 and 7. The association between the treatments regarding the sustained juiciness was also not significant ($P > 0.05$). The chi-square value was 10.04 with df = 8. A score range between 3 and 7 was used in this case, however most judges scored the sustained juiciness of the treatments between 4 and 6.

Figure 11 also shows the frequency distribution of the first bite attribute. The chi-square value was 14.72 with 8 degrees of freedom. The first bite attribute was rated between 4 and 8 with the most scores at 6. The association between the treatments was not significant ($P > 0.05$). For all the attributes mentioned thus far, a higher score is regarded as being positive.

The frequency distribution regarding the residue is shown in Fig. 11 (continued). The association between the treatments was not significant; however, the p-value was relatively low compared to that of the previous attributes. The chi-square value was 13.59 with df = 8. Although a range from 4 to 8 was used to describe the residue, most of the judges scored the residue between 6 and 8. Again a higher score is perceived as positive for the residue attribute as is the case with the pork flavour attribute. Most of the judges used 5 to 7 to indicate the pork flavour. The chi-square for the pork flavour was 3.26 with six degrees of freedom. Again, the association between the treatments was not significant.

The frequency table of metallic taste in Fig. 11 shows that the association between the treatments was not significant ($P > 0.05$). Chi-square for metallic taste was 9.90 (df = 10). Most of the judges used the lower range of the scale, indicating that this unacceptable flavour was barely noticeable. Most of the judges rated the metallic taste as 1. The range from 2 to 4 was also used frequently.

Table 7. The effect of bacteriocin treatment (1:1:1) on the different attributes of sensory evaluation

Attribute	Treatment			[3]LSD (p = 0.05)	[4]MSE (df = 84)
	Control	[1]B_2	[2]B_4		
Aroma (rank mean)	5.7 (1.8)[a]	5.9 (2.0)[ab]	6.1 (2.2)[b]	0.3774	0.613095
Initial Juiciness (rank mean)	5.5 (1.9)	5.6 (2.0)	5.7 (2.1)	0.2877	0.356293
Sustained Juiciness (rank mean)	4.8 (1.7)[a]	5.1 (2.1)[ab]	5.2 (2.2)[b]	0.3850	0.637755
First Bite (rank mean)	5.4 (1.7)[a]	5.9 (2.2)[b]	5.9 (2.1)[b]	0.4072	0.713435
Residue (rank mean)	6.5 (1.8)	6.8 (2.0)	6.9 (2.2)	0.3703	0.590136
Pork Flavour (rank mean)	6.1 (2.1)	6.0 (2.0)	6.0 (1.9)	0.3365	0.487245
Metallic Taste (rank mean)	1.4 (1.8)[a]	1.7 (2.0)[ab]	1.9 (2.2)[b]	0.3423	0.504252

[1]B_2 - Sample treated with bacteriocins two days before sensory evaluation

[2]B_4 - Sample treated with bacteriocins four days before sensory evaluation

[3]Tukey's Least Significant Difference at a 5% significance level

[4]MSE - Mean Square Error

[a]Rank means in the same row with the same letter(s) do not differ significantly at a 5% significance level according to Tukey's LSD

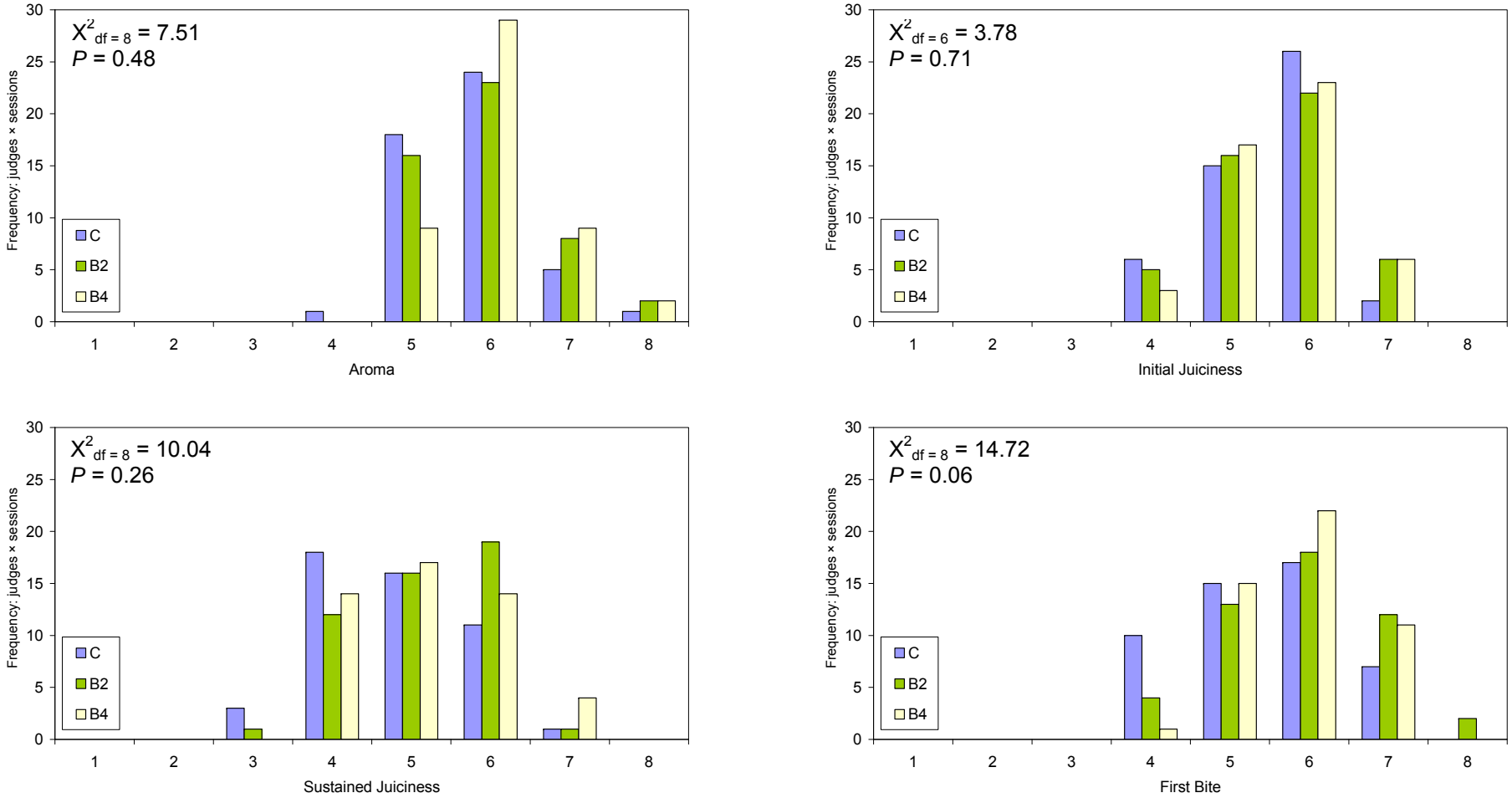
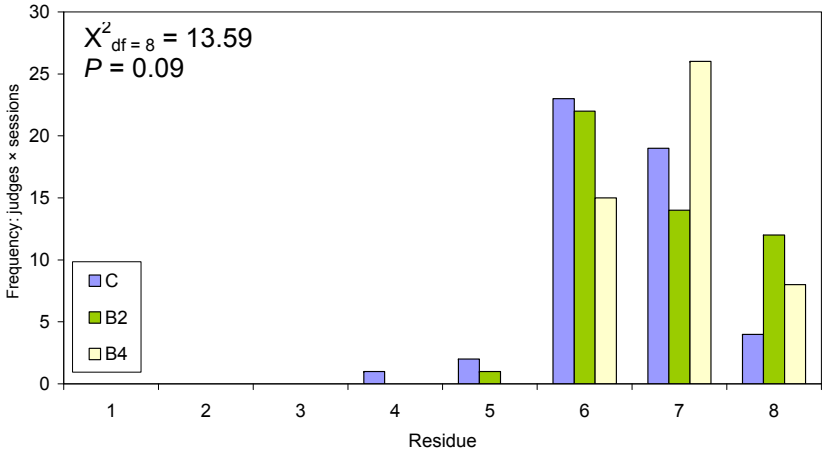


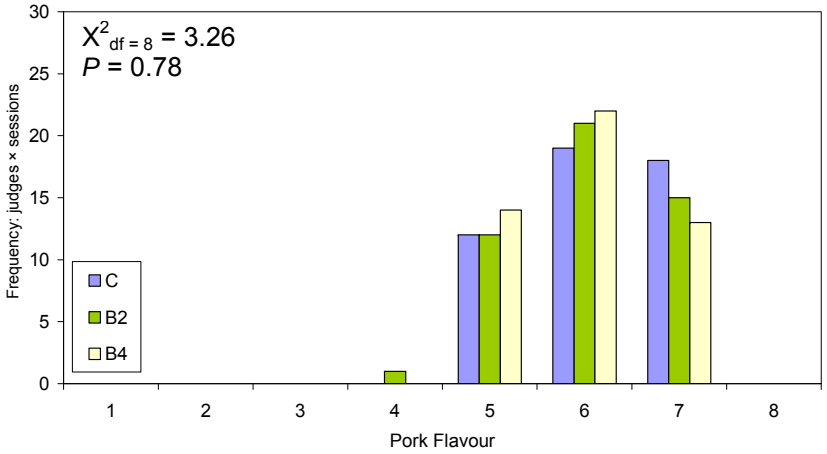
Figure 11. Contingency distributions of the scores awarded for the Aroma (A), Initial Juiciness (B), Sustained Juiciness (C) and First Bite-attributes (D) by the seven-member panel in the descriptive sensory evaluation study.

where C = Control

B_2 = Two-day treated sample

B_4 = Four-day treated sample





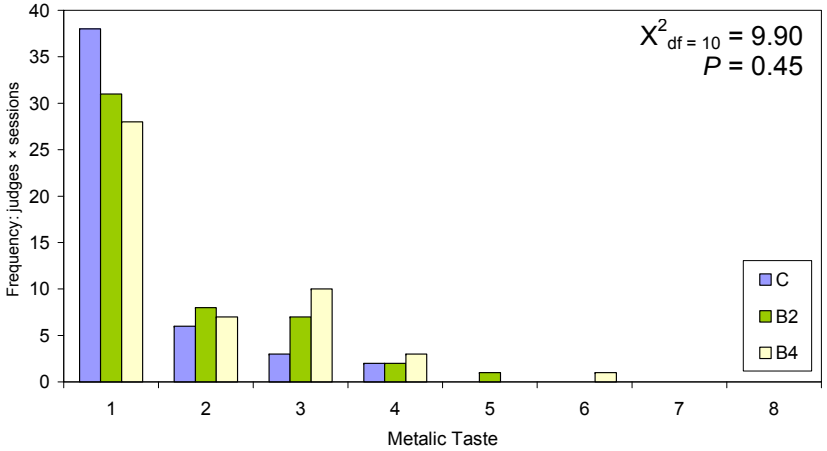


Figure 11 (continued). Contingency distributions of the scores awarded for the Residue (E), Pork Flavour (F) and Metallic Taste-attributes (G) by the seven-member panel in the descriptive sensory evaluation study.

where C = Control

B_2 = Two-day treated sample

B_4 = Four-day treated sample

CONCLUSIONS

Bacteriocins produced by *Lactobacillus curvatus* DF38, *Lb. plantarum* 423 and *Lb. casei* LHS are able to extend the shelf-life of pork loin chops by up to 2 d as shown in a pilot study. The treatment of pork spiked with *Leuconostoc mesenteroides* subsp. *mesenteroides,* a *Bacillus* sp. or a mixture (1:1) of the two, with curvacin, plantaricin, caseicin or a mixture (1:1:1) did not differ significantly from each other or the untreated samples. The total aerobic cell count only changed significantly over time.

The meat colour evaluation indicated that the bacteriocin treated sample was darker (L*-values) than the control sample, however it only differed significantly ($P \leq 0.05$) directly after application where after the treated and untreated samples did not differ significantly ($P > 0.05$). The a*-values of the treated and untreated samples differed significantly from day 0 up to day 7. After day 7 there was a drastic drop in the a*-value, probably as a result of microbial spoilage. The a*-values from day 8 onward did not differ significantly. The b*-values of the treated and untreated samples did differ significantly throughout the study.

The sensory evaluation indicated that there were no significant difference ($P \leq 0.05$) between the control (C) and two day-treated sample (B_2) and sample B_2 and the four day-treated sample (B_4), regarding the aroma, sustained juiciness, first bite and metallic taste attributes, however C and B_4 for these attributes differed significantly. The difference in the aroma and first bite attributes could be due to enzymatic action, as the samples were not kept at 4ºC for the same period of time. The metallic taste was more prominent for B_4 than B_2; however, the average rating was below 2, which was described as “practically no metallic taste”. The initial juiciness, residue and pork flavour of the treated samples and the control did not differ significantly ($P > 0.05$).

Although the microbiological shelf-life of pork loin chops was extended by up to 2 d, it is important that further research focusing on means to eliminate the potentially negative sensory effects (darker colour, metallic taste). Further purification of the bacteriocins is an option; however an alternative growth medium might be a more economical option.

Presently in South Africa, it is illegal to add substances, such as bacteriocins, to fresh meat (Anon., 1972). However, as shown in this investigation, the bacteriocins tested did increase the shelf-life of the pork. These investigations have to be extended to include other bacteriocins and applying the bacteriocins to other meat species. If these results are positive, steps can be initiated to have the use of bacteriocins made legal in South Africa.

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APPENDIX

To Chapter Five

Tables A1 – A5 are given in this Appendix. The data recorded was placed in a separate appendix to simplify the discussion section of this chapter.

Table A1. Colony forming units per gram of a pork sample treated with a mixture of curvacin DF 38, plantaricin 423 and caseicin LHS over a period of 12 d.

Time (d)	Mixture	Log_{10} Mixture	Mixture	Log_{10} Control
0	3.50×10^3	3.54	2.70×10^3	3.43
2	1.00×10^4	4.00	7.00×10^5	5.85
4	2.30×10^5	5.36	2.00×10^6	6.30
6	6.50×10^5	5.81	5.50×10^6	6.74
8	1.92×10^7	7.28	9.80×10^8	8.99
10	1.14×10^8	8.06	7.24×10^9	9.86
12	2.09×10^9	9.32	1.35×10^{10}	1.01

Table A2. Total colony forming units per gram of pork sample spiked with *Leuconostoc mesenteroides* subsp. *mesenteroides* and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins.

Time (d)	Mixture		Curvacin DF 38		Plantaricin 423		Caseicin LHS		Control	
	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$
0	3.70×10^{7}	7.57	3.90×10^{7}	7.59	1.07×10^{7}	7.03	6.70×10^{7}	7.83	4.10×10^{7}	7.61
0	2.51×10^{6}	6.40	3.70×10^{7}	7.57	2.40×10^{6}	6.38	2.40×10^{6}	6.38	2.70×10^{6}	6.43
0	2.80×10^{4}	4.45	2.20×10^{4}	4.34	1.40×10^{6}	6.15	1.70×10^{6}	6.23	5.00×10^{5}	5.70
2	3.90×10^{7}	7.59	7.80×10^{7}	7.89	1.47×10^{7}	7.17	1.83×10^{8}	8.26	1.70×10^{8}	8.23
2	2.70×10^{7}	7.43	4.60×10^{6}	6.66	8.60×10^{6}	6.93	3.40×10^{6}	6.53	3.11×10^{8}	8.49
2	1.67×10^{7}	7.22	2.45×10^{6}	6.39	1.72×10^{6}	6.24	3.70×10^{6}	6.57	2.49×10^{6}	6.40
4	1.66×10^{8}	8.22	5.60×10^{8}	8.75	4.80×10^{7}	7.68	5.40×10^{8}	8.73	4.40×10^{8}	8.64
4	3.00×10^{7}	7.48	1.10×10^{7}	7.04	4.40×10^{6}	6.64	8.00×10^{6}	6.90	.	.
4	1.03×10^{6}	6.01	1.43×10^{6}	6.16	1.31×10^{6}	6.12	1.14×10^{6}	6.06	2.60×10^{6}	6.41
6	2.20×10^{9}	9.34	4.20×10^{9}	9.62	9.10×10^{8}	8.96	1.20×10^{9}	9.08	2.15×10^{9}	9.33
6	2.49×10^{7}	7.40	2.26×10^{8}	8.35	1.09×10^{8}	8.04	2.11×10^{7}	7.32	1.41×10^{8}	8.15
6	1.02×10^{7}	7.01	8.10×10^{6}	6.91	1.80×10^{8}	8.26	5.10×10^{7}	7.71	8.00×10^{6}	6.90
8	3.90×10^{9}	9.59	2.71×10^{9}	9.43	8.80×10^{8}	8.94	1.11×10^{9}	9.05	2.04×10^{9}	9.31
8	1.17×10^{8}	8.07	4.80×10^{8}	8.68	2.12×10^{7}	7.33	3.50×10^{8}	8.54	2.82×10^{8}	8.45
8	2.60×10^{8}	8.41	1.24×10^{9}	9.09	5.30×10^{8}	8.72	8.70×10^{8}	8.94	.	.

Table A3. Total colony forming units per gram of pork sample spiked with *Bacillus* sp. and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins.

Time (d)	Mixture		Curvacin DF 38		Plantaricin 423		Caseicin LHS		Control	
	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$	$cfu.g^{-1}$	Log_{10} $cfu.g^{-1}$
0	1.80×10^{8}	8.26	3.00×10^{8}	8.48	1.85×10^{8}	8.27	1.17×10^{8}	8.07	1.50×10^{8}	8.18
0	1.28×10^{7}	7.11	7.60×10^{6}	6.88	3.00×10^{7}	7.48	1.53×10^{7}	7.18	2.50×10^{7}	7.40
0	1.00×10^{6}	6.00	1.00×10^{5}	5.00	1.00×10^{5}	5.00	9.00×10^{5}	5.95	2.00×10^{6}	6.30
2	6.20×10^{7}	7.79	1.40×10^{8}	8.15	5.00×10^{7}	7.70	1.00×10^{8}	8.00	1.35×10^{8}	8.13
2	6.40×10^{7}	6.81	8.40×10^{6}	6.92	5.00×10^{6}	6.70	2.20×10^{7}	7.34	2.50×10^{7}	7.40
2	1.50×10^{5}	5.18	2.00×10^{5}	5.30	8.40×10^{5}	5.92	4.00×10^{4}	4.60	7.00×10^{4}	4.85
4	9.10×10^{7}	7.96	8.70×10^{7}	7.94	5.00×10^{7}	7.70	6.30×10^{7}	7.80	7.60×10^{8}	8.88
4	6.50×10^{6}	6.81	9.00×10^{6}	6.95	1.42×10^{7}	7.15	7.80×10^{6}	6.89	2.60×10^{7}	7.41
4	2.08×10^{6}	6.32	.	.	2.50×10^{6}	6.40	9.00×10^{4}	4.95	8.00×10^{4}	4.90
6	1.84×10^{8}	8.26	1.53×10^{8}	8.18	4.70×10^{7}	7.67	.	.	6.00×10^{8}	8.78
6	1.60×10^{7}	7.20	2.25×10^{7}	7.35	8.40×10^{6}	6.92	1.83×10^{7}	7.26	9.30×10^{7}	7.97
6	7.10×10^{7}	7.85	2.00×10^{6}	6.30	2.31×10^{7}	7.36	.	.	.	.
8	3.29×10^{7}	7.52	9.85×10^{8}	8.99	2.67×10^{7}	7.43	4.66×10^{7}	7.67	.	.
8	4.40×10^{7}	7.64	8.30×10^{8}	8.92	2.40×10^{7}	7.38	3.90×10^{7}	7.59	.	.
8	1.99×10^{9}	9.30	8.20×10^{8}	8.91	1.90×10^{9}	9.28	1.46×10^{9}	9.16	4.50×10^{8}	8.65

Table A4. Total colony forming units per gram of pork sample spiked with *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Bacillus* sp. and treated with curvacin DF 38, plantaricin 423, caseicin LHS or a mixture of the three bacteriocins.

Time (d)	Mixture		Curvacin DF 38		Plantaricin 423		Caseicin LHS		Control	
	cfu.g^{-1}	Log_{10} cfu.g^{-1}	cfu.g^{-1}	Log_{10} cfu.g^{-1}	cfu.g^{-1}	Log_{10} cfu.g^{-1}	cfu.g^{-1}	Log_{10} cfu.g^{-1}	cfu.g^{-1}	Log_{10} cfu.g^{-1}
0	3.90×10^{8}	8.59	9.00×10^{7}	7.95	9.60×10^{7}	7.98	7.50×10^{7}	7.88	7.70×10^{7}	7.89
0	9.40×10^{6}	6.97	2.39×10^{7}	7.38	1.24×10^{7}	7.09	1.77×10^{7}	7.25	1.23×10^{7}	7.09
0	1.50×10^{6}	6.18	1.30×10^{5}	5.11	9.00×10^{5}	5.95	9.00×10^{5}	5.95	8.30×10^{6}	6.92
2	3.90×10^{8}	8.59	2.93×10^{8}	8.47	2.92×10^{8}	8.47	7.50×10^{7}	7.88	2.63×10^{8}	8.42
2	8.90×10^{6}	6.95	6.50×10^{6}	6.81	2.50×10^{7}	7.40	4.90×10^{6}	6.69	9.10×10^{6}	6.96
2	5.60×10^{5}	5.75	5.10×10^{5}	5.71	6.10×10^{5}	5.79	1.59×10^{6}	6.20	6.20×10^{5}	5.79
4	1.45×10^{9}	9.16	2.64×10^{8}	8.42	3.67×10^{9}	9.56	3.30×10^{8}	8.52	7.50×10^{8}	8.88
4	6.50×10^{6}	6.81	1.80×10^{7}	7.26	6.00×10^{7}	7.78	8.20×10^{6}	6.91	2.53×10^{7}	7.40
4	4.60×10^{5}	5.66	1.97×10^{6}	6.29	1.10×10^{6}	6.04	1.19×10^{6}	6.08	1.64×10^{6}	6.21
6	2.93×10^{9}	9.47	1.17×10^{9}	9.07	3.70×10^{9}	9.57	2.00×10^{9}	9.30	1.22×10^{9}	9.09
6	3.20×10^{7}	7.51	2.29×10^{8}	8.36	2.14×10^{7}	7.33	1.21×10^{8}	8.08	8.30×10^{7}	7.92
6	1.90×10^{7}	7.28	2.90×10^{7}	7.46	2.80×10^{7}	7.45	7.30×10^{6}	6.86	2.47×10^{7}	7.39
8	1.93×10^{9}	9.29	2.09×10^{9}	9.32	2.71×10^{9}	9.43	1.80×10^{8}	8.26	3.20×10^{10}	10.50
8	2.77×10^{7}	7.44	3.80×10^{9}	9.58	8.60×10^{7}	7.93	1.56×10^{7}	7.19	1.67×10^{8}	8.22
8	1.99×10^{9}	9.30	8.20×10^{8}	8.91	1.90×10^{9}	9.28	1.46×10^{9}	9.16	1.62×10^{9}	9.21

Table A5. Change in bL*, ba*, bb* bh_{ab} and bC* (treated with a mixture of curvacin, plantaricin and caseicin) and cL*, ca*, cb*, ch_{ab} and cC* (control) over 14 d.

Time (d)	bL*	cL*	ba*	ca*	bb*	cb*	bh	ch	bC*	cC*
1	49.03	55.98	7.86	9.84	14.78	16.59	62.00	59.33	16.74	19.29
1	50.31	58.23	5.57	9.29	14.09	17.11	68.43	61.50	15.15	19.47
1	49.72	60.27	6.19	8.97	13.44	16.61	65.27	61.63	14.80	18.88
1	50.60	.	5.35	.	14.35	.	69.55	.	15.31	.
1	52.28	.	5.66	.	14.57	.	68.77	.	15.63	.
2	49.45	55.69	7.87	9.63	15.11	16.12	62.49	59.15	17.04	18.78
2	51.05	59.40	5.62	8.13	12.92	16.77	66.49	64.14	14.09	18.64
2	51.44	60.60	5.85	8.07	13.13	15.90	65.98	63.09	14.37	17.83
2	52.56	.	5.75	.	12.95	.	66.06	.	14.17	.
2	53.30	.	6.40	.	14.90	.	66.76	.	16.22	.
3	50.89	55.71	7.45	9.59	13.34	15.98	60.82	59.03	15.28	18.64
3	50.75	59.71	5.55	7.80	13.23	16.31	67.24	64.44	14.35	18.08
3	50.89	61.05	5.54	7.81	13.87	16.20	68.23	64.26	14.94	17.98
3	51.71	.	6.06	.	13.02	.	65.04	.	14.36	.
3	53.19	.	6.57	.	14.89	.	66.19	.	16.28	.
4	50.58	56.72	7.65	8.72	14.31	15.80	61.87	61.11	16.23	18.05
4	50.87	60.11	5.59	7.25	13.36	16.33	67.29	66.06	14.48	17.87
4	51.18	61.33	6.07	7.70	13.71	15.82	66.12	64.05	14.99	17.59
4	52.25	.	6.06	.	12.98	.	64.97	.	14.32	.
4	53.87	.	6.47	.	14.55	.	66.03	.	15.92	.

Table A5 (continued). Change in bL*, ba*, bb* bh_{ab} and bC* (treated with a mixture of curvacin, plantaricin and caseicin) and cL*, ca*, cb*, ch_{ab} and cC* (control) over 14 d.

Time (d)	bL*	cL*	ba*	ca*	bb*	cb*	bh	ch	bC*	cC*
5	50.91	56.32	7.77	8.88	13.59	15.82	60.24	60.69	15.65	18.14
5	51.50	59.66	5.90	7.35	13.41	16.23	66.25	65.64	14.65	17.82
5	51.79	61.54	5.73	7.57	13.69	15.38	67.29	63.79	14.84	17.14
5	52.44	.	5.96	.	13.29	.	65.85	.	14.57	.
5	54.16	.	6.28	.	14.38	.	66.41	.	15.69	.
6	51.55	55.86	7.13	8.23	12.88	16.21	61.03	63.08	14.72	18.18
6	51.90	59.96	5.38	6.97	13.70	15.96	68.56	66.41	14.72	17.42
6	51.89	60.02	5.92	6.22	13.48	16.26	66.29	69.07	14.72	17.41
6	53.00	.	5.69	.	13.39	.	66.98	.	14.55	.
6	54.18	.	6.58	.	14.43	.	65.49	.	15.86	.
7	51.81	56.56	6.25	8.12	13.11	16.61	64.51	63.95	14.52	18.49
7	51.57	60.59	5.82	6.64	13.59	15.83	66.82	67.24	14.78	17.17
7	52.98	61.68	5.46	6.38	13.79	16.19	68.40	68.49	14.83	17.40
7	53.11	.	5.05	.	12.98	.	68.74	.	13.93	.
7	55.12	.	4.87	.	13.73	.	70.47	.	14.57	.
8	52.33	57.82	6.09	7.59	12.43	15.85	63.90	64.41	13.84	17.57
8	51.78	60.32	5.26	6.51	13.22	15.96	68.30	67.81	14.23	17.24
8	52.80	61.28	5.29	6.33	13.29	16.11	68.30	68.55	14.30	17.31
8	53.94	.	4.99	.	13.25	.	69.36	.	14.16	.
8	55.46	.	4.56	.	14.19	.	72.19	.	14.90	.

Table A5 (continued). Change in bL*, ba*, bb* bh_{ab} and bC* (treated with a mixture of curvacin, plantaricin and caseicin) and cL*, ca*, cb*, ch_{ab} and cC* (control) over 14 d.

Time (d)	bL*	cL*	ba*	ca*	bb*	cb*	bh	ch	bC*	cC*
9	53.49	56.25	4.01	5.21	11.18	14.84	70.27	70.65	11.88	15.73
9	52.79	59.79	2.92	3.67	12.02	14.74	76.35	76.02	12.37	15.19
9	55.02	52.23	3.49	3.48	12.17	14.90	74.00	76.85	12.66	15.30
9	54.84	.	4.57	.	12.79	.	70.34	.	13.58	.
9	54.08	.	2.21	.	10.96	.	78.60	.	11.18	.
10	51.78	54.88	3.50	3.53	10.91	13.62	72.21	75.47	11.46	14.07
10	52.78	59.61	2.19	2.29	10.72	14.38	78.45	80.95	10.94	14.56
10	53.97	61.66	2.89	2.17	11.72	14.95	76.15	81.74	12.07	15.11
10	53.98	.	2.60	.	12.12	.	77.89	.	12.40	.
10	54.16	.	2.24	.	11.35	.	78.84	.	11.57	.
11	51.14	55.73	2.92	3.95	10.64	12.85	74.65	72.91	11.03	13.44
11	53.01	59.41	1.60	3.26	10.97	13.77	81.70	76.68	11.09	14.15
11	53.30	61.15	2.53	3.12	12.13	14.05	78.22	77.48	12.39	14.39
11	53.49	.	2.46	.	11.60	.	78.03	.	11.86	.
11	54.17	.	2.01	.	11.67	.	80.23	.	11.84	.
14	52.51	55.14	2.91	4.94	9.27	11.47	72.57	66.70	9.72	12.49
14	54.34	58.28	1.90	4.75	10.20	12.70	79.45	69.49	10.38	13.56
14	53.87	60.89	1.77	4.05	9.63	13.13	79.59	72.86	9.79	13.74
14	51.58	.	2.84	.	10.57	.	74.96	.	10.94	.
14	54.33	.	2.19	.	9.89	.	77.51	.	10.13	.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Consumers are, as a consequence of market globalisation, demanding high quality meat products that have a relatively long shelf-life and the appearance of minimally processed food (Hugas *et al*., 2002; Ross *et al*., 2002). The use of chemical preservatives, currently employed to limit the number of micro-organisms capable of growing in foods, is increasingly being questioned in terms of their effect on human health (Kennedy *et al*., 2000). To accommodate the demands of the consumer without compromising the safety of the product, new preservation technologies in the meat and food industry are necessary (Hugas *et al*., 2002; Ross *et al*., 2002). The aim of this study was thus to determine the activity of bacteriocins produced by *Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *Pediococcus pentosaceus* ATCC 43201 against micro-organisms isolated from red meat for the possible preservation of fresh red meat at refrigerated temperatures.

The microbiological evaluation of beef, lamb and pork loin chops obtained from local retailers, revealed a high number of viable cells per gram (cfu.g^{-1}) ranging from 80 cfu.g^{-1} to 1.4 × 10^{8} cfu.g^{-1} three days after the sell-by date at 4ºC. In some cases the aerobic count reached the microbial spoilage limit of 1 × 10^{7} cfu.g^{-1} (ICMSF, 1986). Fifty three percent of the isolates were Gram-negative, 35% Gram-positive and 12% identified as yeast. From the results, it was clear that the microbial population of the meat was greatly influenced by the origin, i.e. the retailer, rather than by the meat species. This observation confirms the statement by Merck (undated), that most of the microbes on the meat are acquired during the dressing process. To prevent or lower the level of contamination of the meat before packaging, systems like HACCP (Kennedy *et al*., 2000; Panisello *et al.*, 2000) should be implemented. To prevent the possible microbial increase of the spoilage and potentially pathogenic microbes in the consumers' refrigerator and the subsequent extension of the shelf-life of the meat, a preservative of some kind is required.

Bacteriocins are a bio-active, heterogeneous group of ribosomally synthesized antimicrobial proteins, peptides or peptide complexes that are extracellularly released and inhibitory or lethal to other, mostly taxonomically closely related bacteria, but also bacteria confined within the same ecological niche. Since bacteriocins vary in activity spectrum, mode of action, molecular weight, genetic origin and biochemical properties, each bacteriocin must be studied before being applied to any food product. Thus, the

bacteriocins produced by the *E. faecalis* BFE 1071, *Lb. curvatus* DF 38, *Lb. plantarum* 423, *Lb. casei* LHS, *Lb. salivarius* 241 and *P. pentosaceus* ATCC 43201 strains were screened for activity against the microbes isolated from red meat samples that were obtained from the commercial retailers. The results showed that 16 to 21% of the meat isolates were sensitive to these bacteriocins, including members of *Klebsiella*, *Shigella*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus* and *Bacillus* as well as some yeast. This indicated that bacteriocins might be efficient in extending the shelf-life of red meat.

Three bacteriocins that were produced by the *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS strains were found to be stable and were most active against the meat isolates. The inhibitory spectrum, size, sensitivity to heat, pH, NaCl and impact of proteolytic enzymes were determined. According to the size (2.35 and 3.4 kDa) and the heat stability (60 min at 100ºC) these bacteriocins were classified as Class I lantibiotics using the classification of Klaenhammer (1993), De Vuyst & Vandamme (1994) and Cleveland *et al.* (2001). The bacteriocins were also found to be stable in buffers ranging from pH 2 to 10 and in NaCl concentrations ranging from 0.1 to 10.0%. All three bacteriocins were sensitive to Proteinase K, pepsin and trypsin. The curvacin DF 38 bacteriocin was also sensitive to amylase, suggesting that it might be glycosylated, which means that it could also be placed in the Class IV bacteriocin group, i.e. complex bacteriocins with one or two moieties of carbohydrate or lipids.

Most of the bacteriocin research conducted in this study, was done in broth systems, where the bacteriocins were effective in inhibiting the target organisms, however in food products, where the bacteriocin will eventually be added to serve as a preservative, the activity may be affected by changes in bacteriocin solubility and charge, the binding of the bacteriocin to the food components, the inactivation by proteases and changes in the cell envelope of the target organisms as a response to the environmental factors. Since both the chemical composition as well as the physical conditions of the food product may have a significant influence on the bacteriocin's bacteriocidic activity (Gänzle *et al.*, 1999; Leroy & De Vuyst, 1999; Cleveland *et al*., 2001; Työppönen *et al*., 2003) it is important to evaluate these factors in the designated food product.

To determine the efficiency of the bacteriocins produced by the *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS strains on meat, pork loin fillets was used as evaluation product. In a pilot study, a combination of the bacteriocins produced by *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS was found to efficiently extend the shelf-life of pork loin fillets by up to two days when compared to an untreated sample.

The addition of the bacteriocins was also evaluated in terms of sensory characteristics. A trained sensory panel experienced a metallic taste with the pork

sample that had been treated with the bacteriocins. It was found that a four-day treatment with the bacteriocins and the untreated sample differed significantly regarding the aroma, sustained juiciness and metal taste attributes ($P \leq 0.05$). The control and bacteriocin treatment for two days and the bacteriocin treatments for two and four days did not differ significantly. The metallic taste experienced by the sensory panel could possibly be due to the bitter metallic taste of the yeast extract in the MRS medium. Further purification of these antimicrobial peptides and the possible development of an alternative growth medium for the purpose of bacteriocin production specifically, might eliminate the metallic taste associated with the bacteriocin treated samples. The meat colour evaluation indicated that the bacteriocin treated samples were overall darker than the control samples. Although the difference was relatively small, they may be eliminated by the further purification of these antimicrobial peptides.

Bacteriocins produced by lactic acid bacteria may be effective as a preservative on fresh red meat at refrigerated temperatures. It is also recommended that further purification steps be implements to eliminate any unacceptable tastes. The bacteriocins produced by *Lb. curvatus* DF 38, *Lb. plantarum* 423 and *Lb. casei* LHS are very stable under different environmental conditions, which may broaden the preserving options.

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