Expression of genes encoding bacteriocin ST4SA as well as stress proteins by *Enterococcus mundtii* ST4SA exposed to gastro-intestinal conditions, as recorded by real-time polymerase chain reaction (PCR).

by

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Thesis presented for the Masters Degree in Microbiology at the University of Stellenbosch

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DECLARATION

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

Signature: _____ Date: _____

Summary

The tolerance of *Enterococcus mundtii* ST4SA to stressful gastro-intestinal conditions in humans and animals is vital to its success as a probiotic. The need for new effective probiotics with stronger inhibitory (bacteriocin) activity has arisen due to the increasing number of antibiotic resistant pathogens. Enterococci are used in the fermentation of sausages and olives, cheese making and as probiotics. Their role as opportunistic pathogens in humans makes them a controversial probiotic (Moreno *et al.,* 2005). Enterococci occur naturally in the gastro-intestinal tract which renders them intrinsic acid and bile resistance characteristics. *E. mundtii* ST4SA produces a 3950 Da broad-spectrum antibacterial peptide active against Gram-positive and Gram-negative bacteria, and viruses. The bacteria include *Enterococcus faecalis, Streptococcus* spp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pneumoniae* and *Staphylococcus aureus. E. mundtii* ST4SA inactivates the herpes simplex viruses HSV-1 (strain F) and HSV-2 (strain G), a measles virus (strain MV/BRAZIL/001/91, an attenuated strain of MV), and a polio virus (PV3, strain Sabin).

This study focuses on the genetic stability of *E. mundtii* ST4SA genes when exposed to stress factors in the human and animal gastrointestinal tract. Based on results obtained by real-time PCR, the expression of genes encoding bacST4SA, RecA, GroES and 23S rRNA by *E. mundtii* ST4SA were not affected when the cells were exposed to acid, bile and pancreatic juice. This suggests that these genes of *E. mundtii* ST4SA will remain stable in the intestine. This could indicate that other genes of *E. mundtii* ST4SA could remain stable in the host. Further studies on the stability of genes encoding antibiotic resistance and virulence factors should be conducted to determine their stability and expression in the host in stress conditions. Concluded from this study, *E. mundtii* ST4SA is an excellent probiotic strain.

Opsomming

Enterococcus mundtii ST4SA se weerstandsvermoë teen stresvolle gastrointestinale kondisies is essensieel vir die sukses van hierdie organisme as ‘n probiotikum. Die aanvraag vir nuwe, meer effektiewe probiotika met sterker inhibitoriese (bakteriosien) aktiwiteit is as gevolg van die toename in antibiotikum weerstandbiedende patogene. Enterococci word algemeen gebruik as probiotika, sowel as in die fermentasie van worse, olywe en kaas. Hulle rol as oppertunistiese patogene in mense veroorsaak kontroversie as gevolg van hul toenemende gebruik as probiotika. Enterococci is deel van die natuurlike mikroflora in die gastrointestinale weg van mense en diere. Dit verleen aan hierdie spesies ‘n natuurlike weerstandsvermoë teen maagsure, galsoute en pankreatiese afskeidings. *E. mundtii* ST4SA produseer ‘n 3950 Da wye spektrum anti-bakteriese peptied, aktief teen Gram positiewe en Gram negatiewe bakterieë sowel as virusse. Hierdie bakterieë sluit *Enterococcus faecalis, Streptococcus* spp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pneumoniae* en *Staphylococcus aureus* in. *E. mundtii* ST4SA inaktiveer die herpes simpleks virus HSV-1 en HSV-2, ‘n masels virus (MV/BRAZIL/001/91), en ‘n polio virus (PV3, stam Sabin).

Hierdie studie fokus op die genetiese stabiliteit van *E. mundtii* ST4SA gene, wanneer hulle blootgestel word aan stress faktore in die mens en dier gastrointestinale weg. “Intydse” PKR data gebasseer op die uitdrukking van die bacST4SA, RecA, GroES en 23S rRNA gene in stresvolle kondisies dui aan dat *E. mundtii* ST4SA nie geaffekteer word wanneer die sel blootgestel word aan suur, gal en pankreatiese vloeistowwe nie. Hierdie resultate dui aan dat hierdie gene van *E. mundtii* ST4SA stabiel sal bly in die intestinale weg van die mens en dier. Dit kan aandui dat ander gene van *E. mundtii* ST4SA soos die wat kodeer vir virulensie faktore en antibiotikum se weerstandsvermoë stabiel mag bly in die gasheer. Verdere studies wat fokus op die stabiliteit van gene wat kodeer vir antibiotikum weerstandbiedendheid en virulensie faktore moet uitgevoer word om hulle stabiliteit en uitdrukking in die gasheer te bepaal. Bevindings van hierdie studie dui aan dat *E. mundtii* ST4SA goeie potensiaal het as ‘n probiotikum.

Biographical sketch

Monique Granger was born in Cape Town on the 30th of April 1982. She matriculated from Monument Park High School in 2000. In 2003 she obtained her B.Sc degree in molecular and cellular biology at Stellenbosch University, majoring in Microbiology, Biochemistry and Genetics. She was awarded her B.Sc Hons degree *cum laude* in 2004 from Stellenbosch University.

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Contents

INTRODUCTION

Lactic acid bacteria were among the first microorganisms to be used in food manufacturing, contributing to flavour, texture and preservation of fermented foods (De Vuyst and Vandamme, 1994). They are used in various processes, including fermented milk products, vegetables and meat, cheeses, and wine fermentation. Recently, the health industry has gained interest in lactic acid bacteria. Health promoting benefits of fermented foods containing lactic acid bacteria has been known since the early nineteen hundreds (Metchnikoff, 1908). Lactic acid bacteria produce bacteriocins that has numerous applications and possibilities in the food and health industry (Ennahar *et al.*, 2000). Bacteriocins may replace antibiotics in the near future, overcoming the problem of multi-drug resistant pathogens. The medical industry focuses on class II bacteriocins because some has potent anti-viral properties (Richard *et al*., 2006).

The intestinal tract is a complex ecosystem, containing at least 50 genera of bacteria and hundreds of species (Finegold *et al*., 1974). Most probiotics reside in the colon where they provide health, therapeutic and nutritional benefits to the host. These include reduction of blood cholesterol, deconjugation of bile acids, improvement of lactose utilization and increased immunity. Probiotics maintain equilibrium between beneficial and potential pathogenic bacteria in the host (Gagnon *et al*., 2004).

The gastrointestinal tract is an oxygen depleted, nutrient rich, and ecologically complex environment (Flahaut *et al.,* 1996). Probiotic bacteria has to survive numerous stress factors such as low pH in the stomach, high levels of pancreatic juice and bile salts in the upper duodenum and competition with other microorganisms in the lower duodenum. For these reasons potential probiotics has to be tested for traits such as bacteriocin production, absorption to epithelial cells, antibiotic resistance, and hemolytic activity. Probiotics has to be non-pathogenic, resistant to acid and bile and colonize the intestinal ecosystem (Gagnon *et al*., 2004).

Enterococcus mundtii occurs naturally in the human intestine. *E. mundtii* ST4V produces a broad-spectrum bacteriocin active against Gram-positive and Gram-negative bacteria and viruses (Todorov *et al.,* 2005). Class II bacteriocins are heat-stable peptides, less than 10kDa in size and

does not contain modified amino acids. They has a highly conserved N-terminal domain and at least one disulfide bridge (Richard *et al*., 2006).

The use of enterococci as probiotics remains a controversial issue. Probiotic benefits of some enterococci are well documented, but the emergence and increased association of enterococci with human disease and multiple antibiotic resistances has raised some concern (Moreno *et al*., 2006). *Enterococcus mundtii*, however, has GRAS (generally regarded as safe) status. This study focuses on the genetic reaction of *Enterococcus mundtii* ST4SA in conditions simulating stress in the gastrointestinal tract. Gene expression of the genes encoding bacST4SA, RecA, GroES and 23S rRNA was recorded by real–time PCR.

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LITERATURE STUDY

1. LACTIC ACID BACTERIA

Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating, microaerophilic bacteria that produce lactate as main end product from the fermentation of carbohydrates. The genera include *Lactococcus, Vagococcus, Leuconostoc, Pediococcus, Aerococcus, Tetragenococcus, Streptococcus, Enterococcus, Lactobacillus, Melissococcus, Oenococcus, Weissella, Carnobacterium* and *Bifidobacterium*. Some LAB, such as *Lactobacillus* spp., *Bifidobacterium bifidum* and *Enterococcus faecium*, form part of the natural gastrointestinal microflora in humans and animals. A number of species are used as starter cultures in the production of yogurt and as probiotics in animal feed and food supplements. LAB promotes aroma and flavour development in foods. The main nutritional and therapeutic effects of these organisms, as described by De Vuyst and Vandamme (1992) are:

- Production of vitamins (e.g. folic acid) and enzymes (e.g. lactase).
- Improvement of the overall quality and nutritional value of food and animal feed.
- Stabilization of intestinal microflora and reduction in colonization of pathogenic bacteria.
- Protection against intestinal and urinary tract infections by production of antimicrobial substances, including bacteriocins.
- Reduction in the level of cholesterol in the blood serum by cholesterol assimilation, bile salt hydrolysis and modulation of the ratio of high-density to low-density lipoprotein.
- Decreasing the risk of developing intestinal cancers such as colon cancer by detoxifying carcinogenic compounds and toxic substances, assisting in the breakdown of anti-nutritional factors (including trypsin inhibitors and glucosinolates) and modulation of feacal procarcinogenic enzymes such as β-glucuronidase, azoreductase and nitroreductase.
- Promotion of tumor suppression by aspecific stimulation of the immune system to produce macrophages (De Vuyst and Vandamme, 1992).

LAB are generally regarded as safe (GRAS) and bacteriocins (antimicrobial peptides) produced by these organisms are considered safe. The use of natural antimicrobial compounds instead of

chemical preservatives such as benzoate and sulfur dioxide are increasing in popularity. Bacteriocins are heat stable, biodegradable, digestible and active at low concentrations (Jack *et al*., 1995). A number of starter cultures has been developed for the food industry. Lactic acid bacteria may also be used to synthesize fine chemicals and antimicrobial compounds of pharmaceutical importance (De Vuyst and Vandamme, 1992). The medical applications of LAB has not been fully explored and could become an important research field, especially with so many strains resistant to antibiotics.

Classification

LAB includes *Enterococcus, Aerococcus, Carnobacterium, Vagococcus, Lactococcus, Lactobacillus, Leuconostoc, Globicatella, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus* and *Weissella*. Enterococci form a distinct cluster with *Vagococcus, Carnobacterium* and *Tetragenococcus* in the clostridial subdivision (Franz *et al*., 1999). LAB important in foods is *Carnobacterium, Enterococcus, Oenococcus, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus, Tetragenococcus, Streptococcus, Weissella* and *Vagococcus* (Vandamme *et al*., 1996).

LAB is classified as either homo- or hetero-fermentative based on the pathways used for hexose metabolism. Homofermentative LAB uses the Embden-Meyerhof-Parnas pathway to convert glucose to lactic acid (Pot *et al*., 1994). Homofermentative LAB, such as *Carnobacterium, Enterococcus, Lactococcus* and *Streptococcus*, do not ferment pentoses or gluconate. Heterofermentative LAB ferment carbohydrates via the 6-phosphogluconate pathway. Facultative heterofermentative LAB ferment hexoses to lactic acid. Obligate heterofermentative lactic acid bacteria convert glucose to lactic acid, CO_2, ethanol or acetic acid. Facultative and obligate heterofermentative species ferment pentoses to lactic acid and acetic acid. Although LAB are microaerophilic, some strains metabolize carbohydrates in the presence of oxygen (Pot *et al*., 1994).

Enterococcus

Enterococci are Gram-positive, asporogenous, catalase-negative, oxidase-negative and facultatively anaerobic. The round-shaped cells occur singly, in pairs or in chains (Moreno *et al*.,

2006). The cells survive heating at 60°C for 30 minutes, grow from 10 to 45°C (optimally at 35°C), and in medium supplemented with 6.5% NaCl and at pH 9.6 (Franz *et al*., 1999). All enterococci, with the exception of *Enterococcus cecorum, Enterococcus columbae, Enterococcus dispar, Enterococcus pseudoavium, Enterococcus saccharolyticus* and *Enterococcus sulfurous*, possess the Lancefield group D antigen. Most enterococci hydrolyze aesculin in the presence of 40% (v/v) bile salts (Hardy and Whiley, 1997). *Enterococcus faecium* and *Enterococcus faecalis* are associated with the human and animal gastrointestinal tract, whereas the pigmented species *Enterococcus mundtii* and *E. casseliflavus* are associated with plants. *E. gallinarum* and *E. casseliflavus* are distinguished from other species by being motile (Schleifer and Kilpper-Bälz, 1987).

Although enterococci, especially *E. faecalis* and *E. faecium* (to a lesser extent) has been associated with clinical infections and some strains has been isolated from patients diagnosed with endocarditis, bacteraemia and urinary tract infections, many strains are used as starter cultures in fermented foods (Franz *et al*. 1999). The best example is *E. faecium* in cheese. Enterococci has also been included in a few probiotic preparations (Stiles and Holzapfel, 1997). Many strains has lipolytic and esterolytic activities (De Vuyst *et al*., 2003).

Bacteriocins (antimicrobial peptides) has been described for almost all enterococci. These peptides, generally known as enterocins, are often located on pheromone responsive conjugative plasmids. Pheromones, cytolysin or hemolysin production, and resistance to phagocytosis and adherence to epithelial cells may all contribute to virulence. Recently, more antibiotic resistant strains, especially against vancomycin, has been described. The association of enterococci with clinical specimens calls for strict rules and regulations when used as starter cultures or probiotics (De Vuyst *et al*., 2003).

Streptococcus

The genus *Streptococcus* originally included enterococci, lactic streptococci, pyogenic streptococci and virulent streptococci. The original classification was based on morphological, physiological, serological and biochemical characteristics (Stiles and Holzapfel, 1997). Differentiation into the genera *Enterococcus*, *Streptococcus* and *Lactococcus* was based on

16SrRNA sequencing and DNA homology (Schleifer and Kilpper-Bälz, 1984, 1987). Currently the genus *Streptococcus* contains the species *S. pyogenes, S. mutans, S. salivarius, S. pneumoniae* and *S. thermophilus. S. thermophilus* is the only species used in food fermentations, specifically dairy products (Stiles and Holzapfel, 1997).

Lactococcus

Lactococci are ovoid-shaped and may form chains. The species include *Lactococcus lactis, Lactococcus garvieae, Lactococcus plantarum, Lactococcus raffinolactis* and *Lactococcus piscium* (Pot *et al*., 1994). *Lactococcus lactis* consists of four subspecies, *viz*. *lactis*, *cremoris, diacetylactis* and *hordiniae*. Strains from the first two subspecies are commonly used in the dairy industry as starter cultures. The cells are non-motile, grow at 10^{o}C but not at 45^{o}C, and produce L(+)-lactic acid from glucose (Stiles and Holzapfel, 1997).

Pediococcus* and *Tetragenococcus

Pediococci are non-motile, tetrad-forming homofermentative cocci (Collins *et al*., 1990). The genus *Pediococcus* consists of obligate homofermenters, as well as facultative and obligate heterofermenters (Stiles and Holzapfel, 1997). *Pediococcus damnosus* is a major spoilage organism in beer, wine and cider, leading to diacetyl/acetoin formation which results in a buttery taste (Garvie, 1986b). *Pediococcus acidilactici* and *Pediococcus pentosaceus* are used as starter cultures in the production of sausage and silage (Hammes *et al*., 1990). Both species produce bacteriocins. Pediococci are usually associated with leuconostocs and lactobacilli (Stiles and Holzapfel, 1997). *Pediococcus halophilus* is the only species that grows in the presence of 18% (w/v) NaCl (Garvie, 1986b) and is used to produce soy sauce. The species has been reclassified as *Tetragenococcus halophilus* based on 16S rRNA sequencing (Collins *et al*., 1990).

Vagococcus

Motile cocci are grouped in the genus *Vagococcus* and belong to serological group N. Concluded from 16SrRNA analyses, vagococci are phylogenetically closer related to *Enterococcus*, *Carnobacterium* and *Listeria* than to *Streptococcus* and *Lactococcus*. Three species has been identified, *viz*. *Vagococcus flavialis, Vagococcus salmoninarum* and *Vagococcus lutrae* (Stiles and Holzapfel, 1997; Lawson *et al*., 1999).

Lactobacillus* and *Carnobacterium

The genus *Lactobacillus* is strictly fermentative and has complex nutritional requirements. Lactobacilli are widespread in nature. The genus is divided into three groups based on fermentative characteristics, *viz*. obligate homofermentative (Group 1), facultative heterofermentative (Group 2) and obligate heterofermentative (group 3) (Stiles and Holzapfel, 1997). Species in Group 1 ferments hexoses to lactic acid. Species in Group 2 ferments hexoses to lactic acid and may produce gas from gluconate, but not from glucose. Obligate homofermentative lactobacilli lack the enzymes glucose 6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate dehydrogenase (6-P-GDH). Group 2 species has both enzymes (Pot *et al*., 1994). Group 3 lactobacilli ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. Gas is produced from glucose. Pentoses are fermented to lactic acid and acetic acid via the pentose phosphoketolase pathway (Pot *et al*., 1994). *Lactobacillus* spp. are the most acid tolerant of all lactic acid bacteria and are used as starter cultures in many different food products (Stiles and Holzapfel, 1997).

Lactobacillus divergens, Lactobacillus carnis and *Lactobacillus piscicola*, originally classified as Group 3, has been reclassified as *Carnobacterium divergens, Carnobacterium carnis* and *Carnobacterium piscicola* (Collins *et al.,* 1987). Fermentation of glucose is predominantly homofermentative and the fatty acid composition differs from that of lactobacilli. *Carnobacterium* spp. are normally isolated from meat and fermented meat products (Collins *et al.*, 1987).

Leuconostoc, Oenococcus* and *Weissella

Members of the genus *Leuconostoc* are facultative anaerobic, heterofermentative cocci. *Leuconostoc* produce D(-) lactate from glucose which differs from the L(+) lactate produced by lactococci and DL- lactate produced by heterofermentative lactobacilli. Argenine is not hydrolyzed. *Leuconostoc* spp. is normally associated with plants and play an important role in the production of sauerkraut (Stiles and Holzapfel, 1997). Diacetyl is produced from the fermentation of citrate and dextrans from sucrose (Daeschel *et al*., 1987; Stiles and Holzapfel, 1997). *Leuconostocs* are also prevalent in vacuum-packed meats (Shaw and Harding, 1984). The genus

consists of *Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum, Leuconostoc mesenteroides* subsp. *cremoris, Leuconostoc paramesenteroides, Leuconostoc lactis, Leuconostoc carnosum, Leuconostoc gelidum, Leuconostoc fallax, Leuconostoc amelibiosum, Leuconostoc citreum, Leuconostoc pseudomesenteroides* and *Leuconostoc argentinum* (Dellaglio *et al*., 1995). *Leuconostoc oenos* has been reclassified as *Oenococcus oeni* (Dicks *et al*., 1995). *Leuconostoc paramesenteroides* has been reclassified as *Weissella paramesenteroides* and grouped with *Lactobacillus hellenica, Lactobacillus viridescens, Lactobacillus confusa, Lactobacillus kandleri, Lactobacillus minor* and *Lactobacillus halotolerans* in the genus *Weissella* (Collins *et al*., 1993).

2. BACTERIOCINS

Many LAB produce bacteriocins (antimicrobial peptides) to compete against other bacteria in the same ecological niche. Bacteriocins are gaining interest in the midst of increased reports of antibiotic resistance. These peptides are cationic and amphiphilic, on average 4 kDa in size, and ribosomally synthesized (Moreno et al., 2006). They vary in spectrum and mode of activity, molecular structure and mass, thermostability, pH stability and genetic determinants (Moreno *et al*., 2006). Bacteriocins are often only active against species closely related to the producer strain, but a number of these peptides are active against a variety of Gram-positive and Gram-negative bacteria, even viruses (Messi et al., 2001). Examples of such broad-spectrum bacteriocins are plantaricin 35d produced by *Lactobacillus plantarum* (Messi *et al*., 2001), bacteriocin ST151BR produced by *Lactobacillus pentosus* ST151BR (Todorov and Dicks, 2004), thermophylin produced by *S. thermophylus* (Ivanova *et al*., 1998), enterocin CRL35 produced by *E. faecium* (Farias *et al*., 1996), peptide AS-48 produced by *E. faecalis* (Abrionel *et al*., 2001), a bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* (Caridi, 2002) and bacteriocin ST4V produced by *E. mundtii* ST4V (Todorov *et al*., 2005).

Classes

Bacteriocins are divided into three classes based on structure, physicochemical and molecular properties (Klaenhammer, 1993). Class I bacteriocins, the lantibiotics, are small, heat stable, cationic, hydrophobic peptides and contain unusual amino acids. These include thioether,

lanthionine and 3-methyl-lanthionine amino acids that are post-translationally modified. Class II bacteriocins are small, heat stable, cationic, hydrophobic peptides that are not post-translationally modified (except for cleavage of the leader peptide). Class II bacteriocins are subdivided into three subclasses. Subclass IIa include pediocin-like bacteriocins with anti-listerial activity with a consensus sequence YGNGV in the N-terminus. Subclass IIb bacteriocins require two polypeptide chains for activity. Subclass IIc are bacteriocins that do not belong to the other two subclasses. Class III bacteriocins are large, heat-labile, hydrophilic proteins (Moreno *et al*., 2006). This review will focus on class IIa bacteriocins, as most enterococci are known to produce class II bacteriocins (De Vuyst and Vandamme, 1994).

Subclass IIa bacteriocins are the largest and most extensively studied. They all inhibit the growth of *Listeria* spp. and are used as bio-preservatives in many different food products (Ennahar *et al*., 2000). The YGNGVXaaC-motif on the N-terminal of these bacteriocins is part of a recognition sequence for a membrane receptor protein. Class IIa bacteriocins has a net positive charge and their iso-electric points vary from 8.3 to 10.0. The C-terminal domain is moderately conserved, hydrophobic or amphiphilic. Class IIa bacteriocins has at least two cysteines with a disulfide bridge. The cysteine residues are in conserved positions and the disulfide bridge forms a six-membered ring over these two residues. Bacteriocins with more than one disulfide bond has a broader spectrum of activity. The N-terminus of class IIa bacteriocins has an amphiphilic characteristic due to β-sheets in a β-hairpin conformation. The C-terminal forms an amphiphilic α-helix, leaving one or two residues in the C-terminal non-helical (Ennahar *et al*., 2000).

The majority of enterococci produce class IIa bacteriocins inhibitory to closely related Gram-positive bacteria (De Vuyst *et al*., 2003). Enterocin AS-48, produced by *E. faecalis* S-48, was the first enterocin to be purified and was defined as a cyclic peptide antibiotic (Galvez *et al*., 1989; Martinez-Beuno *et al*., 1994). The ability of enterococci to inhibit *Listeria* spp. may be explained by the close phylogenetic relatedness between enterococci and listeriae (Moreno *et al*., 2006).

A number of bacteriocins has been described for *E. mundtii*. They are classified as class IIa and include mundticin ATO6 isolated from chicory endive, mundticin KS isolated from grass silage, and enterocin CRL35 from Argentinean cheese (Bennik *et al*., 1998; Kawamoto *et al*., 2002;

Saavedra *et al*., 2004). Mundticin ATO6 and mundticin KS are identical in mature and leader peptides. The mature peptide of enterocin CRL35 is identical to the mundticin KS mature peptide, but differs with two amino acids in the leader peptide (Zendo *et al*., 2005; Kawamoto *et al.*, 2002).

Enterococcus mundtii ST4SA produces a 3950 Da broad-spectrum antibacterial peptide active against Gram-positive and Gram-negative bacteria, including *Enterococcus faecalis, Streptococcus* spp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pneumoniae* and *Staphylococcus aureus* (H. Knoetze, 2006). Treatment of mundticin ST4SA with pepsin, Proteinase K, pronase and trypsin leads to a significant reduction in activity, while α-amylase treatment does not reduce activity (Todorov *et al*., 2005). The genes encoding mundticin ST4SA are located on a 50-kb plasmid (H. Knoetze, 2006). The gene cluster (*mun* locus) consists of three genes *viz*., *munA*, *munB* and *munC*. The structural gene, *munA,* encodes a 58-amino-acid mundticin ST4SA precursor. The leader peptide contains 15 amino acids with a double-glycine processing site. *MunB* encodes a 674-amino-acid (ABC-transporter) protein involved in translocating and processing of the bacteriocin. *MunC* encodes the 98-amino-acid immunity protein. The amino acid sequence, deduced from the sequence of the mundticin ST4SA structural gene, is completely homologous to that of mundticin KS, mundticin AT06 and mundticin QU2. Mundticin ST4SA differs from enterocin CRL35 by two amino acids in the leader peptide, but the mature peptides are completely homologous. The ABC-transporter gene of bacteriocin ST4SA has 98.9% homology to mundticin KS and 99.25% homology to enterocin CRL35. The mundticin ST4SA immunity gene is completely homologous to the immunity gene of enterocin CRL35, and is 96.9% homologous to that of mundticin KS (H. Knoetze, 2006). Mundticin ST4 (Todorov *et al*., 2005) inactivates the herpes simplex viruses HSV-1 (strain F) and HSV-2 (strain G), a measles virus (strain MV/BRAZIL/001/91, an attenuated strain of MV) and a polio virus (PV3, strain Sabin).

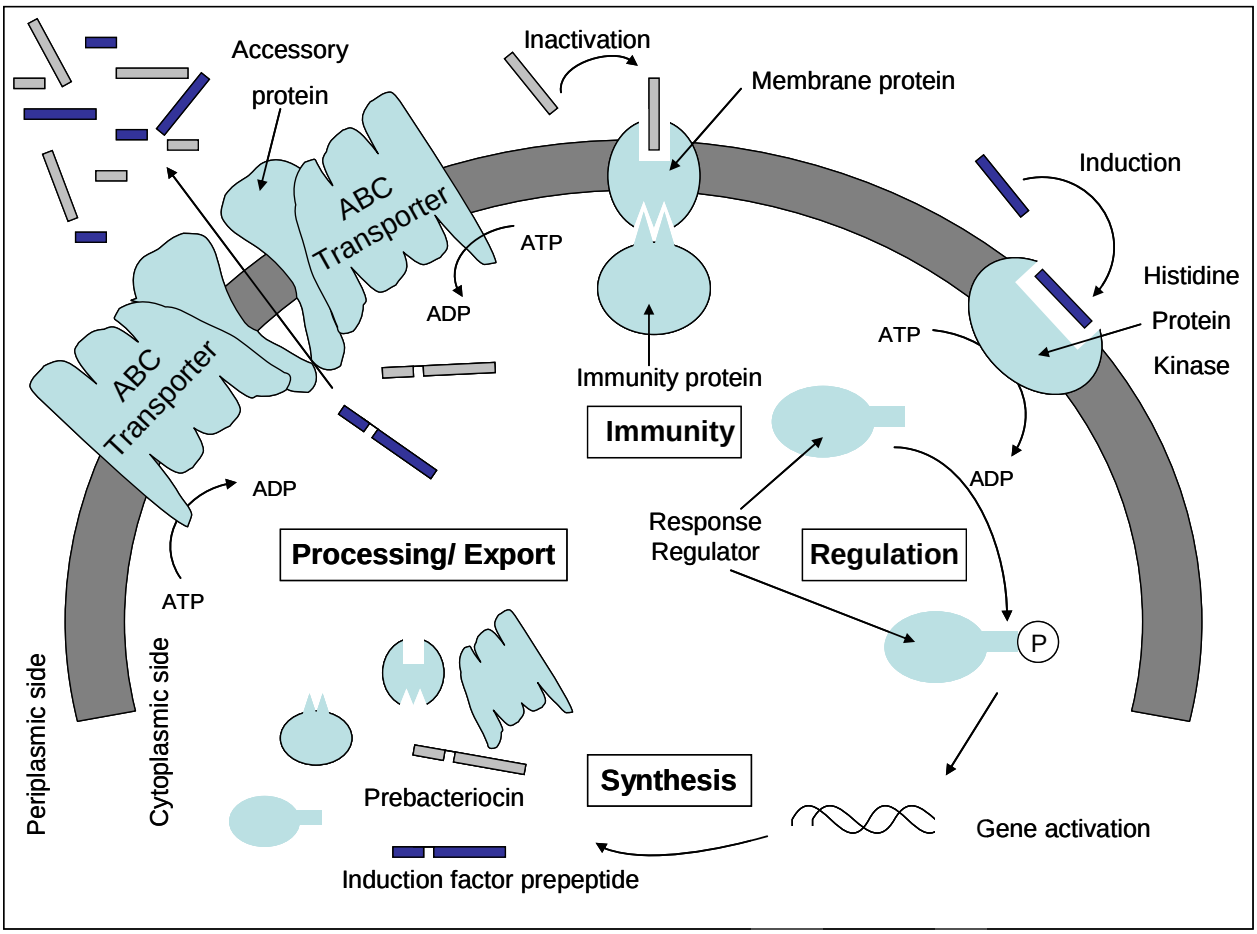

Fig.1. Schematic overview of machinery for the production of class IIa bacteriocins: three component system (histidine protein kinase, response regulator and induction factor), synthesis, processing, excretion and immunity (Ennahar *et al*., 2000; Permission granted from Blackwell Publishing, Oxford, U.K.).

Biosynthesis

Class IIa bacteriocins are formed as ribosomally synthesized precursors or pre-peptides containing an N-terminal leader sequence (Ennahar *et al*., 2000). The leader sequence is removed when the pre-peptide is cleaved at a specific processing site. The active peptide is then exported by membrane translocation. The two conserved glycine residues may serve as recognition signal for transport. A histidine protein kinase, a response regulator and an induction factor are typical three-component system inducers of class IIa bacteriocin genes (Fig.1). Membrane translocation is mediated by two membrane-bound proteins, an ABC transporter and an accessory protein (except for enterocin P). The ABC transporters of Class IIa bacteriocins share no common

structural features. The only common features are the N-terminal region that is a hydrophobic integral membrane domain which carries a 150 amino acid extension and the C-terminal domain that contains a highly conserved ATP binding domain with 200 terminal amino acids. The N-terminal 150 amino acid peptide cleaves the leader peptide at the double-glycine motif. The leader peptide serves as a recognition signal for cleavage and membrane translocation of the mature peptide (Ennahar *et al*., 2000). Non-lanthionine containing bacteriocins has 18 to 24 amino acid residues in their leader sequences. Possible functions of the leader peptides include stabilization of the pre-peptide during translocation, preventing insertion of the peptide into the cell membrane, maintenance of specific conformation during processing, and assisting with the translocation of pre-peptides by specific transport systems (Jack *et al*., 1995).

Mechanisms and mode of action

Class IIa bacteriocins permeabilize sensitive bacterial membranes by pore formation. This results in the dissipation of the proton motive force (PMF), preventing the formation of a pH gradient (Ennahar *et al*., 2000). The intracellular ATP is depleted and blocks amino acid uptake, mediated by active transport. Amino acid leakage can also occur via pores in the membrane. Initial class IIa interaction with a membrane involves electrostatic binding mediated by a putative membrane-bound receptor-type molecule. Ennahar *et al.* (2000) suggested binding between positively charged, polar residues of class IIa bacteriocins and anionic groups in the phospholipid membrane (Fig. 2). Hydrophobic interactions occur in the hydrophobic domain of the C terminal half of the bacteriocin and the lipid acyl chains. Class IIa bacteriocins insert the C-terminal into the target membrane and aggregate to form water-filled pores (Ennahar *et al*., 2000).

The YGNGV motif is recognized by a putative membrane receptor due to a β-turn structure which allows for correct bacteriocin positioning. The N-terminal β-sheet confers an amphiphilic characteristic to class IIa bacteriocins, which is important for bacteriocin-membrane interaction. The N-terminal region is possibly involved in a membrane-surface recognition step through electrostatic interactions. The central oblique orientated α-helical regions, present in nine of the class IIa bacteriocins, facilitates insertion of the C-terminal into the phospholipid bilayer. Class IIa bacteriocins contain at least one disulfide bridge. Bacteriocins with more than one disulfide bridge has a broader spectrum of activity (Ennahar *et al*., 2000). The two disulfide bridges in

pediocin PA-1 forces the positively charged His and Lys residues closer together and forms a positively charged amino acid patch and a tighter junction with negatively charged lipid head groups, thereby increasing bacteriocin activity (Chen *et al*., 1997).

Bacteriocin induced cell-death occurs in a concentration- and time-dependant style. Factors influencing the target cell or medium also play a role. The pH of the medium affects the affinity of the bacteriocin. A decrease from pH 7.5 to pH 6.0 has a positive membrane binding effect and increases pediocin PA-1 activity (Chen *et al*,. 1997). Positively charged class IIa bacteriocins bind to negatively charged phospholipid head groups. Any environmental factor (such as change of pH or medium components) that changes any of these two charges affects bacteriocin binding. The spectrum of activity is species- and strain-specific. Bacteriocin activity depends on the whole sequence. Fragments of the peptide display weak or no activity (Moll *et al*., 1999).

Regulation

Many bacterial pathways are induced by external stimuli which are sensed and signaled by signal transduction systems. Two-component signal transduction systems consist of a sensor located in the cytoplasmic membrane and a cytoplasmic response regulator. The environmental sensor acts as a histidine protein kinase and modifies the response regulator protein which triggers an adaptive response usually by gene regulation. Response regulators bind as dimers to a specific site (direct or inverted repeats) present near the promoter of an operon. This structurally inhibits binding of RNA polymerase to the promoter region. Direct repeats has been reported upstream from the promoters of several inducible bacteriocin operons, suggesting a common mechanism for bacteriocin regulation (Diep *et al*., 1996).

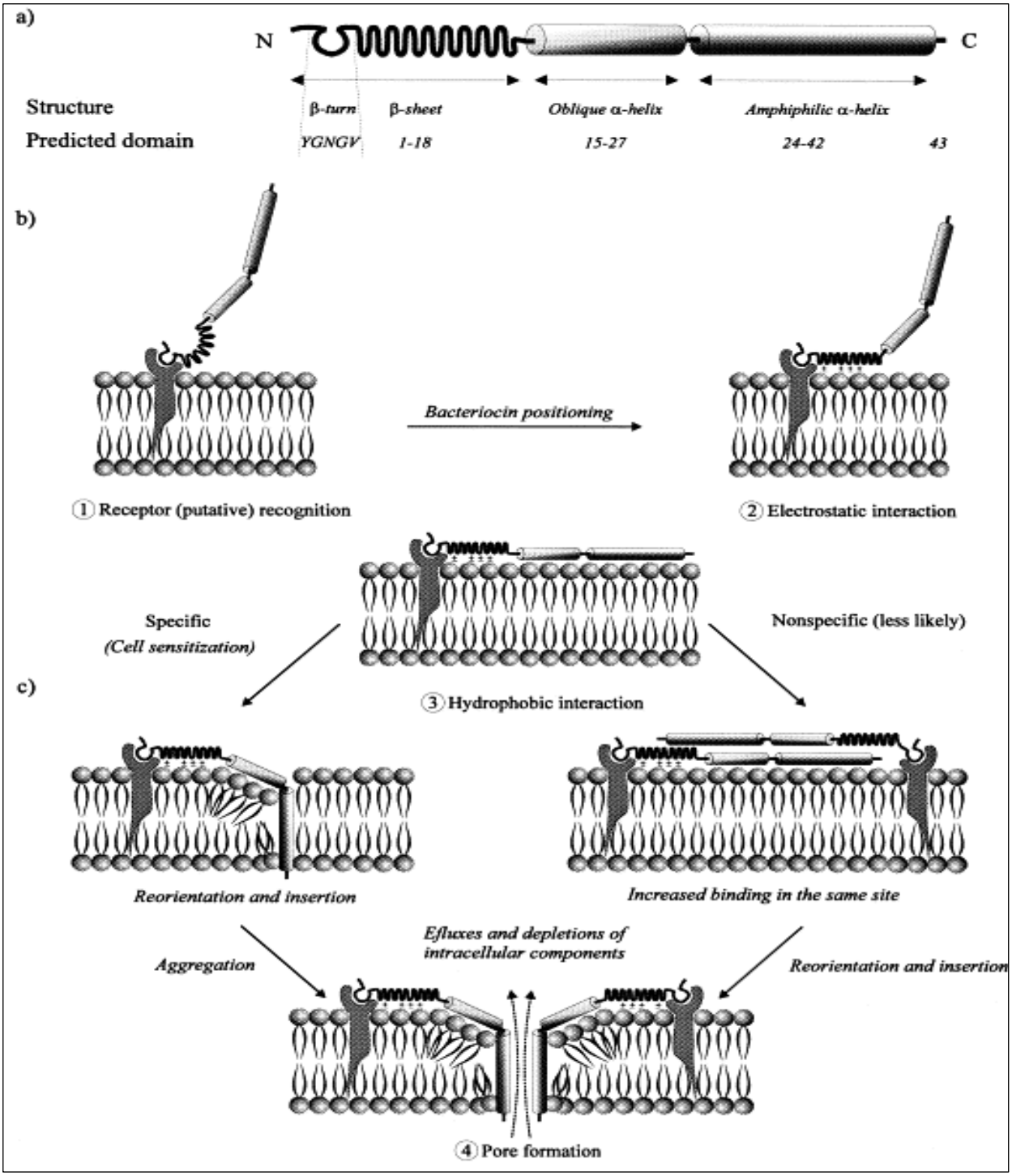

Fig.2. a) Predicted structural domains of class IIa bacteriocins, b) possible interaction of domains with the membrane surface, c) insertion and pore formation by class IIa bacteriocins. The hydrophobic face of the peptide is shaded dark (Ennahar *et al*., 2000; Permission granted from Blackwell Publishing, Oxford, U.K.).

Resistance

Some strains are highly tolerant to bacteriocins. Tolerance can result from exposure to bacteriocins. Some strains of *L. monocytogenes* develop resistance against nisin at high frequencies in commercial media and food products (Ming and Daeschel, 1993). Mutation frequencies depend on the strain and conditions used, particularly the bacteriocin to bacteria ratio. *L. monocytogenes* has the ability to develop resistance to class IIa bacteriocins. Cross-resistance against class IIa bacteriocins, nisin and the class IV leuconocin S has been observed in strains of *L. monocytogenes* and *Clostridium botulinum* (Song and Richard, 1997). The mechanisms involved in bacteriocin resistance are complex and could involve changes in structure, such as fatty acid and phospholipid composition. This renders the cell membrane less fluid and prevents insertion of the bacteriocin. Modifications of this sort are easily reverted when the strain is cultured in the absence of the bacteriocin. Bacteriocin producer strains has immunity genes that confer bacteriocin resistance to closely related bacteriocins. Genetically based resistance (other than immunity proteins) has not been detected in tolerant strains. The long term effects of bacteriocin resistance that may result in stable and viable mutants must be carefully studied (Ennahar *et al*., 2000).

Immunity proteins

Immunity proteins protect bacteriocin producer strains from their own bacteriocins (Ennahar *et al*., 2000). Immunity genes are usually co-transcribed or in close vicinity to the bacteriocin gene. Immunity proteins consist of 88 to 115 amino acid residues and show a high degree of specificity with respect to the bacteriocins they recognize. However, some immunity proteins may confer resistance to other closely related bacteriocins (Ennahar *et al*., 2000). Immunity proteins function by disturbing the interaction between the bacteriocin and a membrane located bacteriocin receptor (Johnsen *et al*., 2004). Immunity proteins are cationic and largely hydrophilic. Previous studies has suggested that these molecules are largely intracellular. Immunity proteins provide total protection against the producer's own bacteriocin and often partial protection against other bacteriocins. LAB genera which produce bacteriocins generally possess one or more immunity genes for class IIa bacteriocins, providing resistance against bacteriocins from closely related species. These proteins render the cell resistant to closely related bacteriocins. Homology between immunity proteins is low, which is surprising considering the similarity between the

corresponding bacteriocins. This suggests that immunity proteins do not interact directly with the bacteriocins but rather with a target or receptor in the cell (Fig.1.) (Ennahar *et al*., 2000). These mechanisms are not fully understood.

3. PROBIOTICS

The concept of probiotics was introduced in 1908 when Metchnikoff described the health promoting benefits of fermented milks (Metchnikoff, 1908). Probiotics are defined as "live microbial food supplements that benefit the health of consumers by maintaining or improving their intestinal microbial balance" (Guarner and Schaafsma, 1998). The Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) Working group report (2002) added to this definition by defining probiotics as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host". The FAO/WHO Working group report defined pre-biotics as "non-digestible food components which has a beneficial effect on human health by selectively stimulating the growth and metabolic activities of one or a limited number of beneficial intestinal bacteria and thus improving the balance of the human intestinal microflora".

The main probiotic bacteria belong to the lactic acid bacteria group. These genera include *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Enterococcus* and *Streptococcus*, although the first two strains are the most commonly used. Other probiotics are *Saccharomyces* and *Propionibacterium* (Vinderola and Reinheimer, 2003). The normal microflora in the human gastrointestinal tract is confined to the distal small bowel and the large bowel. The stomach, duodenum and jejunum are not typically colonized because of the acidity in the stomach and peristaltic movements of the digesta. The epithelial layer is overlaid with mucus and provides a barrier to prevent microbes from disseminating to other organs of the body. Patients suffering from conditions such as lactose intolerance, diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease (Crohn's disease and ulcerative colitis), depressed immune function, cancer and genitourinary tract infections has all been reported to benefit from probiotics (Stanton *et al*., 2005).

Probiotics must has GRAS status, has to survive freeze-drying, spray drying, encapsulation etc. during production and has to be viable and metabolically active in the gastrointestinal tract. Probiotics should not produce off flavour, provide at least one beneficial health promoting effect in humans and attach to the intestinal epithelium (Leverrier *et al*., 2005). Probiotic vectors include fermented milk products, cheese, ice creams, and buttermilks, which are the most popular.

To assess the safety of probiotic strains they must be tested *in vitro* for resistance to gastric acids, bile acid, adhesion to mucus and human epithelial cells, antimicrobial activity against pathogens, competitive exclusion of pathogens, bile salt hydrolase activity and resistance to spermicides (for vaginal use). According to the FAO/WHO Working group report (2002), probiotic strains has to be tested for:

1. Antibiotic resistance
2. D-lactate production and bile salt deconjugation
3. Side effects in human trials
4. Side effects in consumers (post-market)
5. Toxin production
6. Hemolytic activity
7. Lack of infectivity in immuno-compromised humans and animals.

Probiotic containing foods are known as functional foods. Functional foods has health benefits over and above providing basic nutrition and are defined as "foods that can be satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and wellbeing and/or reduction of risk and disease" (Contor, 2001). Some lactic acid bacteria produce secondary metabolites during fermentation that has been associated with health promoting benefits. These metabolites include B vitamins and bioactive peptides released from food proteins (Stanton *et al*., 2005). Production of vitamins by LAB increases the nutritional value of fermented foods. Cobalamin or vitamin B12 is exclusively of microbial origin. It is present in foods such as red meat and milk as a result of rumen microbial action. Vitamin B12 is a co-factor in fatty acid,

carbohydrate, amino acid and nucleic acid metabolism. Intestinal bacteria, such as propionic acid bacteria, contribute to vitamin B12 levels in humans (Stanton *et al*., 2005).

4. STRESS AND GASTRO-INTESTINAL BACTERIA

Acid, bile salts and enzymes represse the growth of most LAB. Probiotic strains prevent cellular and DNA damage by exerting a number of stress responses, such as the SOS, heat shock and stringent response. All of these responses regulate error-prone polymerases. In general these stress responses repair or eliminate damaged macromolecules.

SOS response

The SOS response system is induced by DNA damage and is regulated by the LexA and RecA proteins. The two loosely grouped categories of SOS functions are nucleotide excision repair and translesion synthesis. When the repressor, LexA, is inactivated, an estimated 40 genes are induced, resulting in SOS response. In un-induced cells the SOS-genes are expressed at basal levels (Matic *et al*., 2004). Proteolytic cleavage of LexA is induced upon DNA damage. Single-stranded DNA is then bound to the RecA protein, which in turn acts as a co-protease. Cleavage of LexA also occurs when cells are exposed to UV irradiation. The SOS response is also induced when LexA levels decrease. LexA inactivation increases with an increase in pH and during ageing of colonies (Little, 1991). LexA affinity for SOS box-containing gene sets determines expression of the gene sets. The level of induction is controlled by the amount of single stranded DNA in the cell (Matic *et al*., 2004). The Y-family of polymerases are error-prone DNA polymerases that replicate damaged DNA, but cause frequent mutations. Pol IV, a *dinB* gene product, and Pol V, an *umuDC* operon product, are both repressed by LexA and induced during SOS response. Under normal conditions the mutation rates are kept as low as possible by tightly regulating error-prone polymerases (Foster, 2004). Overproduction of RecA, RecN and RuvAB proteins increases recombination efficiency and capacity. This allows for efficient repair of double-strand breaks and daughter-strand gaps (Matic *et al*., 2004).

General stress response

The alternative sigma factor RpoS (σ^{38} or σ^{s}) is triggered by conditions that terminate growth. This includes starvation, high osmolarity, extreme temperatures, low pH and transition into stationary phase. During stationary phase the RpoS sigma factor is activated and induces a set of genes that direct RNA polymerase to their promoters. The RpoS regulon includes more than 70 genes, of which most encode proteins that help the cell survive the impact from dead cells in the environment. RpoS is considered a master regulator of stress response (Hengge-Aronis, 2002). Translation of *rpoS* mRNA is controlled by a cascade of interacting factors, including Hfq, H-NS, *dsrA* RNA, LeuO and *oxyS* RNA. These factors modulate the stability of secondary structures in the ribosome-binding region of the *rpoS* mRNA (Matic *et al*., 2004). During late stationary phase Pol IV is induced under positive regulation of RpoS. Pol IV is the dominant polymerase under starvation conditions which leads to increases in the error rate of DNA synthesis. Mismatch repair systems correct mismatches of newly synthesized DNA before mutations. During starvation mismatch repair systems are only active at low levels and this leads to genetic variations (Foster, 2004). Different stress conditions affect the mechanisms of σ^{s} control differently. A reduced growth rate stimulates *rpoS* transcription, while low temperature, high osmolarity, acidic pH and some late log-phase signals stimulate translation of *rpoS* mRNA. Exposure to stress stabilizes σ^{s} which rapidly degrades under normal non-stress conditions (Matic *et al*., 2004). The RpoS regulon is expressed *in vitro* during stationary growth at low pH and nutrient limitations.

Heat-shock response

Protein denaturation induces a heat shock response which in turn induces a set of chaperons and proteases involved in refolding and elimination of damaged proteins. During chronic or acute stress, heat shock proteins (Hsps) are up-regulated. They function as molecular chaperones in regulating cellular homeostasis and promoting survival. Apoptosis (programmed cell death) occurs when the stress is too severe. The two main functions of stress proteins in repairing damaged cells are: i) participation in protein folding into correct tertiary structures and incorporation of polypeptides into intracellular membranes or transport across those membranes and ii) functioning in ubiquitin-dependant protein degradation.

The heat shock response induces 30 genes under control of sigma factor RpoH (σ^{32}). The RpoH regulon is induced by temperature and other conditions that result in unfolding of proteins. GroE, part of the RpoH regulon, is induced by DNA damage, oxidative stress, antibiotics, heavy metals, phage infection and carbon source or amino acid starvation. GroE regulates proper protein folding and conformation and is required at all temperatures (Yura *et al.*, 2000). The induction of the RpoS regulon results in the expression of a complex network of genes with no linked function, which increases the cell's capacity to resist a variety of conditions.

Stringent response

Starvation (e.g. amino acid starvation) causes cells to down-regulate the synthesis of stable RNAs (rRNA and tRNA) (Chatterji and Ojha, 2001). Guanosine tetra- and (penta-) phosphate (ppGpp) alters RNA polymerase promoter selectivity so that transcription of stable RNAs is decreased and selected mRNAs are increased. ppGpp is a positive effecter of RpoS and RpoS dependant genes, and increases the ability of RpoS and RpoH to compete with RpoD (σ^{70}) for RNA polymerase (Jishage *et al*., 2002). The stringent response thus enhances induction of the general stress and heat shock responses.

Stationary cells are more tolerant to different stresses than exponential growing cells (Leverrier *et al*., 2005). Starvation of carbohydrates, oxygen or phosphate induces a non-specific sigma-B dependant general stress regulon that causes over-expression of a series of general stress proteins. This leads to a multi-tolerant stage in *Bacillus subtilis* (Hecker and Volker, 1998). This is not true for all bacteria, as starvation causes autolysis in *Lactococcus lactis, Leuconostoc mesenteroides* and *Pediococcus acidilactici* (Leverrier *et al*., 2005).

Stress proteins

Genes for stress proteins are transcribed with increased levels and activity of sigma factor 32 (σ^{32}). The rpoH gene encodes σ^{32} and transcription of σ^{32} is controlled by 20 heat shock proteins, including DnaK, DnaJ, GroEL and GroES (Craig and Gross, 1991). Sigma 32 competes with σ^{70} for RNA polymerase when σ^{70} levels in the cell decreases. This increases the heat shock protein levels (Yura *et al*., 1993).

In principal, stress proteins function as molecular chaperones facilitating protein degradation. Molecular chaperones stabilize the partly denatured sections of proteins and assist in folding and refolding to their native state. Chaperones form complexes with emerging polypeptides on ribosomes once translation is terminated and prevents premature folding. The heat shock protein 70 (Hsp70) family plays a major role in complex formation, transport and polypeptide folding. Binding of unfolded proteins and their release proceeds in an ATP-dependant cycle with hsp70 co-operating with hsp40 and Bag-1 proteins (Fig. 3). Partially unfolded protein associates with the C-terminal domain of Hsp70; co-chaperone hsp40 binds and initiates dissociation of ATP with the N-terminal domain of hsp70, leading to a conformational change (hsp70/ADP complex). Hsp40 then dissociates and the BAG-1 protein binds, initiating an ADP/ATP exchange. BAG-1 then dissociates and bound proteins are released. This cycle is called the hsp70 chaperone machine (Forreiter and Nover, 1998).

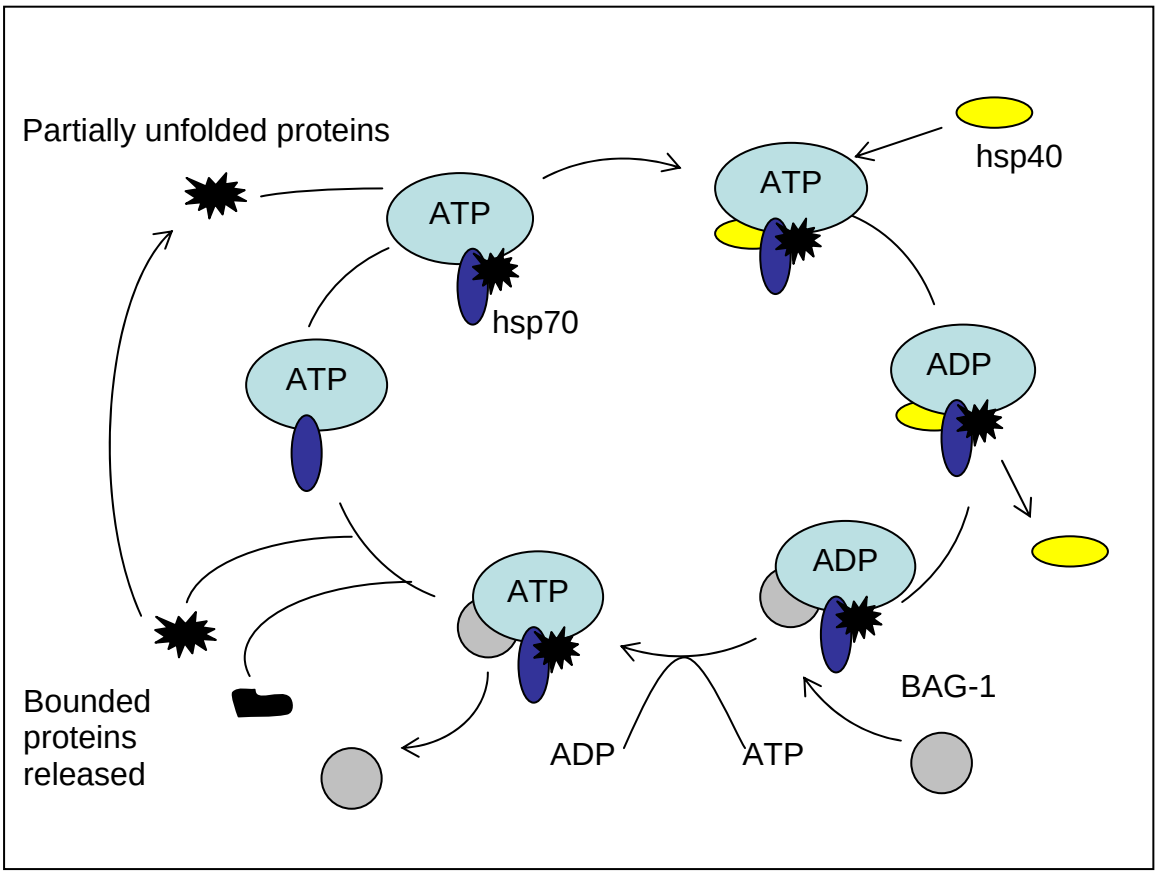

Fig.3. The hsp 70 chaperone machine (adapted from Kopecek *et al*., 2001).

Hydrophobic residues on the surface of partially denatured proteins are recognized by GroEL. The damaged proteins are then introduced into the central cavity of the GroEL heptameric ring formed by 57-kDa units. This "cage" effect protects the protein from proteolytic enzymes and protein folding continues (Baneyx and Gatenby, 1993). Molecular chaperones take part in degradation of irreversibly damaged proteins. The stress protein DegP has a chaperone function at lower temperatures and proteolyitc function at higher temperatures. Ubiquitin binds substrate proteins by binding to the etha-amino groups of lysine residues in substrates. The 26S proteasome (multi-subunit protease) recognizes the ubiquitin side chains that form on the substrate proteins and breaks down the monomers. The ubiquitin is then ready for a following binding cycle (Muller and Schwartz, 1995; Kopecek *et al*., 2001).

Cross-protection has been observed in various studies with different combinations of stress. Bile provides cross protection against heat stress in *E. faecalis*; while in *L. monocytogenes* acid stress provides cross-protection against heat, ethanol and osmotic stress. Ultraviolet radiation stress provides improved ability to survive acid, heat, ethanol and hydrogen peroxide stress in *L. lactis*. Some proteins induced during stress are the same for more than one type of stress, while other proteins are specific for only one type (Kim *et al*., 2001). During stationary phase RpoS induces various genes involved with stationary stress response. These gene products has a multitude of unrelated functions. RpoS is regulated by metabolites such as cAMP, ppGpp and other extra-cellular factors.

Acid

Resistance to human gastric acid is an important trait of probiotic bacteria. Each day, approximately 2.5 liters of gastric juice is secreted in the stomach at a pH of 2 -3 (Vinderola and Reinheimer, 2003). Food in the stomach may has a slight buffering effect against the low pH. Acids passively diffuse through the cell membrane of bacteria and enter the cytoplasm. The acids then dissociate into protons to which the cell membrane is impermeable. This results in intracellular accumulation of protons and lowers the intracellular pH (pH_i). The lower pH_i affects the transmembrane pH-gradient which contributes to the proton motive force and serves as an energy source in various transmembrane transport processes. Acid sensitive enzymes and proteins are also negatively affected by cytoplasmic acidification (Van de Guchte *et al*., 2002).

Sub-lethal acidic environments can lead to an adaptive response and offers protection in later acid exposure. This mechanism is known as the acid tolerance response (ATR). ATR was first described by Goodson and Rowbury (1989) when *E. coli* cells adapted to normally lethal acidic conditions when first grown in conditions of sub-lethal acidity. Adaptation to and survival in low pH conditions are important for food grade and gastrointestinal bacteria. *Propionibacterium freudenreichii* subjected to extreme acid challenge dramatically changed in morphology. The viability of the cells did, however, not decrease and cell integrity was not compromised (Jan *et al*., 2001). Cells that did not adapt to acid conditions underwent dramatic morphological changes and decreased in viability when subjected to acid stress. Adapted cells retained viability and cell integrity (Jan *et al*., 2001). This adaptive response has been reported for several species, including *Oenococcus oeni, Lactobacillus plantarum, Lactococcus lactis* and *Lactobacillus sanfranciscensis* (G-Alegria *et al*., 2004; Alemayehu *et al*., 2000; De Angelis *et al*., 2001). The extra-cellular pH at which bacteria grow ranges from 1.0 to 11.0. The ability to grow at different pH values divide bacteria into three groups *viz.* neutrophiles which grow best at neutral pH, alkalophiles which grow best at alkaline pH, and acidophiles which grow best at low pH. Intracellular pH has to be maintained at approximately neutral. Membrane-bound F_1F_0-ATPase plays an important role to maintain intracellular pH (Amachi *et al*., 1998). The *gadB*-encoded glutamate decarboxylase and the *gadC*-encoded glutamate-γ-aminobutyrate antiporter are additional mechanisms employed to retain intracellular pH. Both mechanisms are important in *Lactococcus lactis,* as shown with mutant strains (Amachi *et al*., 1998; Sanders *et al*., 1998).

Oral streptococci employ numerous mechanisms to tolerate their acidic environment. These mechanisms include ammonia-generating activities, proton-pumping F-ATPase and up-regulation of DNA, and protein repair systems. *Streptococcus mutans* increases the proportion of long chained, mono-unsaturated fatty acids in the membrane, and decreases the short-chained saturated fatty acids in response to acidification. The organism is rendered acid sensitive if the ability to alter fatty acid composition is compromised (Fozo and Quivey, 2004). Membrane alterations (fatty acid or phospholipid compositions) are common adaptation mechanisms for many bacteria in response to environmental stresses.

F_1F_0-ATPase is a multimeric enzyme that synthesizes ATP by using protons or pumping protons out of the cell with the energy provided by ATP hydrolysis. Lactic acid bacteria increase their F_1F_0-ATPase activity at low pH to maintain the transmembrane pH gradient. The *atp* operon (encoding the F_1F_0-ATPase) encodes five subunits (α, β, γ, δ, ε) of the cytoplasmic F_1 complex and three subunits (a, b, c) of the F_0 membrane proton channel. In lactic acid bacteria the *atp* operons differ in their genetic organization from other bacteria, but the significance of this feature is unknown. Studies suggest that several ATPases with different optimum pHs exist and that a K^+-ATPase may also be involved in maintaining optimum intracellular pH (Van de Guchte *et al*., 2002). During environmental stress the cell membrane is usually the first target and changes in the fatty acid composition of the membrane is a general response.

Bile

A probiotic bacterium must be able to resist the deleterious effects of bile to successfully colonize the intestine. The human liver secretes as much as one liter of bile per day which is released into the intestinal tract exposing bacteria to a serious challenge. The relevant bile concentrations in humans range from 0.3% to 0.5% (v/v). Bile is isotonic with plasma and has an osmolarity of approximately 300 mOsm/ kg, which is attributable to the osmotic activity of the inorganic ions. Bile is amphipatic and plays a key role in the solubilization and emulsification of lipids. It also functions as an excretory fluid by eliminating substances that cannot be excreted in urine (because of bound proteins or insolubility). Bile can affect the phospholipids and proteins in the bacterial cell membrane and disrupt cellular homeostasis (Begley *et al*., 2004).

Bile is synthesized in the pericentral hepatocytes of the liver and is secreted into thin channels called bile canaliculi, which drain into bile ducts that merge to form hepatic ducts. Bile leaves the liver through the common hepatic duct that joins the cystic duct from the gal bladder to form the common bile duct (Begley *et al*., 2004). Bile then enters the duodenum at a junction regulated by the sphincter of Oddi. Chyme from an ingested meal enters the duodenum and acid and partially digested fats stimulate cholecystokinin and secretin secretion. Secretin stimulates biliary ducts to secrete bicarbonate and water which expands the volume of the bile. Cholecystokinin stimulates contractions of the gallbladder and the common bile duct. The gallbladder contracts, the sphincter

of Oddi relaxes and up to 80% of the gallbladder contents are released into the duodenum (Begley *et al*., 2004).

Bile is yellow-green in colour and consists of organic and inorganic substances, including bile acids, cholesterol, phospholipids (mainly phosphatidylcholine) and the pigment biliverdin. Mucus and immunoglobulin A are secreted into bile to prevent bacterial growth and adhesion. Tocopherol may also be present to prevent oxidative damage to the biliary and small intestine. Endogenous substances (endobiotics) such as lipovitamins (biologically active forms of vitamin D2), water-soluble vitamins (mainly vitamin B12, folic acid and pyrodoxine), estrogenic steroids, progesterone, testosterone, corticosteroids and essential trace metals may be secreted into the bile and undergo enterohepatic cycling (Carey and Duane, 1994). Many exogenous substances (xenobiotics) are also secreted into bile (e.g. commonly used drugs and antimicrobial substances) and undergo some degree of enterohepatic cycling. All bile acids are conjugated before secretion as N-acyl amidates (peptide linkage) with glycine (glycoconjugated) or taurine (tauroconjugated). The ratio of glycoconjugates to tauroconjugates in human bile is usually 3:1. Conjugation lowers the pK_a of the terminal acidic group and allows the bile acids to be freely soluble over a wide range of ionic strengths, calcium concentrations and pH values.

Bacteria in the caecum and colon transform conjugated bile acids. The main alterations include deconjugation (cleavage of the amino acid side chain), 7α-dehydroxylation (replacement of a hydroxyl group with hydrogen) and 7α-dehydrogenation. Other modifications include hydroxylation (replacement of hydrogen with a hydroxyl group), epimerization (inversion of the stereochemistry of the hydroxyl groups at C-3, C-7 and C-12), oxidation (expulsion of H_2) and reduction (insertion of H_2). Primary bile acids (from cholesterol in the liver) are modified by bacterial enzymes in the intestine into secondary bile acids. Some of these secondary bile acids are potentially mutagenic and toxic (Begley *et al*., 2004). Bile salt hydrolases (BSHs) are catalytic enzymes that hydrolyze the amide bond between the amino acid side chain and the C-24 position of the steroid moiety of bile acids. The enzymes are intracellular and oxygen insensitive, has a slightly acidic optimal pH (between pH 5 and 6), their activity is coupled to biomass production and they are not regulated by bile salts. Three hypotheses has been formulated to explain the ecological significance of BSH activity. The first hypothesis suggests a nutritional

advantage on hydrolytic strains. The liberated amino acids can be used as carbon, nitrogen and energy sources but some studies (Tannock *et al*., 1989) has shown that this is not a universal function of BSHs. The second hypothesis proposes that BSHs facilitate incorporation of cholesterol or bile into the bacterial membrane. This may strengthen the membranes, affect fluidity or charge, and could lead to increased protection against host defense mechanisms. Finally, deconjugation of bile salts may be a detoxification mechanism where BSH enzymes play a role in bile tolerance and consequently survival in the gastrointestinal tract. The exact role of BSHs is, however, unknown. BSH-active bacteria in the intestine has a positive cholesterol lowering effect on the host, but modification of unconjugated bile salts may generate toxic compounds that has a negative effect on the host (Begley *et al*., 2004).

Bacterial membranes are more resistant to the negative effects of bile after acid adaptation, entry into stationary phase or increased osmolarity. Loci in bile sensitive mutants that are disrupted when coming into contact with bile are associated with the maintenance of membrane integrity. Bile alters membrane integrity and electron microscopy has shown that cells become shrunken and empty after exposure to bile, while enzyme assays has indicated leakage of intracellular material (Begley *et al*., 2004). High bile salt concentrations rapidly dissolve membrane lipids and dissociate integral membrane proteins. Low concentrations may disrupt membrane integrity through subtle effects on membrane permeability and fluidity, altered activity of membrane bound enzymes and transmembrane flux of divalent cations (Heuman *et al*., 1996). Conjugated bile acids are strong acids and are fully ionized at physiological pH values and remain in the outer hemileaflet of the lipid bilayer. Unconjugated bile acids flip-flop passively across the lipid bilayer and enter the cell (Cabral *et al*., 1987).

Membrane structure and composition plays a major role in bile resistance. Altering membrane characteristics such as charge, lipid fluidity and hydrophobicity, or injury of the cell membrane (by freezing) may significantly increase susceptibility to bile. Carbon dioxide significantly reduces tolerance to bile salts in cells. Temperature downshifts (from 37^{o} to 25^{o}) that alter fatty acid composition renders cells more resistant to bile. Growth in the presence of Tween 80 enhances bile tolerance in some strains and produces strain-specific changes in fatty acid composition (Begley *et al*., 2004). Bile acids also disturb macromolecule stability; alter the

conformation of proteins inducing misfolding and denaturation, and cause oxidative stress through generation of oxygen-free radicals. Molecular chaperones such as DnaK and GroESL has been shown to be induced by bile stress. Other promoters induced by bile include those that are also induced by oxidative stress (*mic*F and *osm*Y in *E. coli*). Bile chelates calcium and iron, leading to low intracellular calcium and iron concentrations (Begley *et al*., 2004).

Gram-negative bacteria are inherently more resistant to bile than Gram-positive bacteria. Bile salts are often used in the selective enrichment of growth media for Gram-negative bacteria. Gram-negative bacteria possess multi drug resistance (MDR) transporters that extrude bile from the cell along with other toxic compounds such as antibiotics and organic solvents. The role of these efflux systems in bile resistance in Gram-positive bacteria has not been determined (Van de Guchte *et al*., 2002). Activity of MDR transporters depends on the proton motive force or ATP hydrolysis and is part of the ABC transporter super-family. Resistance to bile is one of the selective criteria for probiotic Gram-positive bacteria. Bile tolerance is strain specific. Various studies has shown the variability of tolerance within a species and genus. Bile stress is complex and a variety of proteins are involved, many of which will preside over cell envelope architecture or maintenance of intracellular homeostasis. Proteins that transport bile salts and enzymes that modify and transform bile salts are also likely to play central roles. Studies with Two-dimensional-PAGE analysis revealed an increase in production of 45 proteins in *E. faecalis* during bile salt treatment (Flahaut *et al*., 1996). Seven general stress proteins were identified including the molecular chaperones DnaK, GroEL and Ohr (an organic hydroperoxide resistance protein). DNA repair proteins (MutS and SbcC), oxidative response (NifJ), transcription regulation, dGTP hydrolysis (Dgt), membrane composition (YvaG) and cell wall synthesis (SagA) proteins are encoded in loci that are disrupted in bile sensitive mutants (Begley *et al*., 2004).

Mechanisms used by bacteria to respond to bile may be similar to mechanisms used for other stress responses. A two-component system consisting of a histidine kinase and a cytoplasmic response regulator could be used to sense bile. Histidine kinase measures the presence of the environmental parameter (bile), senses a change and signals a response regulator which then instructs the cell to respond. Binding of signal molecules such as bile leads to a conformational

change in the sensing protein which then activates gene transcription. Disruption of cell membrane integrity or accumulation of damaged proteins as a result of exposure to bile could lead to an indirect response and induce genes involved in stress responses (Begley *et al*., 2004).

Enteric pathogens also use bile as an environmental cue to determine location and influence regulation of virulence genes. During colonization and infection of a host, bacteria continuously monitor their environment. Virulence factors are regulated by environmental factors such as acid, temperature and osmolarity. It is also likely that some of the gene products involved in bile tolerance will assist in survival and colonization of the intestinal tract, functioning as virulence factors (Mekalanos, 1992). *Enterococcus faecalis* grown in bile has altered physicochemical surface properties which results in increased invasion of biliary drain materials (Waar *et al*., 2002). Research on bile may explain chronic infections such as *Salmonella typhi* in the gallbladder and provide insight into pathogenic survival mechanisms *in vivo*. Antimicrobial agents combined with bile salts are very effective in the topical treatment of sexually transmitted diseases (Herold *et al*., 1999). Compounds could be designed to inhibit bile-efflux pumps, but it should be borne in mind that this could inhibit the natural intestinal microflora. Studying the relationship of bile stresses and other stresses can lead to better probiotic development. Pre-exposing strains to sub lethal bile conditions may also improve bile tolerance when ingested. Stimulation of BSH activity in probiotic strains lead to lower host cholesterol levels, which can be used as a biological alternative to other cholesterol lowering drugs. Research on bile may lead to development of better probiotics. Knowledge on regulatory pathways controlling bile stress is limited.

Human Pancreatic juice

Pancreatic juice has a pH above 8 and contains electrolytes and enzymes. Pancreatic juice has an intrinsic antimicrobial activity. However, infections are caused by Gram-negative bacteria such as *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and other pathogens such as *Enterococcus faecalis, Proteus mirabilis* and *Staphylococcus aureus.*

5. REAL-TIME POLYMERASE CHAIN REACTION

Kary Mullis revolutionized molecular science when he developed the polymerase chain reaction (PCR) method in the 1980s allowing the amplification of specific pieces of DNA to more than a billion fold. The first demonstration of real-time PCR was by Higuchi and co-workers in 1993, when they added ethidium bromide to the PCR reaction and ran the reaction under ultraviolet light enabling them to visualize and record the accumulation of DNA during the run (Highuchi *et al*., 1993). Real-time PCR was developed with the help of real-time videography in the early 1990s. The methods involve the use of fluorogenic probes that reports the concentration (level) of amplified DNA in each cycle. Quantitative PCR refers to the ability to quantify the initial amount of a specific DNA sequence.

DNA polymerase limits PCR because it uses DNA as template. Reverse transcriptase enzymes overcome this problem by generating a complementary cDNA strand from a RNA template. These reverse transcriptase enzymes are used by retroviruses in nature to generate DNA from viral RNA. Under specific reaction conditions the level of cDNA generated by reverse transcription is proportional to the level of its RNA template. cDNA can be used as template in real-time PCR to determine changes in expression levels of specific genes. This is called real-time (RT-PCR). Because of the sensitivity and accuracy of this method even slight changes in expression levels are detected (Valasek and Repa, 2005). When using DNA-binding dyes such as SYBR green I, a two step protocol may be preferred because it is easier to eliminate primer-dimer formation through manipulation of melting temperatures. One-step RT-PCR minimizes experimental variation, since both enzymatic reactions (from cDNA synthesis to PCR amplification) take place in the same tube. RNA used during one step procedures as a template is rapidly degradable and this method may not be ideal when analyzing samples over a period of time.

One-step protocols are also reported to be less sensitive than two-step protocols. During two-step reactions reverse transcription and PCR occur in separate tubes. The reverse transcriptase process is a highly variable reaction and using dilutions from the same cDNA template ensures that reactions from subsequent assays has the same amount of template as those assayed earlier. Two-

step real-time PCR data is more reproducible than one-step RT-PCR procedures. However, two-step procedures allow for more cross contamination in RT-PCR (Wong and Medrano, 2005).

Synthesis of cDNA from RNA is an important step in real-time RT-PCR and can, if not conducted under stringent conditions, lead to variation in reproducibility. Variability in RNA quality results from the unstability of RNA once extracted and the fact that the dynamic state of cells differ in different biological samples. The efficiency of RNA to cDNA conversion is dependant on template abundance. Different priming methods can be used to synthesize cDNA, e.g. with random primers, oligo-dT, target gene-specific primers or a combination of oligo-dT and random primers. Random primers prime the RT reaction at multiple origins along every RNA template and produce more than one cDNA target per original mRNA target. The rRNA (ribosomal RNA) is the largest part of total RNA and cDNA may be synthesized un-proportionately if the mRNA target is present at low levels (Bustin *et al*., 2005). Random primers has been shown to overestimate mRNA copy numbers by as much as 19-fold (Zhang and Byrne, 1999). Oligo-dT priming is more accurate than random hexamers and is the best method when trying to amplify several target mRNAs from a limited RNA sample. Oligo-dT priming requires full length RNA, with no secondary structures present and RT may fail if the primer binding site is at the extreme 5'-end of a long mRNA. Some RT-PCR reactions use both oligo-dT and random hexamer primers. Target-specific primers synthesize the most specific cDNA and may result in the greatest sensitivity for quantitative assays. It may be restrictive when only limited levels of RNA are available. This method requires separate priming reactions for each target and the same preparation cannot be used to amplify other targets at a later stage. Multiplexing (amplifying more than one target in a single reaction tube) is possible when experiments are carefully designed and optimized. If the target is present at very low amounts, random priming may be a better option than specific priming. Primers must be designed to maximum amplification efficiencies and be target-specific for the PCR step. Melting curve analysis and SYBR green I chemistry can be used to verify specificity. Primer design software is available to assist in optimum primer design (Bustin *et al*., 2005).

DNA amplification is monitored by using fluorescent probes. Fluorescent probes include DNA binding dyes such as ethidium bromide and SYBR green I, hydrolysis probes (5'-nuclease

probes), molecular beacons, hybridization probes, sunrise and scorpion primers and peptide nucleic acid (PNA) light up probes (Fig.3). Each type of probe has a unique method of fluorescence. SYBR green I has a 1000-fold greater fluorescence when bound to the minor groove of dsDNA compared to when in solution. SYBR green I binds dsDNA and fluorescence increases in proportion to the increase in dsDNA. This method's specificity relies on its primers. To ensure specificity, a dissociation curve of the amplified product is analyzed to determine the melting point. Hydrolysis probes are sequence-specific dually fluorophore labeled DNA oligonucleotides. One fluorophore is the quencher (acceptor, such as TAMRA, DABCYL and BHQ) and the other the reporter (donor, including FAM, VIC, NED, etc.). When the quencher and reporter are both attached to the same short oligonucleotide the quencher absorbs the signal from the reporter. This is an example of fluorescence resonance energy transfer. During amplification the oligonucleotide is excised by DNA polymerase (5' nuclease activity) and the reporter and quencher separates, resulting in the release of a fluorescent signal. This increases the reporter signal in correspondence to the amplification of the DNA. Hydrolysis probes allow for simple identification of point mutations (Valasek and Repa, 2005). Hybridization probes consist of two probes. One of the probes has a fluorescein donor on its 3' end and emits a green fluorescent light when excited by the light source. An acceptor fluorophore is attached to the 5' end and emission spectrums overlap. The acceptor fluorophore must be blocked at its 3' end to prevent extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of red fluorescent light. The two dyes are separate in solution and only background fluorescence is emitted by the donor. During the annealing step, both probes hybridize to their target sequence in a head to tail arrangement. In this close proximity the fluorescein transfers its energy at a high efficiency. The fluorescent signal is only detected when both probes hybridize to their correct target sequences (Bustin, 2000). TaqMan probes are sequence-specific oligonucleotides carrying a fluorophore and a quencher dye. The TaqMan probe hybridizes to the template between the standard PCR primers. The 5' dye molecule (such as 6-FAM or 6-carboxyfluorescein) is released from the probe and the quenching effect (via FRET) when in close proximity to a quenching molecule such as TAMRA (6-carboxy tetramethyl rhodamine) (Ginzinger, 2002). Molecular beacons are dual labeled probes with a fluorophore attached to the 5' end and a quencher dye attached at the 3' end. The ends of the probes are complementary. When the probe is in solution the two ends of the probe hybridize

and form a stem-and-loop structure (hairpin) with the fluorophore in close proximity (Fig.4.). This quenches the fluorescent signal. When the probe binds to the target sequence the stem opens and the fluorophore and quencher separates to generate a fluorescent signal (Critical factors for successful real-time PCR, 2004).

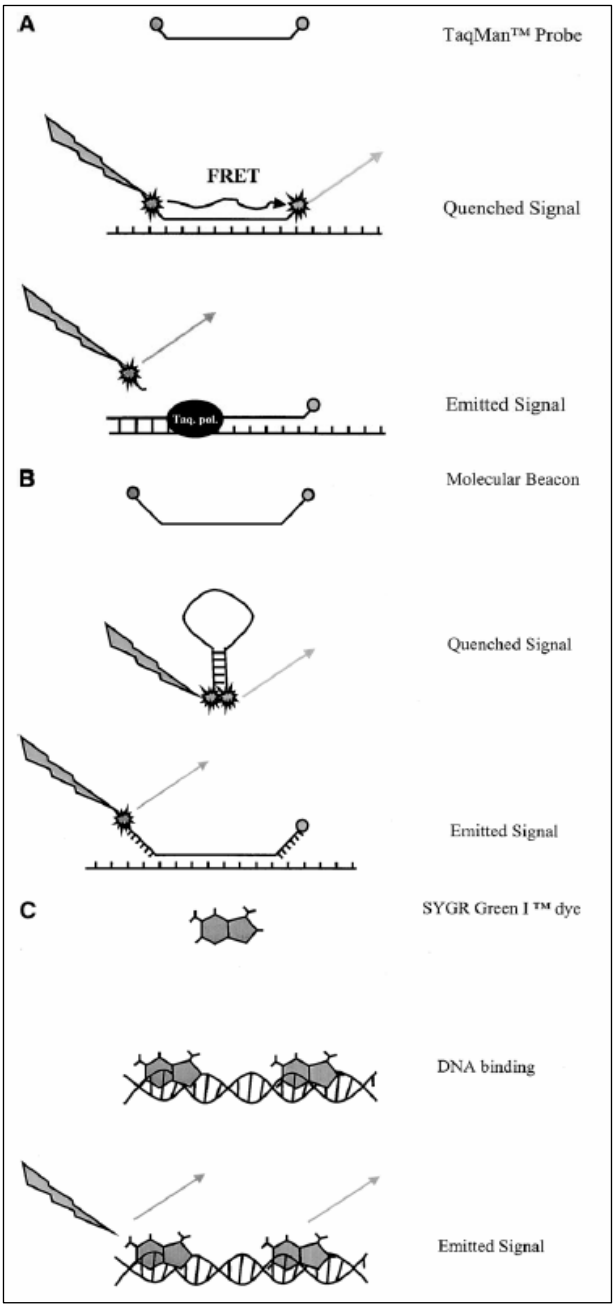

Fig. 4. Commonly used methods to generate a fluorescent signal in real-time PCR (Ginzinger, 2002). A) The TaqMan probe is used in a 5' nuclease assay to generate a signal using the 5' to 3'

exonuclease activity of Taq DNA polymerase. B) Molecular beacons use quenching action through a direct transfer of energy. A fluorescent signal is generated only when it is hybridized to the template when the distance of the 5' dye molecule from the quencher is adequately apart. A hairpin structure ensures the quenching effect. C) SYBR green I DNA binding dye binds to double-stranded DNA and emits a fluorescent signal when exited by the light source when bound. Primers has to be optimized to prevent the formation of primer dimers (Ginzinger, 2002).

Fluorescent values are recorded during every cycle and represent the amount of product amplified at a specific stage in the amplification reaction. The more template present at the beginning of the reaction, the fewer cycles it takes to first record the fluorescent signal as statistically significant above background (Gibson *et al*., 1996). This point is the C_t (crossing point) or threshold cycle and occurs during exponential phase of amplification. This value is representative of the starting copy number in the original template and is used to calculate experimental results. The components become limiting during the plateau phase, but because the measurements are made during the beginning stages of the exponential phase this does not affect the quantification. Any quantification based on measurements of overall product yield will therefore be intrinsically unreliable. The C_t values are translated into a quantitative result by the construction of a standard curve. Standard curves are easily generated because of the linear response of fluorescence over a large dynamic range (Bustin, 2000).

The instrumentation of real-time PCR must be able to detect the fluorescent signal and record the progress of the PCR. Three methods to supply excitation energy for fluorophores are by lamp, light emitting diode (LED) and laser. Lamps are broad spectrum emission devices, while LEDs and lasers are narrow spectrum devices. Detectors include charge coupled device cameras, photomultiplier tubes and other photodetectors. Multiple discrete wavelengths can be measured at once allowing running of multiple assays in a single reaction tube. The thermocycler used to perform the PCR must also be able to maintain a constant temperature among all sample wells. This is accomplished by using heated air or a heating block. Computer hardware and data-acquisition and analysis software offers graphical outputs of assay results.

Quantification of RNA can be done by using absolute or relative quantification. Absolute quantification determines the absolute amount of target (copy number or concentration). Relative quantification determines the ratio between the amount of target and an endogenous reference molecule (housekeeping gene). During absolute quantification an external standard is used to express the level of a gene as an absolute copy number. The standards are usually RNA molecules of known copy number and concentration. A standard curve of a range of known RNA standards is used to determine the concentrations of unknown samples by comparing the corresponding C_t values. Standards should be appropriate for the type of nucleic acid to be quantified. Standard and target molecules must has similar amplification efficiencies. Relative quantification determines the ratio between the amount of target and an endogenous reference gene that is present in all samples. The ratio can then be used to compare samples. Housekeeping (maintenance) genes are usually chosen as reference genes (Critical factors for successful real-time PCR, 2004).

Normalization serves to minimize errors resulting from sample to sample variations and variations in starting material. A cellular RNA that serves as an internal reference gene is used to normalize other RNA values. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatments. The reference gene should also be expressed at roughly the same level as the RNA under study. Commonly used endogenous reference genes are the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (Bustin, 2000). Unfortunately no such stably expressed gene is known for bacteria, although many groups has proposed using certain genes such as the 16S rRNA or 23S rRNA genes. These genes are not optimal because rRNA is expressed at higher levels than mRNA and rRNA is closely dependant on the physiological status of the bacteria (Fey *et al*., 2004).

The target and reference gene is amplified from the same sample. Amplification efficiencies determine the method of data analysis. If the amplification efficiencies of the target and reference genes are comparable (less than 10% difference) one standard curve can be constructed for both genes and the C_t values used to determine the amount of target from the standard curve. Alternatively the comparative method ($\Delta\Delta C_t$ method) can be used, were the standard curves are

only used to determine amplification efficiencies initially and the amount of target is calculated by using the C_t values. The amplification efficiencies of the target gene and the reference gene must be approximately equal (Critical factors for successful real-time PCR, 2004). Amplification efficiency of the reaction is important to consider in the method of analysis. Without the appropriate correction for efficiency an overestimation of the starting concentration may be made. Amplification efficiency is calculated from a standard curve with the formula: exponential amplification = $10^{(-1/slope)}$. The amplification efficiency is relatively stable in the early exponential phase and gradually declines to zero because of the depletion of PCR components. Calculating the efficiencies from raw data collection is reported to be more accurate than when collected from a standard curve (Wong and Medrano, 2005). Various other methods of quantification are available, such as the Pfaffl model (Pfaffl, 2001) which combines the gene quantification and normalization into a single calculation using a mathematical model and the Q-Gene method, a software program calculating the mean normalized gene expression with standard errors using two different mathematical models (Muller *et al*., 2002).

Most quantification methods in molecular biology are time consuming, labor intensive, insensitive, non-quantitative, require radio-activity, or has great cross contamination probability (Reischl *et al*., 2002). Real–time PCR assays are 10 000 to 100 000 times more sensitive than RNase protection assays, 1 000 times more sensitive than dot blot hybridization, and can even detect a single copy of a specific transcript. Real-time PCR can also detect differences between samples as small as 23%, requires much less RNA template than other methods of gene analysis and with a relatively high throughput (Wong and Medrano, 2005). The major disadvantage of RT-PCR is that it requires expensive equipment and reagents as well as sound understanding of the techniques and experimental design. Reproducibility of RT-PCR is influenced by the need to use two enzymatic steps, the exponential nature of the PCR and the use of small quantities of target molecules. The biggest limitation of RT-PCR is human error such as improper assay development, incorrect data analysis, and unwarranted conclusions. Real time PCR gene expression analysis measures mRNA levels and only suggests possible changes in protein levels or functions, rather than demonstrating them. Conclusions based on data derived from RT-PCR are best made when the biological context is well understood.

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SHORT COMMUNICATION

Antimicrobial action of bacteriocin ST4SA against gastro-intestinal bacteria

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Abstract

Bacteriocin ST4SA (bacST4SA), produced by *Enterococcus mundtii* ST4SA, inhibits the growth of *Enterococcus faecium* HKLHS. The antimicrobial effect of bacST4SA against strain HKLHS was tested at conditions simulating the human intestinal tract. *E. faecium* HKLHS grown in the presence of a combination of bile and pancreatic juice was more sensitive to bacST4SA treatment. BacST4SA was added to cells at a final concentration of 12800 AU/ml. The level of bacST4SA did not have a significant impact on HKLHS cells. Cells were more sensitive to bacST4SA when in early log phavee. Low concentrations (12800 AU/ml) of bacST4SA had the same inhibiting effect as higher concentrations (25600 AU/ml and higher), indicating that low levels of bacST4SA are effective for treatment of infections. Treatment of *E. faecium* HKLHS cells with bacST4SA resulted in leakage of β-galactosidase. Adsorption of bacST4SA to *E. faecium* HKLHS cells, was maximal at temperature ranges between 25^{o}C and 37^{o}C (75%) Acidic pH levels of pH 4.0 to 6.0 did not have a significant effect on adsorption, while higher pH levels of pH 8.0 to10.0 increased adsorption to 87.5%. No effect on adsorption of bacST4SA to *E. faecium* HKLHS cells was observed in the presence of 0.3%, 0,5% and 1,0% ox-bile, respectively. Treatment of cells with pancreatic juice (3.0%. 5.0% and 10.0%) showed a significant increase in adsorption (100%) for all three conditions. The presence of salts such as sodium chloride, magnesium chloride and potassium chloride did not have an effect on adsorption of bacST4SA to *E. faecium* HKLHS. Results indicate that *E. mundtii* ST4SA may inhibit *E. faecium* HKLHS in the human and animal gastro-intestinal tract.

Introduction

Probiotics are defined as "live microbial food supplements that benefit the health of consumers by maintaining or improving their intestinal microbial balance" (Guarner and Schaafsma, 1998). Lactose intolerance, irritable bowel syndrome, diarrhea, gastroenteritis and inflammatory bowel disease may be alleviated by probiotic strains of *Bifidobacterium, Propionibacterium, Lactobacillus, Lactococcus, Enterococcus* and *Streptococcus* spp. (Stanton et al., 2005).

Some strains of *Enterococcus faecium* have been associated with infections and are considered as emerging pathogens (Moreno *et al*., 2006). They have become important in community- and hospital-acquired infections and super-infections such as bacteraemia, urinary tract and intra-abdominal infections (Moreno *et al.,* 2005).

Enterococcus mundtii ST4SA produces a 3950 Da class IIa bacteriocin, active against Gram-positive and Gram-negative bacteria (Knoetze, 2006) and viruses, including the herpes simplex viruses HSV-1 (strain F) and HSV-2 (strain G), measles virus strain MV/BRAZIL/001/91 (an attenuated strain of MV) and polio virus PV3, strain Sabin (Todorov *et al*., 2005), *Acinetobacter haumanii* 16, *Bacillus cereus* LMG 13569, *Clostridium tyrobutyricum* LMG 13571, *Enterococcus faecalis* LMG 13566, E77, E80, E90, E92, FA2, 20 and 21, *Klebsiella pneumoniae* 31, *Lactobacillus sakei* LMG 13558, *Propionibacterium* sp. LMG 13574, *Pseudomonas aeruginosa* 1, 7, *Staphylococcus aureus* 6,13 and 36, *Streptococcus caprinus* TS1 and TS2, *Streptococcus pneumo*niae 3, 4, 27 and 29 (De Kwaadsteniet *et al*., 2005).

Bacteriocins provide probiotic bacteria with a competitive edge to ensure survival in the gastro-intestinal tract. Bacteriocins are small ribosomally synthesized, cationic, amphiphilic antimicrobial peptides, which vary in spectrum and mode of activity (Moreno *et al*., 2006). Bacteriocins are usually only active against species closely related to the producer strain (Moreno *et al*., 2006). However, recently a number of broad-spectrum bacteriocins have been described, *viz*. plantaricin 35d produced by *Lactobacillus plantarum* (Messi *et al*., 2001), bacteriocin ST151BR produced by

Lactobacillus pentosus ST151BR (Todorov and Dicks, 2004), enterocin CRL35 produced by *Enterococcus faecium* (Wachsman *et al*., 1999), thermophylin produced by *Streptococcus thermophylus* (Ivanova *et al*., 1998), *Lactobacillus plantarum* sp. isolated from ben saalga (Omar *et al.,* 2006) and bacteriocin ST4V produced by *Enterococcus mundtii* ST4V (Todorov *et al*., 2005). Other bacteriocins active against gram negative bacteria are the bacteriocin from *Lactobacillus paracasei* HL32 active against *Pophyromonas gingivalis* (Pangsomboon *et al.*, 2006), ST28MS and ST26MS produced by *Lactobacillus plantarum* isolated from molasses (Todorov and Dicks, 2004), broadspectrum bacteriocins isolated from boza: ST194BZ, ST242BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ, ST664BZ, ST712BZ and bozacin 14 (von Mollendorff *et al*., 2006) and bacteriocin produced by *Lactobacillus plantarum* ATCC8014 (Lash *et al.*, 2005).

Bacteriocins are divided into four classes based on structure, physicochemical properties and molecular properties (Klaenhammer, 1993). Class I bacteriocins are small, heat stable, cationic, hydrophobic peptides and contain unusual amino acids. Class II bacteriocins are subdivided into three classes. Subclass IIa include pediocin-like bacteriocins with anti-listerial activity with a consensus sequence YGNGV in the N terminus. Subclass IIb bacteriocins require two polypeptide chains for activity. Subclass IIc are bacteriocins that do not belong to the other two classes. Class III bacteriocins are large, heat-labile, hydrophilic proteins. Class IV comprises complex proteins that depend on a carbohydrate or lipid moiety to be fully functional (Moreno *et al*., 2006; Nes *et al.*,1996).

Bacteriocins interact with the bacterial membrane in various ways. Specific targets involved in pore formation have been identified, e.g. the nisin and epidermin family of lantibiotics target the membrane bound cell wall precursor lipid II (Linnett and Strominger, 1973), while the duramycin family binds to phosphoethanolamine which inhibits phospholipase A2 function (Nes and Holo, 2000). The intracellular ATP levels are depleted and amino acid leakage occurs via pores in the membrane. Interaction with the membrane involves electrostatic binding mediated by a putative membrane-bound

receptor-type protein. The positively charged polar residues of the bacteriocin binds with the anionic phospholipid head groups in the membrane. The C-terminus of the bacteriocin inserts into the target membrane and aggregates to form water-filled pores (Ennahar *et al*., 2000). Most class II bacteriocins permeabilize sensitive bacterial cell walls by dissipating the proton motive force (PMF) and pore formation. Class IIa bacteriocin activity may depend on a mannose permease of the phosphotransferase system (PTS) as a target (Ennahar *et al.,* 2000). Subclass IIb specific targets have not yet been identified. Subclass IIc bacteriocins act via membrane permeabilization, specific inhibition of septum formation and pheromone activity (Hechard and Sahl, 2002). Bacteriocins act in a time and concentration dependant manner (Ennahar *et al*., 2000).

E. faecium is a common intestinal microbe in the human and animal gut (Devriese and Pot, 1995). It is also resistant to several antibiotics, such as ampicillin, streptogramins, macrolides, chloramphenicol and quinolones (Klare *et al*., 2001). *E. mundtii* ST4SA will have to compete with this organism in the intestine.

In this study, we focused on the action of bacST4SA on *E. faecium* as a model intestinal organism in simulated gastro-intestinal conditions.

Materials and Methods

Bacterial strains and growth conditions. *Enterococcus mundtii* ST4SA isolated from soya beans, produces bacteriocin bacST4SA (Todorov *et al*., 2005; Knoetze, 2006). *Enterococcus faecium* HKLHS was used as sensitive intestinal organism (Knoetze, 2006). Strains were stored in the presence of glycerol (15%) at -80^{o}C. All strains were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37^{o}C. Oxbile (OXOID, England) and pancreatic juice (Sigma Aldrich, USA) were added to simulate intestinal conditions.

Bacteriocin bioassay. Bacteriocin activity tests were performed on cell-free supernatants by using the agar-spot test method (Todorov and Dicks, 2005). Cell-free supernatants

were adjusted to pH 6.0 with sterile 1 M NaOH before testing. Target microorganisms were cultured in BHI (Biolab, Biolab diagnostics, Midrand, South Africa). Antimicrobial activity was expressed as arbitrary units (AU)/ml, calculated as follows: a^b x 100, where "a" represents the dilution factor and "b" the last dilution that produces an inhibition zone of at least 2 mm in diameter. Activity was expressed per ml by multiplication with 100 (Todorov and Dicks, 2005).

Bacteriocin ST4SA production and purification. An overnight culture of ST4SA was harvested (8000 × g, 4°C, 15 min) and the supernatant adjusted to pH 6.0 with NaOH, treated for 10 min at 80°C, filter-sterilized and stored at 4°C. Ammonium sulfate was gently added to the cell-free supernatant at 4° C to a saturation level of 70% (SAMBROOK *et al*., 1989). After 4 h of vigorous stirring, the precipitate was collected (20 000 x g, 1 h, 4° C), the pellet re-suspended in one-tenth volume 25 mM ammonium acetate (pH 6.5) and desalted against sterile MilliQ water using a 1.0 kDa cut-off Spectra/Por dialysis membrane (Spectrum Inc., CA, USA).

Activity of bacteriocin ST4SA against *Enterococcus faecium* HKLHS. *Enterococcus faecium* HKLHS was grown to early log, mid-exponential, and stationary phavee in MRS broth (Biolab) and MRS broth, supplemented with 1% bile (v/v), 10% pancreatic juice, a combination of 1% bile (v/v) and 10% pancreatic juice, and MRS broth (Biolab) adjusted to pH 5.5. Cells (10ml) were harvested (8 000 x g, 5 min, 4°C), washed twice in 10ml physiological water, diluted and plated onto MRS agar. Incubation was at 37°C. To each of these samples bacST4SA was added (final concentration 25 600 AU/ml) and incubated at 25°C. The number of viable cells were determined by plating onto MRS agar. In a separate experiment, bacST4SA was added at 51 200, 25 600 and 12 800 AU/ml (final concentrations) to 3h, 6h and 12h-old cultures of *E. faecium* HKLHS in MRS broth (Biolab) supplemented with 1% bile (v/v) and 10% pancreatic juice (v/v). Optical density readings (600nm) were recorded every hour for 12 hours. An un-treated cell culture was used as control. The most sensitive growth condition (MRS broth supplemented with both 10% pancreatic juice and 1% bile) was further tested to determine if more diluted bacteriocin will have the same inhibiting effect on *E. faecium* HKLHS (Fig 3.A-C).

Adsorption of bacteriocin ST4SA to *Enterococcus faecium* HKLHS in the presence of oxbile and pancreatic juice. *Enterococcus faecium* HKLHS was grown overnight in MRS broth at 37°C. Cells were harvested (8000 × g, 10 min, 4°C), washed twice with 25mM phosphate buffer (pH 6.5) and resuspended in sterile phosphate buffer to the original volume. Oxbile was added to cell suspensions to final concentrations of 1%, 0.5% and 0.3% (v/v). Pancreatic juice was added to yield final concentrations of 3%, 5% and 10% (v/v). To these cell suspensions an equal volume of 25 600 AU/ml bacST4SA was added (final concentration of 12 800 AU/ml). Cells suspended in sterile distilled water served as control. Incubation was for 1 h at 37°C. The cells were harvested (8000 × g, 10 min, 4°C) and bacST4SA activity determined as described before.

Adsorption of bacST4SA to *E. faecium* HKLHS target cells were performed according to the method described by Yildirim et al. (2002). The target strain *E. faecium* HKLHS was grown overnight in 10ml MRS or BHI broth at 37°C and then harvested (10 000 x *g*, 15 min, 4°C). The cells were washed twice with 10 ml sterile 5 mM phosphate buffer (pH 6.5) and re-suspended to the original volume in the same buffer. The cell suspension (0.7 ml) was treated with 0.7 ml bacteriocin ST4SA (25 600 AU/ml) and incubated for 1h at 37°C. Cells were then harvested (10 000 x *g*, 15 min, 25°C) and the activity of unbound bacteriocins in the supernatant determined as described before.

The percentage adsorption of bacteriocin ST4SA to the target cells was calculated according to the following formula:

$$\% \text{ adsorption} = 100 - \left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}} \times 100 \right)$$

Effect of pH and temperature on the adsorption of bacteriocin ST4SA. Bacteriocin ST4SA (25 600 AU/ml) was added to *E. faecium* HKLHS to a final concentration of 12 800 AU/ml. The cells were incubated for 1h at 4, 10, 25, 30, 37, 45 and 60°C and, in a separate experiment, at pH 2.0, 4.0, 6.0, 8.0 and 10.0. The cells were then harvested (10 000 x *g*, 15 min, 25°C), the pH of the cell-free supernatant adjusted to 6.0 with sterile 1M NaOH and bacteriocin activity determined as described before.

Effect of SDS, inorganic salts and organic compounds on the adsorption of bacteriocin ST4SA to *E. faecium* HKLHS. Eighteen-h-old cells of *E. faecium* HKLHS were treated with 1% (m/v) NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, $(NH_4)_3C_6H_5O_7$, CH_3COONa, Na_2CO_3, EDTA ($C_{10}H_{16}O_8N_2$) and SDS, 1% (v/v) Triton X-100, Triton X-114 and β-mercaptoethanol, and 80% ethanol, methanol and chloroform, respectively. Bacteriocin ST4SA (25 600 AU/ml) was added to the treated cells, as described before, and incubated for 1h at 37°C. The cells were then harvested (10 000 x *g*, 15 min, 25°C) and the activity of the bacteriocins in the cell-free supernatant determined as described before.

Cell lysis. *Enterococcus faecium* HKLHS were grown in MRS broth and MRS broth supplemented with 1% (v/v) oxbile, 10% (v/v) pancreatic juice and a combination of 1% (v/v) oxbile and 10% (v/v) pancreatic juice, respectively. BacST4SA was added to 3h old cultures (final concentrations = 12 800 AU/ml) in the same growth conditions. Optical density readings (600nm) were taken every hour for 8 hours.

The level of β-galactosidase secreted from damaged cells was determined as follows. Ten ml of a log-phavee culture of *E. faecium* HKLHS (OD_{600} = 0.8 – 1.0) was harvested, the cells washed twice with 10 ml 0.03 M sodium phosphate buffer (pH 6.5), the pellet re-suspended into 4 ml of the same buffer and divided into two equal volumes. The cell suspension was treated with bacteriocin ST4SA (final concentrations = 12 800 AU/ml). After 5 min of incubation at 25°C, 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside), dissolved in 0.03 M sodium phosphate buffer (pH 6.8), was added to each of the cell suspensions and the cells incubated for 10 min at 37°C. The β-galactosidase reaction was stopped by adding 2.0 ml 0.1 M sodium carbonate. The cells were harvested (10 000 x *g*, 15 min, 25°C) and absorbance readings of the cell-free supernatants recorded at 420 nm. Controls were cells of *E. faecium* HKLHS prepared the same way, but not treated with bacteriocin ST4SA and the cells of *E. faecium* HKLHS mechanically broken with glass beads after 5 min (Hsu et al., 2005; Nagy et al., 2005). The resultant levels of β-galactosidase were determined from the standard curve prepared with β-galactosidase (Sigma-Aldrich, USA).

Results

Bacteriocin production and activity. Activity of bacteriocin ST4SA produced by *Enterococcus mundtii* ST4SA in MRS broth was recorded as 25 600 AU/ml in the cell-free supernatant (one AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition). After ammonium sulphate precipitation, an activity of 102 400 AU/ml was recorded. After dialysis the activity remained at 102 400 AU/ml.

Determination of the effect of bacST4SA on *E. faecium* at different growth phavees. *Enterococcus faecium* HKLHS growth was completely inhibited when ~ 3.0×10^6 CFU/ml were treated with bacST4SA after 3 h (early log phase), 6 h (mid-exponential phase) and 12 h (stationary phase) of growth in MRS broth supplemented with 10% (v/v) pancreatic juice, 1% (v/v) bile, both 10% (v/v) pancreatic juice and 1% (v/v) bile and at pH 5.5, respectively (Fig. 1.A-O, Fig. 2A-D). Cells cultured in MRS broth supplemented with 10% (v/v) pancreatic juice and 1% (v/v) bile (in all growth phases) were the most sensitive to bacteriocin treatment. Low concentrations (12800 AU/ml) of bacST4SA had the same inhibiting effect as higher concentrations (25600 AU/ml and higher), indicating that low levels of bacST4SA were effective for treatment of infections.

Adsorption of bacST4SA to *E. faecium* HKLHS in the presence of ox-bile and pancreatic juice. Adsorption of bacST4SA to *E. faecium* HKLHS cells were maximal at temperature ranges between 25^{o}C and 37^{o}C (75%), but significantly reduced at temperatures of 4^{o}C, 10^{o}C, 45^{o}C and 60^{o}C. Acidic pH levels of pH 4.0 to 6.0 did not have a significant effect on adsorption, while higher pH levels of pH 8.0 to10.0 increased adsorption to 87.5%. The presence of salts such as sodium chloride, magnesium chloride and potassium chloride did not have an effect on adsorption of bacST4SA to *E. faecium* HKLHS.

The addition of K_2HPO_4, KH_2PO_4, KI, tris-HCl, and Na_2CO_3 to *E. faecium* HKLHS cells increased adsorption of bacST4SA to 87.5% (Table 1). *E. faecium* HKLHS

cells treated with 2-mercaptoetanol increased adsorption to 87.5%, while other organic solvents such as 80% ethanol and methanol had no effect on adsorption. Treatment of cell walls with chloroform and SDS increased adsorption to 87.5%, while treatment with Triton X-100 and Triton X-114 increased adsorption to 93.75% (Table 1).

E. faecium HKLHS cells in the presence of 0.3%, 0,5% and 1,0% ox-bile, respectively, showed no effect on adsorption of bacST4SA. Treatment of cells with pancreatic juice (3.0%. 5.0% and 10.0%) showed a significant increase in adsorption (100%) for all three conditions (Table 1).

Effect of bacST4SA on *E. faecium* HKLHS cell membrane permeability. The level of β-galactosidase activity secreted from damaged cells was determined. Leakage of β-galactosidase was observed when *E. faecium* HKLHS was treated with bacST4SA. This level of β-galactosidase leakage was significantly higher than when cells were treated with buffer or when cells were mechanically destroyed.

Cell lysis of *E. faecium* HKLHS by bacST4SA. Growth of *E. faecium* HKLHS was significantly inhibited by the addition of bacST4SA after 3 h of growth. *E. faecium* HKLHS cells grown in MRS broth were the most sensitive to bacST4SA treatment. A 20% final concentration of bacST4SA was used. Pancreatic enzymes can inhibit bacST4SA as these enzymes denature the protein. Pancreatic enzymes had a less significant effect when bacST4SA concentrations were higher. Higher concentrations of bacST4SA showed a more significant loss of viability in *E. faecium* HKLHS cells.

Discussion

E. faecium HKLHS cultured in MRS broth supplemented with both 10% (v/v) pancreatic juice and 1% (v/v) bile (in all growth phavees) was more sensitive to bacteriocin treatment than cells cultured in MRS broth, supplemented with 1% bile (v/v), 10% pancreatic juice and pH 5.5, respectively. This suggests that *E. faecium* HKLHS may be sensitive to bacteriocin ST4SA produced by *Enterococcus mundtii* ST4SA in the

human gastro-intestinal tract, although other factors such as enzymes and the immune system response may also play a role. The bile content in the human gastro-intestinal tract ranges from 0.3% to 0.5% (v/v) (Begley *et al*., 2004). Pancreatic juice have a pH of 8.0 and is present at 0.9% (v/v) in the human GIT. BacST4SA remained active while in the presence of bile and pancreatic juice. *E. mundtii* ST4SA should be able to inhibit colonization of *E. faecium* HKLHS in gastro-intestinal conditions.

Low concentrations (12800 AU/ml) of bacST4SA had the same inhibiting effect as higher concentrations (25600 AU/ml and higher), suggesting that low levels of bacST4SA may be sufficient for treatment of infections. Low levels of bacST4SA may thus be effective in controlling the colonization of pathogens in the GIT and control the microbial population.

The effect of oxbile and pancreatic juice on bacteria is an important consideration when developing a probiotic organism. Probiotic bacteria have to survive intestinal conditions and their bacteriocin activity should not be significantly inhibited by intestinal conditions. Probiotic bacteria should be able to compete successfully with other intestinal bacteria and inhibit pathogens.

Effective binding of bacteriocins to target cells are important for probiotic bacteria to kill pathogens in the intestine. Adsorption of bacteriocins to sensitive target cells have been described for pediocin N5p produced by *Pediococcus pentosaceus* (Manca de Nadra *et al*., 1998), buchnericin LB of *Lactobacillus buchneri* (Yildirim *et al.*, 2002) and plantaricin 423 of *Lactobacillus plantarum* 423 (Todorov and Dicks, 2006).

Treatment of the sensitive target strain with 1% (w/v) SDS increased the adsorption of pediocin N5p, while treatment of cells had no effect on the adsorption of buchnericin LB (Yildirim *et al*., 2002). Todorov and Dicks (2006) reported that plantaricin 423 adsorption was affected by environmental factors such as Mn^{2+} (increased binding by 80 and 100%) and 1% SDS (increased adsorption by 25%). They also reported that adsorption conditions was strain specific, affected by pH, but not temperature or contact time (Todorov and Dicks, 2006). Yildirim and co-workers

reported that buchnericin LB only adsorbed to gram-positive bacteria and not gram-negative bacteria. Adsorption was pH dependant but not time and temperature dependant. Treatment of cells with organic solvents, detergents and enzymes did not affect resultant binding of buchnericin LB (Yildirim *et al*., 2002). Pediocin N5p from *Pediococcus pentosaceus* adsorbs to both gram-positive and gram-negative bacteria. Mg^{2+} and Mn^{2+} increases pediocin binding by 80 and 100% respectively, while treatment of cells with 1% SDS and proteolytic enzymes increased binding by 25 and 100% (de Nadra *et al*., 1998). *E. faecium* HKLHS cells treated with organic solvents and detergents increased adsorption of bacST4SA. The presence of salts such as sodium chloride, magnesium chloride and potassium chloride did not have an effect on adsorption of bacST4SA to *E. faecium* HKLHS. These results differ slightly form the results of these previously mentioned studies and indicate that adsorption conditions are strain specific.

Adsorption of bacST4SA to *E. faecium* HKLHS cells were maximal at temperature ranges between 25^{o}C and 37^{o}C (75%), which is close to the natural body temperature of humans. pH levels of pH 8.0 to10.0 increased adsorption to 87.5%. pH levels in the intestine are alkaline, which would indicate that adsorption of bacST4SA will be optimal in the intestine. Adsorption of bacST4SA was enhanced when in the presence pancreatic juice. This may be due to pancreatic enzymes making the cell membrane more sensitive. Bile had no significant effect on the adsorption of bacST4SA. These results indicate that bacST4SA should be able to successfully adsorb to bacterial cell membranes in the intestine. β-galactosidase leakage was significantly higher in the presence of bacST4SA than when cells were treated with buffer and when cells were mechanically destroyed. This indicates that bacST4SA effectively disrupts the cell membrane of *E. faecium* HKLHS cells, resulting in intracellular leakage and cell death.

The ability of probiotic bacteria to inhibit the growth of pathogenic bacteria in the gut by production of bacteriocins and other antimicrobial substances are well-known. *E. mundtii* ST4SA have proven to be effective in inhibiting growth of *E. faecalis* HKLHS in simulated intestinal conditions and should be able to inhibit colonization in the gastrointestinal tract of humans and animals, thereby preventing disease.

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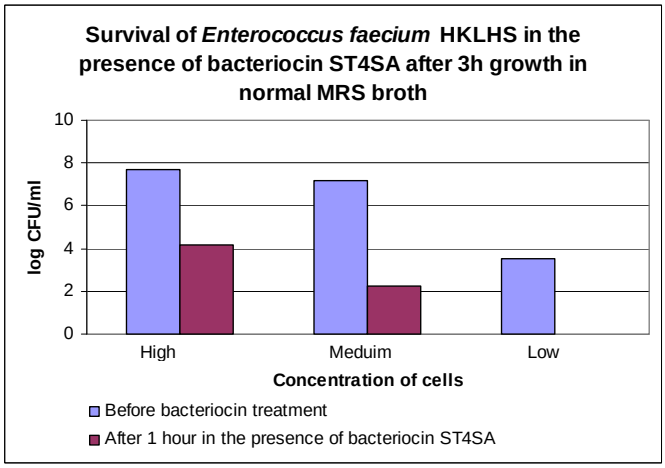
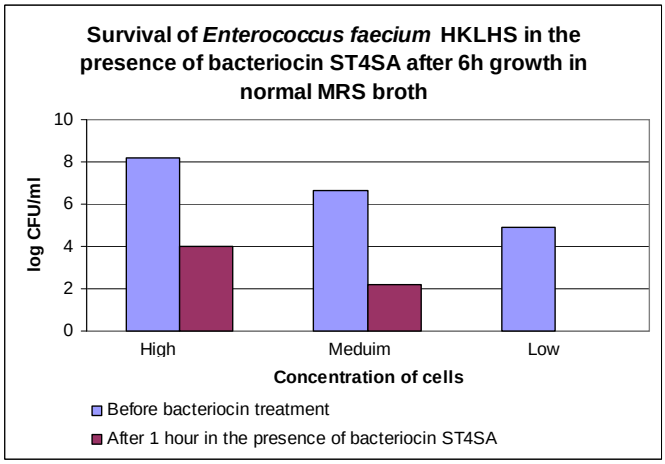
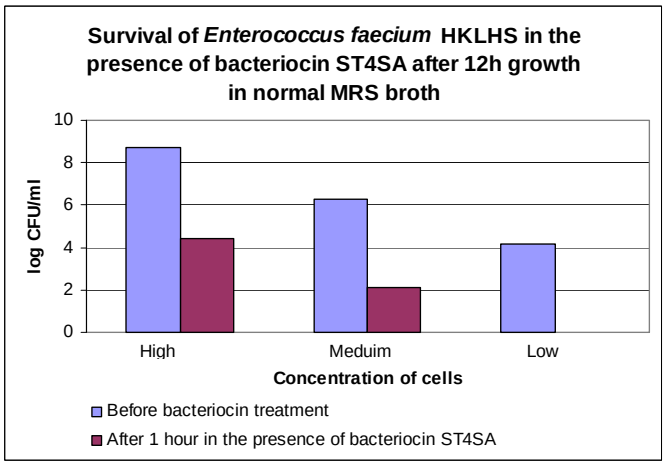

Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 3h growth in normal MRS broth
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

B.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 6h growth in normal MRS broth
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

C.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log CFU/ml
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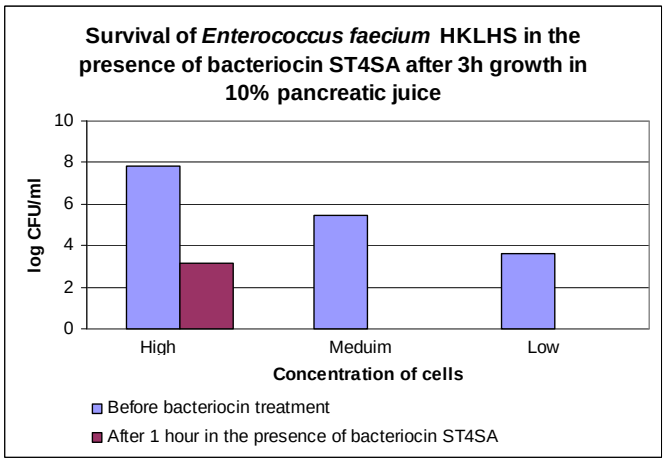
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

D.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log CFU/ml
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8
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4
2
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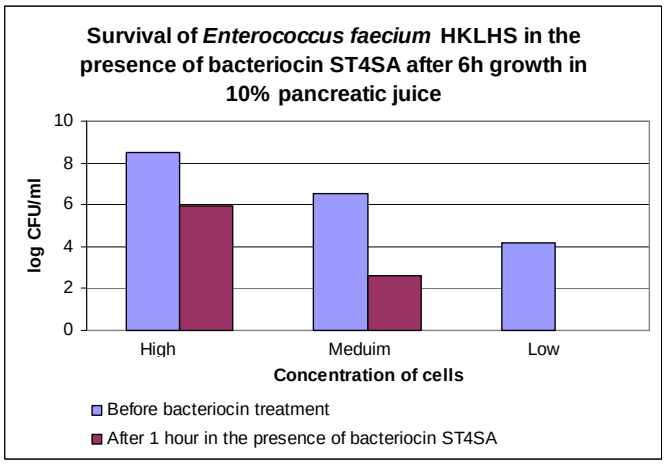
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

E.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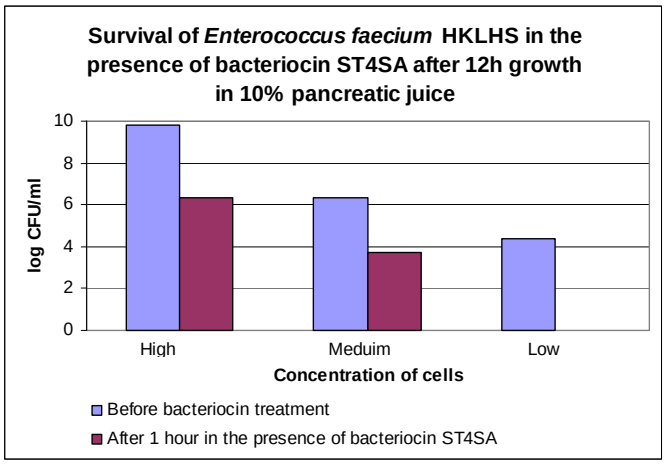
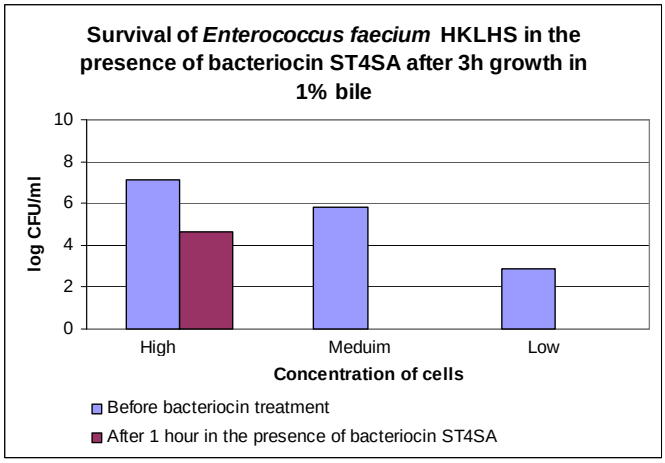
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

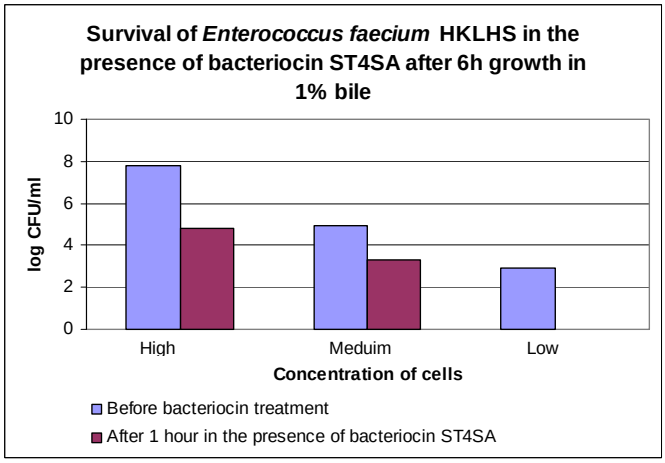
F.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 12h growth in 10% pancreatic juice
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

G.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 3h growth in 1% bile
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

H.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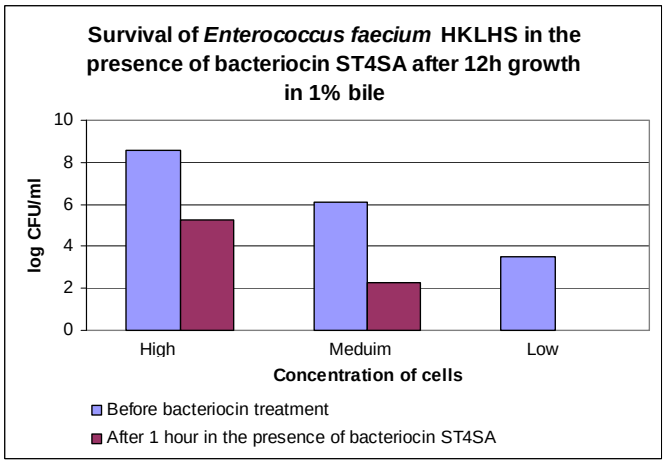
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

I.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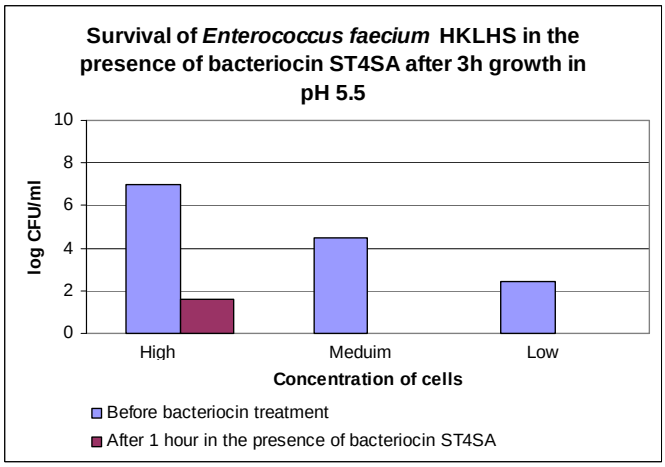
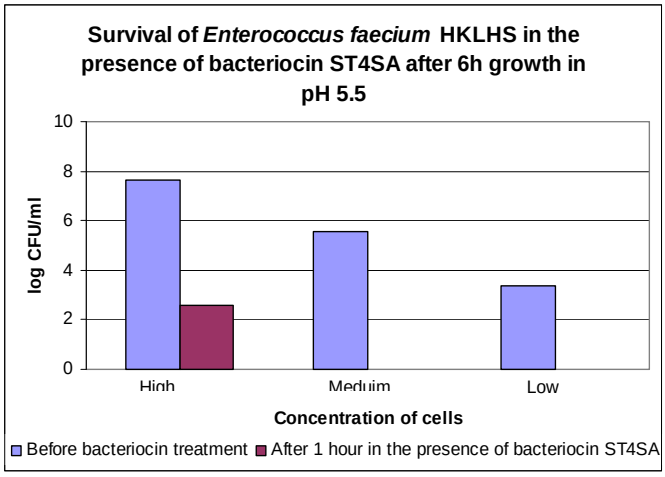
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

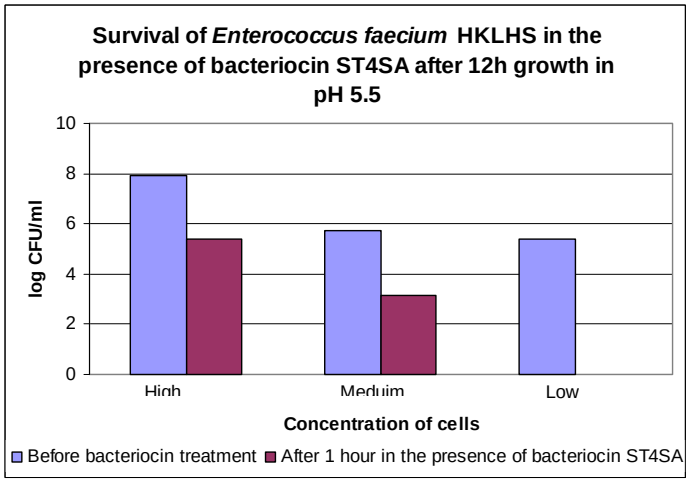
J.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 3h growth in pH 5.5
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

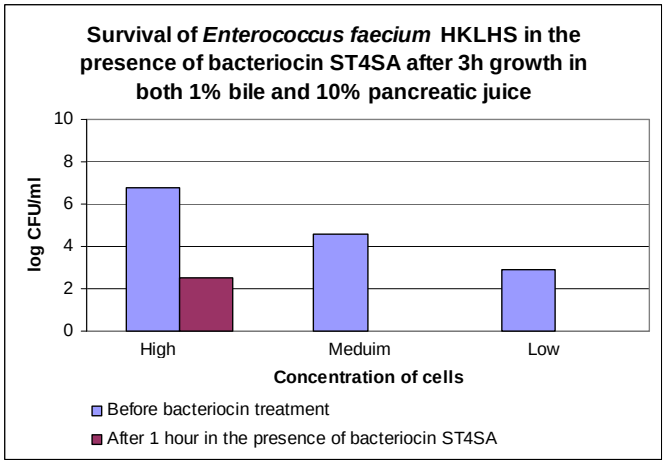
K.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 6h growth in pH 5.5
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

L.


Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA after 12h growth in pH 5.5
log CFU/ml
10
8
6
4
2
0
High
Meduim
Low
Concentration of cells
Before bacteriocin treatment
After 1 hour in the presence of bacteriocin ST4SA

M.



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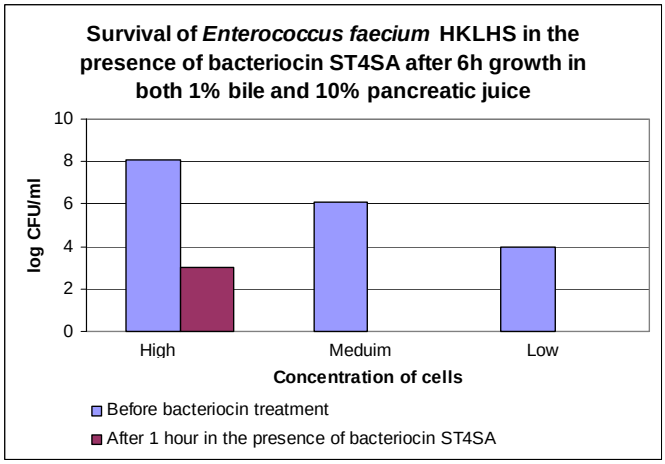

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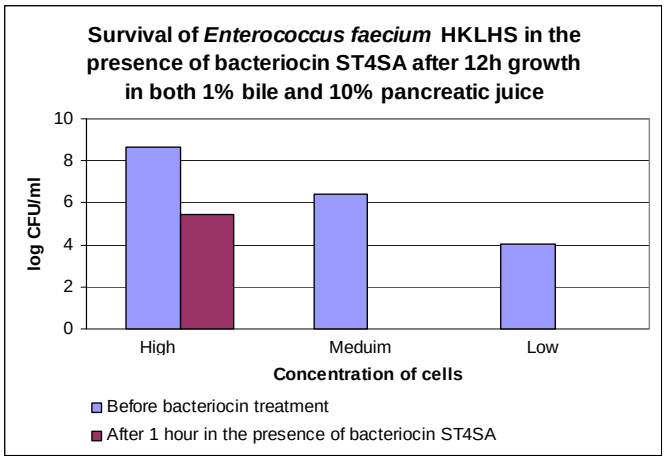
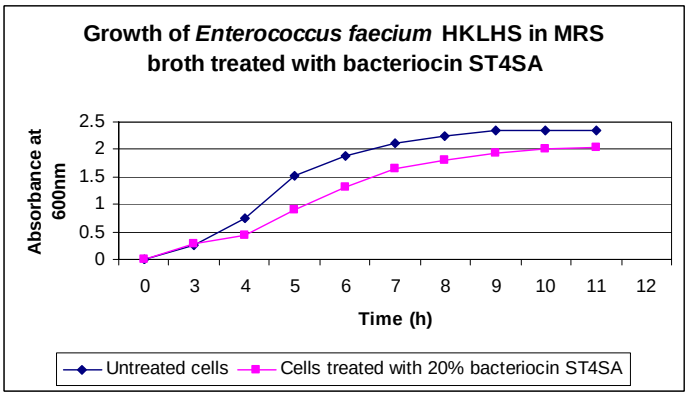


Fig. 1. A-O: Survival of *Enterococcus faecium* HKLHS in the presence of bacteriocin ST4SA.

A.



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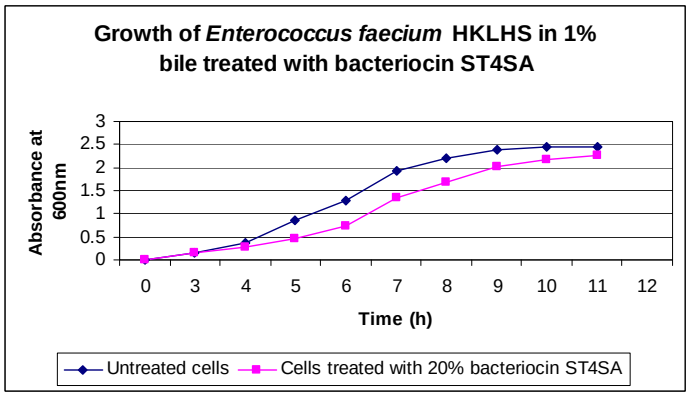

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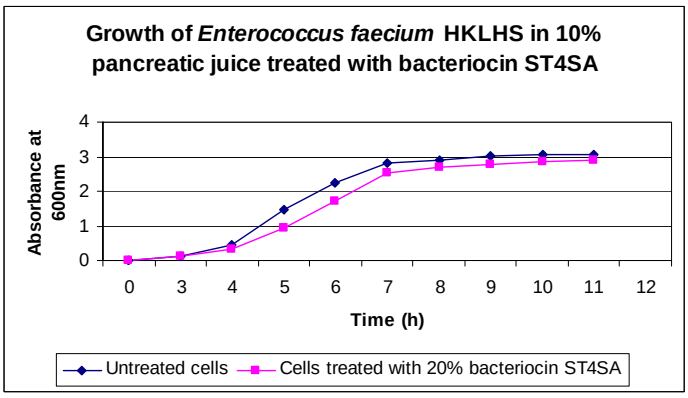


D.

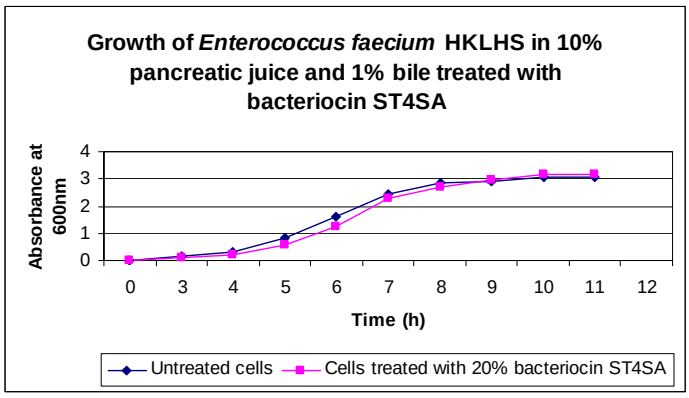
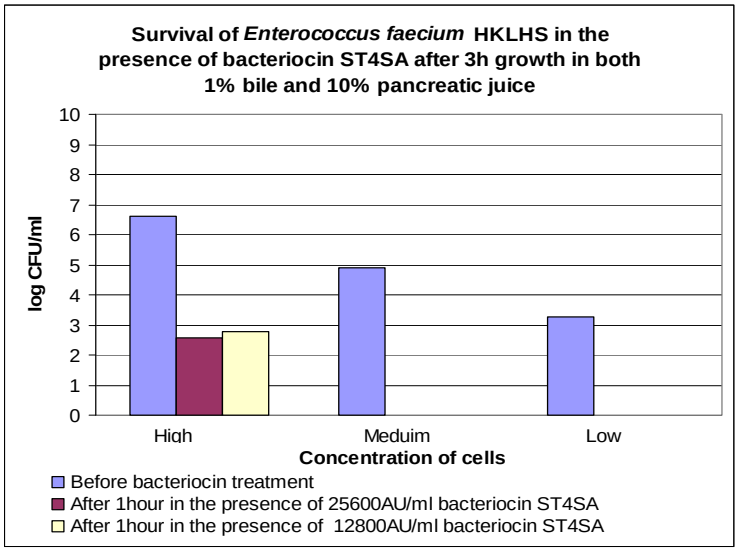


Fig. 2. A – D: *Enterococcus faecium* HKLHS treated with bacteriocin ST4SA in different growth conditions

A.



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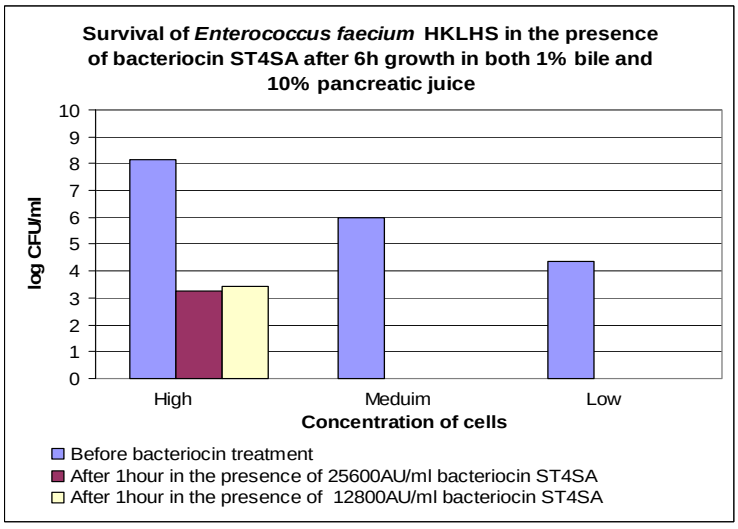

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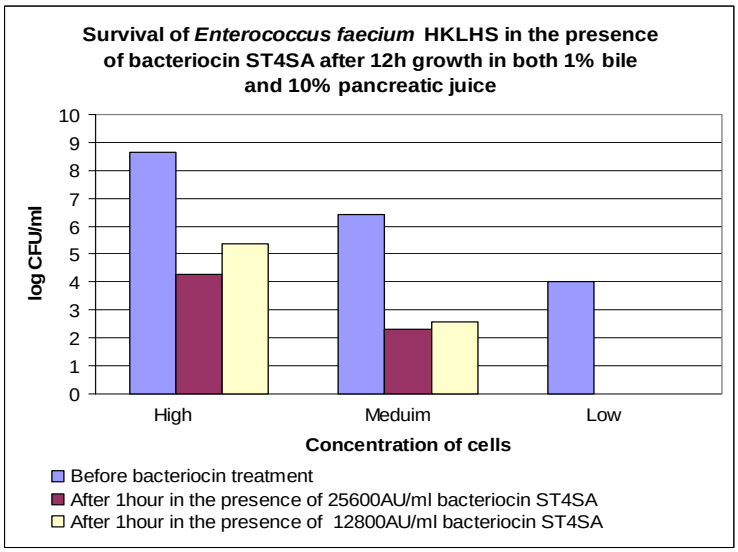
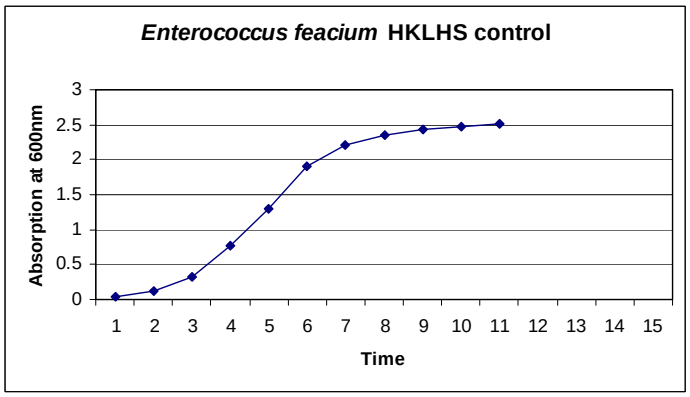


Fig. 3. A, B and C. *Enterococcus faecium* HKLHS in 1% bile and 10% pancreatic juice treated with diluted bacteriocin ST4SA

A.



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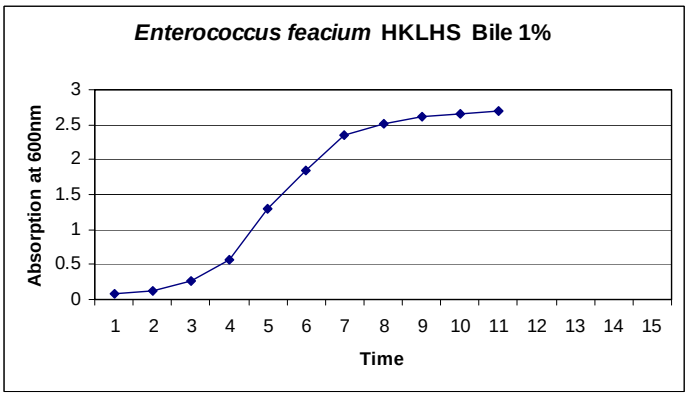

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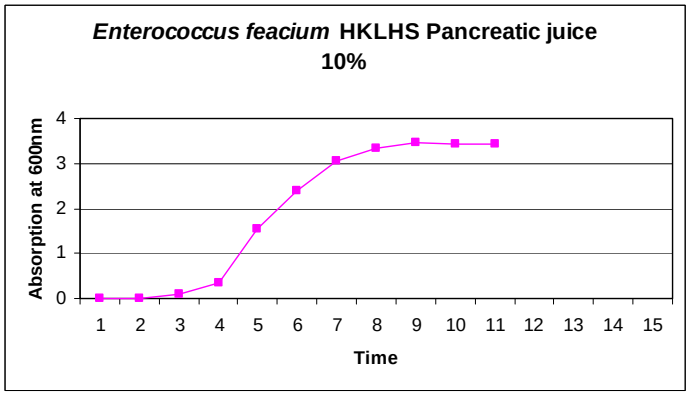
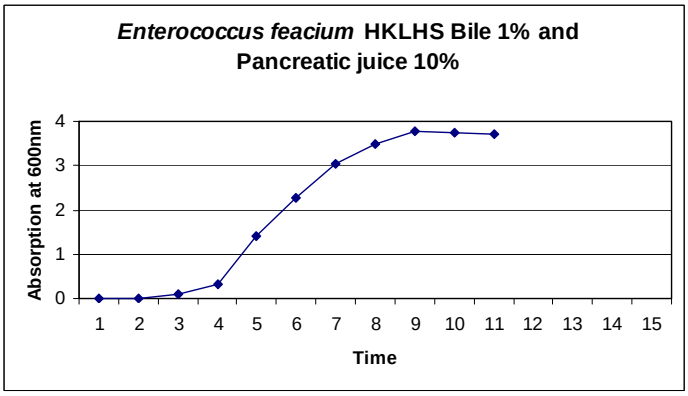


D.



E.

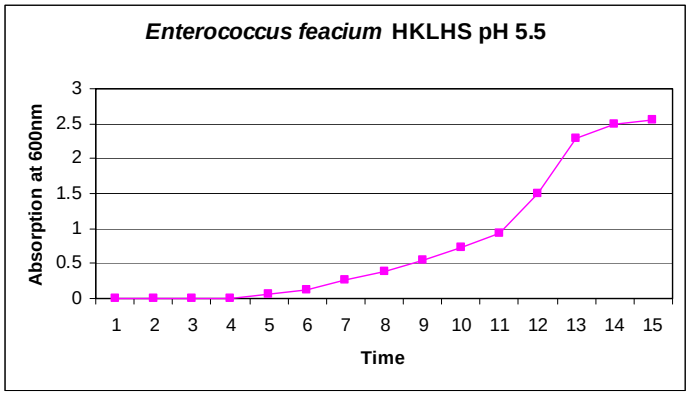


Fig. 4. A-E: *Enterococcus faecium* HKLHS adsorption in different growth conditions.

Table 1. Effect of pH, temperature, SDS, inorganic salts, solvents, ox-bile and pancreatic juice on the adsorption of bacteriocin ST4SA to target cells of *E. faecium* HKLHS (expressed as a percentage value)

Tested factors	**Bacteriocin ST4SA adsorption to *E. faecium* HKLHS (%)**
No chemical added	75
NaCl	75
K_2HPO_4	87.5
KH_2PO_4	87.5
$MgCl_2$	75
KCl	75
KI	87.5
Tris-HCl	87.5
NH_4-citrate	75
Na-acetate	75
Na_2CO_3	87.5
EDTA (-Na)	75
SDS	87.5
Triton X-100	93.75
Triton X-114	93.75
2-mercaptoetanol	87.5
80% ethanol	75
Methanol	75
Chlorophorm	87.5
Ox bile 0.3%	75
Ox bile 0.5%	75
Ox bile 1.0%	75
Pancreatic juice 3.0%	100
Pancreatic juice 5.0%	100
Pancreatic juice 10.0%	100
4 ^oC	50
10 ^oC	50
25 ^oC	75
30 ^oC	75
37 ^oC	75
45 ^oC	50
60 ^oC	50
pH 2.0	50
pH 4.0	75
pH 6.0	75
pH 8.0	87.5
pH 10.0	87.5

Table 2. Levels of β-galactosidase recorded after treatment of *E. faecium* HKLHS with bacteriocin ST4SA

	bacST4SA	**Controls**		
	Treated HKLHS with bacST4SA	HKLHS + buffer	Mechanical destroyed HKLHS + buffer	Cell free supernatant of ST4SA (bacteriocin)
β-galactosidase activity (420 nm)	0.185	0.023	0.168	0.069
β-galactosidase activity (Enzyme Units)	1.01×10^{-1}	6.80×10^{-3}	9.62×10^{-2}	3.44×10^{-2}

Effect of gastro-intestinal conditions on *Enterococcus mundtii* ST4SA and production of bacteriocin ST4SA, as recorded by real-time PCR

M. Granger, C. A. van Reenen, L. M.T. Dicks
Department of Microbiology, Stellenbosch University, 7600 Stellenbosch, South Africa

Abstract

Enterococcus mundtii ST4SA, isolated from soya-beans, produces a 3950 Da bacteriocin (bacST4SA) active against a variety of Gram-positive and Gram-negative bacteria, including human pathogens. In this study, the effect of gastro-intestinal conditions on the survival of strain ST4SA and production of bacST4SA was studied. Strain ST4SA was cultured in MRS broth at different pH and in MRS broth supplemented with bile, pancreatic enzymes, and contents of the stomach and small intestine of pigs, respectively. After 12 and 24h at 37^{o}C, cells were harvested, RNA isolated and cDNA prepared. Expression of the genes encoding bacST4SA, RecA, GroES and 23S rRNA was studied by real-time PCR (RT-PCR). No significant up- or down-regulation of the genes were recorded when cells were cultured under conditions of stress, except at pH 3.5 in which case only the RecA and GroES genes were up-regulated. Growth of *E. mundtii* ST4SA and production of bacST4SA are not affected by stress conditions normally found in the gastro-intestinal tract. Strain ST4SA could thus be used as a probiotic in humans and animals.

Introduction

Lactic acid bacteria (LAB) produce antimicrobial substances that include bacteriocins, lactic acid, acetic acid, hydrogen peroxide and antibiotics (O'Sullivan *et al.*, 2002). Strains with antimicrobial properties are used as starter cultures in a variety of fermented meat, dairy and vegetable products to extend shelf life (O'Sullivan *et al*., 2002). Many of these strains are marketed as probiotics. However, for these strains to survive conditions in the human and animal gastro-intestinal tract, they have to survive low pH, high levels of bile salts and pancreatic enzymes, and compete with other intestinal micro-organisms for adhesion to epithelial cells and mucus (Leverrier *et al*.,

2005). Constitutive production of a broad-spectrum bacteriocin should provide the strain with a competitive advantage over pathogens.

Bacteriocins are small ribosomally synthesized, cationic, amphiphilic, antimicrobial peptides, naturally produced by microorganisms (Moreno *et al.,* 2006). Bacteriocins of lactic acid bacteria vary in spectrum and mode of activity, molecular structure and mass, thermostability, pH range for optimal activity and genetic determinants (Moreno *et al*., 2005). These peptides are often only active against closely related species, but a few, including bacST4SA produced by *E. mundtii* ST4SA, are active against Gram-positive and Gram-negative bacteria and viruses (Todorov *et al*., 2005). Other broad-spectrum bacteriocins include plantaricin 35d produced by *Lactobacillus plantarum* (Messi *et al*., 2001), bacteriocin ST151BR of *Lactobacillus pentosus* ST151BR (Todorov and Dicks, 2004), thermophylin of *Streptococcus thermophylus* (Ivanova *et al*., 1998), enterocin CRL35 of *Enterococcus faecium* (Farias *et al*., 1996), peptide AS-48 of *Enterococcus faecalis* (Abrionel *et al*., 2001) and a bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* (Caridi, 2002).

Enterococcus mundtii ST4SA produces a 3950 Da class IIa broad-spectrum antibacterial peptide (bacST4SA) active against Gram-positive and Gram-negative bacteria, including *Enterococcus faecalis, Streptococcus* spp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus pneumoniae* and *Staphylococcus aureus.* BacST4SA inactivates the herpes simplex viruses HSV-1 (strain F) and HSV-2 (strain G), a measles virus (strain MV/BRAZIL/001/91, an attenuated strain of MV) and a polio virus (PV3, strain Sabin) (Todorov *et al*., 2005). The genes encoding bacST4SA are located on a 50-kb plasmid (H. Knoetze, 2006).

Class IIa bacteriocins have strong activity against *Listeria* and possess the consensus sequence YGNGV in their N-terminus (Ennahar *et al*., 2000). This YGNGVXaaC-motif is part of a recognition sequence for a membrane receptor protein. Class IIa bacteriocins have a net positive charge and their iso-electric points vary from 8.3 to 10.0. The C-terminal domain is moderately conserved, hydrophobic or

amphiphilic. Class IIa bacteriocins have at least two cysteines with a disulfide bridge (Ennahar *et al*., 2000). These bacteriocins form pores in sensitive cell membranes and leads to dissipation of the proton motif force (PMF) (Ennahar *et al*., 2000). Mundticin, produced by some strains of *E. mundtii,* completely dissipates the transmembrane potential (Bennik *et al*., 1998).

ST4SA is encoded in a gene cluster (*mun* locus) that consists of three genes; *munA, munB* and *munC*. The structural gene, *munA*, encodes a 58-amino-acid mundticin ST4SA precursor. The leader peptide contains 15 amino acids with a double-glycine processing site. *MunB* encodes a 674-amino-acid protein (ABC-transporter) that is involved in translocating and processing of the bacteriocin. *MunC* encodes a 98-amino-acid immunity protein against bacST4SA. The amino acid sequence of *munA* is completely homologous to other class IIa bacteriocins, e.g. mundticin KS, mundticin AT06 and mundticin QU2 (H. Knoetze, 2006). BacST4SA and enterocin CRL35 differ with two amino acids in their leader peptides, but the mature bacteriocins are completely homologous (Minahk et al., 2000). The ABC-transporter gene of bacteriocin ST4SA is 98.9% homologous to mundticin KS and 99.25% homologous to enterocin CRL35 (Kawamoto et al., 2002; Minahk et al., 2000). *MunC* is completely homologous to the immunity gene of enterocin CRL35 and 96.9% homologous to the immunity gene of mundticin KS (H. Knoetze, 2006).

Gene translation is usually studied by hybridization (Northern blots), RNAase protection assays and cDNA arrays. These methods are time-consuming and less sensitive than reverse transcription real-time (RT)-PCR (Bustin, 2000). In the latter case, amplification of genes is quantified by fluorescent probes. The assay is rapid and detects a single copy of a specific transcript (Wong and Medrano, 2005).

This study was conducted to determine if strain ST4SA would be able to survive conditions in the gastro-intestinal tract. Production of bacST4SA and stress proteins was studied by RT-PCR.

Materials and Methods

1. Bacterial strains and growth conditions

Enterococcus mundtii ST4SA and the sensitive strain (*Lactobacillus casei* LHS_3), were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C.

2. Growth of strain ST4SA and production of bacST4SA under stress

Strain ST4SA was inoculated (1.0%, v/v) into MRS broth (Biolab) adjusted to pH 5.5, 4.5, 3.5, 2.5 and 1.5, MRS broth (Biolab) supplemented with 0.3% (v/v), 0.5% (v/v) and 1.0% (v/v) bile, and 0.9%, 5.0% and 10.0% (v/v) pancreatic juice, respectively. Growth in unmodified MRS broth (Biolab) served as control. The experiment was repeated in skim milk supplemented with 4.0% (v/v) bile. Changes in cell density (OD_{600}) and culture pH were recorded every 2 h for 24 h. Cell numbers were determined every 4 h by plating onto MRS agar. Plates were incubated at 37°C and colonies counted after 48h.

In a separate experiment, strain ST4SA was inoculated (1.0%, v/v) into MRS broth (Biolab) adjusted to pH 5.5 and 3.5, MRS broth (Biolab) supplemented with 0.5% (v/v) bile, 3.0% (v/v) pancreatic juice, and a combination of 1.0% (v/v) bile and 10.0% (v/v) pancreatic juice, respectively. Growth in unmodified MRS broth (Biolab) served as control. Cultures were incubated at 37°C and bacST4SA activity recorded after 6, 12, 18 and 24h. At each sampling time, cells were harvested (8000 × g, 4^oC, 15 min), the supernatant adjusted to pH 6.0 with 1N NaOH, treated for 10 min at 80^oC, filter-sterilized and stored at 4^oC. BacST4SA production was recorded by using the spot plate technique (Ivanova *et al*. 1998). Activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition of the indicator strain (Ivanova *et al*., 1998).

In another experiment, strain ST4SA was inoculated (1.0%, v/v) in MRS broth (Biolab) with active-growing cells of *L. casei* LHS_3 (10^6 CFU/ml) and autoclaved cells of LHS_3, respectively. Strain ST4SA inoculated into sterile MRS broth (Biolab) served as control. Incubation was at 37°C. BacST4SA activity was recorded after 8, 12, 24 and 48 h, as described before. All experiments were done in triplicate.

3. Expression of genes encoding bacST4SA and stress proteins

Strain ST4SA was inoculated (1%, v/v) into MRS broth (Biolab) adjusted to pH 5.5 and 3.5, MRS broth (Biolab) supplemented with 0.5% (v/v) bile, 3.0% (v/v) pancreatic juice, and a combination of 1.0% (v/v) bile and 10.0% (v/v) pancreatic juice, respectively. Growth in unmodified MRS broth (Biolab) served as control. After 12 and 24 h of growth, cells were harvested (8000 × g, 4°C, 15 min), treated with RNAprotect Bacteria Reagent (Qiagen), and total RNA isolated using the Qiagen RNeasy kit (Qiagen, Southern Cross Biothechnology, South Africa), according to the manufacturers' instructions. Purified RNA was suspended in 30µl 0.1% DMPC (dimethylpyrocarbonate)-treated water. RNA concentrations were measured at 260nm using a NanoDrop spectrophotometer.

RNA samples were treated with four units of TURBO DNase (Ambion, Southern Cross biotechnology), as described by the supplier. Absence of chromosomal DNA contamination was confirmed by PCR. Primers listed in Table 1 were used. cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Randburg, South Africa), as described by the supplier.

Primers used in real-time PCR were designed from sequences listed in GenBank, using Primer Designer version 1.01 (Scientific and Educational Software) and obtained from Inqaba Biotechnology (Pretoria, South Africa). The primers are listed in Table 1.

Real-time PCR was performed using a Roche LightCycler Instrument with SYBR^R green JumpStart™ Taq readymix (Sigma-Aldrich, South Africa) in LightCycler capillaries. Initial denaturation of cDNA was at 95°C for 15 min, followed by 50 cycles

of amplification at 50°C for 10 sec and 72°C for 15 sec. Fluorescence was recorded at each annealing step. A melting curve was performed at the end of each run from 95°C to 45°C ($0.2^{o}Cs^{-1}$). Amplification efficiency (*E*) was determined by running a standard curve with serial dilutions of cDNA. The following formula was used: $E = [10^{(1/slope)}-1] \times 100$, where "slope" refers to the slope of the standard curve. A threshold cycle value (Ct) was determined for each sample, which represents the number of cycles required to reach a level where the fluorescent signal is statistically higher than the background. Results were analyzed using the comparative critical threshold method whereby the RNA level was adjusted to a reference relative to an internal calibrated target RNA (reference gene), as described by Desroche and co-workers (2005). The following equations were used:

ΔC_t = C_t of internal control – C_t of gene of interest.

$\Delta\Delta C_t$ = ΔC_t of sample - ΔC_t of calibrator.

Relative expression level = $2^{\Delta\Delta Ct}$.

Genes were considered significantly up- or down-regulated if the relative expression level was at least two-fold lower or higher than that of the reference gene. Readings were in triplicate on each of the three independent cultures.

In a separate experiment, 50 ml of stomach and small intestine content was collected immediately after slaughtering of pigs and inoculated with strain ST4SA (1%, v/v, from 1×10^{6} cfu/ml). The pH of the stomach and small intestine was 5.5 and 7.5, respectively. The control was strain ST4SA cultured in MRS broth (Biolab). All samples were incubated at 37°C. Total RNA was isolated from 12 and 24h old cells, cDNA prepared and real-time PCR performed as described before.

Results and Discussion

Growth of *E. mundtii* ST4SA in the presence of 0.3% (v/v) bile was inhibited for the first 6h, but recovered during the following 18h (Table 2). Growth in the presence of 0.5% (v/v) bile was inhibited for the first 6h, followed by a slow recovery during the next 6 to 12h (Table 2). Growth was severely inhibited in the presence of 1.0% (v/v) bile (Table 2). After 24h of growth, an eight-fold reduction in bacST4SA activity was

recorded in the presence of 0.3% (v/v) and 0.5% (v/v) bile, and a 16-fold reduction in the presence of 1.0% (v/v) bile (Table 2).

Growth of strain ST4SA was inhibited at pH 2.5 (Table 2). This may not be a true reflection of growth in the stomach, as ingested food has a buffering capacity. Concluded from the low levels of bacST4SA recorded at pH 2.5 (1 600 AU/ml), the cells remained viable for at least 24h. Growth at pH 5.5 was more profound than at pH 3.5 and cell density increased with incubation (Table 2). BacST4SA production was repressed at pH 3.5 (1 600 AU/ml), but increased eight-fold to 12 800 AU/ml at pH 5.5 (Table 2).

Variations in pancreatic juice (5% and 10%, v/v) had no significant impact on the growth of strain ST4SA. BacST4SA production in the presence of 5% (v/v) pancreatic juice was very low (100 AU/ml). No activity was recorded in the presence of 10% (v/v) pancreatic juice (Table 2).

In the human body, approximately 2.5 liters of gastric juice is secreted in the stomach each day, at pH of 2-3 (Vinderola and Reinheimer, 2003). Food in the stomach may have a buffering effect against the low pH. Acids can passively diffuse through the cell membrane of bacteria and enter the cytoplasm. Sub-lethal acidic environments can lead to an adaptive response and offer protection to subsequent exposure to acids (Jan *et al.*, 2001). This mechanism is known as the acid tolerance response (ATR) and was first described by Goodson and Rowbury (1989) when *E. coli* cells adapted to normally lethal acidic conditions when first grown in conditions of sub-lethal acidity.

The human liver secretes as much as one liter of bile per day which is released into the intestinal tract, exposing bacteria to a serious challenge. The relevant bile concentrations in humans range from 0.3% to 0.5% (v/v). Bile is isotonic with plasma and has an osmolarity of approximately 300 mOsm/ kg, which is attributable to the osmotic activity of the inorganic ions. Bile is amphipatic and plays a key role in the solubilization and emulsification of lipids. Bile can affect the phospholipids and proteins in the bacterial cell membrane and disrupt cellular homeostasis (Begley *et al*., 2004).

Bacterial membranes are more resistant to bile after acid adaptation, at the onset of stationary growth or in the presence of increased osmolarity. Pancreatic juice has a pH above 8.0 and contains electrolytes and enzymes, which gives it an intrinsic antimicrobial activity.

Concluded from results obtained in this study, strain ST4SA will survive passage through the stomach (low pH) and even produce low levels of bacST4SA, probably not higher than 1 600 AU/ml. Growth in the small intestine may be stimulated by a slightly higher pH. Despite this, the activity of bacST4SA is not expected to increase in the first part of the small intestine (duodenum), due to the inhibitory effect of pancreatic enzymes. Higher levels of bile in the duodenum will most probably also inhibit growth. However, strain ST4SA may proliferate in the second part of the small intestine (jejunum and ileum) and produce higher levels of bacST4SA, estimated to be as high as 25 600 AU/ml. Better growth and increased bacST4SA production are expected in the colon at higher pH and lower bile concentrations.

Growth of *E. mundtii* ST4SA in intestinal fluids from the porcine gut was similar to the results from laboratory media. Intestinal fluids from the stomach and small intestine were used, but no significant differences in gene expression levels were detected for the bacteriocin gene when compared to the controls. The intestinal fluids contained significant amounts of unidentified natural intestinal microbiota, including strains of *E. mundtii* ST4SA. These naturally occurring strains did not significantly impact on the expression level of the genes tested and also indicates that *E. mundtii* ST4SA is a natural intestinal bacterium and therefore makes it more suitable for use as a probiotic.

BacST4SA production did not increase when strain ST4SA was grown in the presence of viable cells of *L. casei* LHS_3 (Table 3). This could be because *L. casei* LHS_3 does not inhibit the growth of strain ST4SA. Strains that produce inhibiting substances might stimulate bacteriocin production. Production was lower in the presence of non-viable cells of LHS_3 (Table 3). This could be due to inhibiting substances present in cell

lysates. Based on these results, strain ST4SA should be able to successfully compete with other microorganisms in the gastrointestinal tract.

No significant up- or down-regulation of *bac*A, *rec*A and *gro*ES genes were recorded, except at pH 3.5 (Addendum A). In this case, the GroES and RecA genes were up-regulated. This is in agreement with previous studies on lactic acid bacteria (Beltramo *et al*., 2006). *bac*A was up-regulated (nine-fold) after 24h in highly acidic conditions. Low bacST4SA production at low pH was due to very low growth rate in acid conditions. Very acidic conditions significantly lowered the growth rate of *E. mundtii* ST4SA. No significant change in bacST4SA expression was recorded at any of the other conditions, suggesting that bacST4SA is constituently expressed.

To the best of our knowledge, this is the first report of bacteriocin gene expression in lactic acid bacteria with real-time PCR. Gene expression levels were stable in the presence of porcine intestinal fluids. No significant up-regulation was detected for any of the genes. This suggests that bacST4SA production is affected by factors such as media components and enzymatic inhibition. *E. mundtii* ST4SA may be used as a probiotic in humans and animals.

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Table 1. Primers used in reverse transcription real-time PCR

Primer set	Sequence	Product size (bp)	Tm (^{o}C)	Gene	Genbank sequence accession number
Mundticin FW	5′ –ATGTCACAAGTAGTAGGTGG- 3′	123	58.0	Mundticin gene	AB 066267
Mundticin RV	5′ –AGCTAAATTCGCAGCAGA- 3′	123	55.0		AB 066267
GroES FW	5′ –GTGTTCAATTGATCAAAAAC- 3′	169	52.0	GroES gene	AY 328532
GroES RV	5′ –GCATCTTCAATACGTAGTTT- 3′	169	54.2		AY 328532
Mundt 23SrRNA FW	5′ –ATTCGATTCCCTGAGTAGCG- 3′	159	60.4	23SrRNA sub-unit gene	AJ 295311
Mundt 23SrRNA RV	5′ –TCCAAATTTCATCTACGGGG- 3′	159	58.4		AJ 295311
RecA FW	5′ –GGACATTGTCGGTAACCGTA- 3′	196	60.4	RecA gene	AJ 621710
RecA RV	5′ –CTTGACCGATGCGATCTTCT- 3′	196	60.4		AJ 621710

Table 2. Growth of *E. mundtii* ST4SA and bacST4SA production by cells exposed to different stress conditions

Absorption (OD600) of *E. mundtii* ST4SA					**Bacteriocin activity (AU*/ ml)**			
Time	**6h**	**12h**	**18h**	**24h**	**6h**	**12h**	**18h**	**24h**
MRS broth (Biolab)	2.24	3.41	3.65	3.71	51200	51200	409600	409600
0.3% Bile	1.05	3.08	3.56	3.55	25600	25600	25600	51200
0.5% Bile	0.9	2.45	3.3	2.7	25600	25600	25600	51200
1.0% Bile	0.89	1.11	1.52	1.5	12800	12800	25600	25600
pH 2.5	0.05	0.11	0.2	0.11	1600	1600	1600	1600
pH 3.5	0.09	0.66	3.22	3.3	1600	1600	1600	1600
pH 5.5	0.44	2.8	3.7	3.94	1600	3200	12800	12800
Pancreatic juice 5%	2.94	3.52	3.29	3.59	100	100	100	0
Pancreatic juice 10%	2.96	3.58	3.48	3.61	0	0	0	0

*AU= Arbitrary units, one AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition.

Table 3. Bacteriocin activity in the presence of *L. casei* LHS_3

Time	***E. mundtii* ST4SA**		
	Control	**Viable cells of *L. casei* LHS_3**	**Dead cells of *L. casei* LHS_3**
8h	51200	25600	400
12h	409600	409600	6400
24h	409600	409600	12800
48h	204800	204800	6400

GENERAL DISCUSSION AND CONCLUSION

A probiotic bacterium has to be tested for a variety of traits such as adhesion to mucus and epithelial cells, resistance to bile and gastric acids, antimicrobial activity against pathogens, bile salt hydrolase activity and resistance to spermicides. Survival of stress conditions during passage through the gastro-intestinal tract is essential for successful colonization in the gastro intestinal tract. Cellular stress responses are up-regulated after exposure to stressful conditions. Bacteria change their patterns of gene expression so that stress is relieved (Foster, 2004). Bacteria have several stress responses that increase genetic variation and can lead to a selective advantage. These mechanisms include the heat-shock response, the general stress response, the SOS response and the stringent response. The genes selected for this study was the *recA* and *groES* genes. *RecA* gene expression increases under stress conditions when bound to single stranded DNA, as part of the SOS stress response. The *groES* gene, part of the groE regulon, is also induced when DNA damage occurs. In this study the effects of bile, acid and pancreatic enzymes on the growth and gene expression of *Enterococcus mundtii* ST4SA was tested.

Enteric microbes encounter life-threatening levels of inorganic acids (H^+) in the stomach and a combination of inorganic and organic acids (volatile fatty acids) when in the small intestine. The acid stress barrier is the first host defense system against pathogens. The average pH in the stomach of fasting humans is approximately pH 2. Probiotic microbes have to develop acid resistance mechanisms to survive passage through the stomach. *E. mundtii* ST4SA survives gastric acids and remains viable when reaching the small intestine. Growth was not recorded at pH 2.5, but cells remained viable for 24h and multiplied when inoculated onto MRS agar. Growth of *E. mundtii* ST4SA was not severely inhibited at higher pH levels. Real-time PCR studies showed an increase in the expression of *groES* and *recA*, indicating that the cells experienced stress at low pH. This is expected because of DNA damage that may occur during acid conditions. The bacteriocin gene, *bacA*, was up-regulated nine-fold after 24 h at pH 3.5. The presence of food in the stomach and intestine creates micro-environments which enhances the survival of bacteria as they are not in direct contact with toxic agents.

Bile is antimicrobial in nature because of its strong detergent action against lipids. The physiological concentrations of bile in humans range from 0.3% to 0.5% (Vinderola and Reinheimer, 2003). Adaptation to bile salts leads to cross-protection towards acid and heat stress (Flahaut *et al.,* 1996). Bile affects the phospholipids and proteins in the bacterial cell membrane and disrupts cellular homeostasis (Begley *et al.*, 2004). *E. mundtii* ST4SA resists bile concentrations as high as 1.0% (v/v). Growth of *E. mundtii* ST4SA was slightly inhibited when grown in the presence of 0.3% (v/v) bile, but recovered after 6h. Growth in the presence of 1% (v/v) bile was severely reduced, but cells remained viable. This suggests that *E. mundtii* ST4SA will survive passage through the small intestine to lower parts of the duodenum to colonize the intestine. Bile tolerance is strain specific and tolerances of species cannot be generalized (Begley *et al.*, 2004). Encapsulation of probiotic strains may also increase survival. Previous research suggests that bile resistance could be coupled to an ABC transporter. No real evidence of this has been found and further research should be done to establish the mechanisms of bile resistance. Bile salt hydrolase activity may also play a part in bile resistance but it has not been established to what degree. Previous studies in lactobacilli have not supported this hypothesis (Moser and Savage, 2001). This should also be studied further in *E. mundtii* ST4SA.

Pancreatic juices possess an intrinsic antimicrobial activity against several bacteria. It is also the main pathway for antibiotic secretion from the pancreas. Bertazzoni Minelli and co-workers (1996) have shown that pancreatic juice has an intrinsic antimicrobial effect against Gram-negative bacteria, and is bactericidal at high concentrations. Pancreatic enzyme concentrations had no significant effect on *E. mundtii* ST4SA, but did inhibit bacteriocin production. This inhibition was not apparent with real-time PCR. This suggests that inhibition could have been due to inactivation of the bacteriocin protein by proteolytic enzymes, rather than suppression of the bacteriocin genes.

Growth of *E. mundtii* ST4SA in intestinal fluids from the porcine gut was similar to the results from laboratory media. Intestinal fluids from the stomach and small intestine were used, but no significant differences in gene expression levels were detected for the

bacteriocin gene when compared to the controls. The intestinal fluids contained significant amounts of unidentified natural intestinal microbiota, including strains of *E. mundtii* ST4SA. These naturally occurring strains did not significantly impact on the expression level of the genes tested and also indicates that *E. mundtii* ST4SA is a natural intestinal bacterium and therefore makes it more suitable for use as a probiotic.

The host gastrointestinal tract is exposed to numerous foreign antigens. It is embedded with a complex network of immunological and non-immunological mechanisms, termed "the mucosal barrier". This barrier protects the host from potentially harmful pathogens and allows for toleration of resident microbes to allow for absorption and utilization of nutrients (Acheson, 2004). The mucosal immune system has to discriminate between harmful and beneficial agents. Some pathogens have developed ways to bypass the defense of the mucosal immune system, while other opportunistic pathogens take advantage of factors such as stress or antibiotic use which weakens the immune system. In these cases it would be beneficial for the host to have another mechanism of protection in the gut. Probiotic microbes can prevent disease in the gut and help to maintain a healthy mucosal immune system. Production of bacteriocins can inhibit the proliferation of pathogens in the intestine. *Enterococcus mundtii* ST4SA produces a broad spectrum bacteriocin with the ability to inhibit Gram-positive and Gram-negative bacteria and even viruses (Todorov *et al*., 2005). Bacteriocin BacST4SA activity and efficacy was tested against the common intestinal opportunistic pathogen *Enterococcus faecium* HKLHS. When there was only a very small amount of *E. faecium* HKLHS cells present, growth was completely inhibited by bacST4SA. Early log phase cells were significantly inhibited by bacST4SA in the presence of pancreatic juice and bile. Supplementation with bile and pancreatic juice rendered cells more sensitive to bacteriocin treatment. This suggests that pathogenic organisms will be susceptible to antimicrobial treatment when in the gut. The concentration of the pathogenic cells had a bigger effect on survival than the concentration of the bacteriocin. This suggests that treatment of infections with bacteriocin producing probiotics should start early in the disease before the pathogenic microbes colonize the intestine. A constant intake of probiotics on a daily basis will aid in the prevention of infections by controlling the growth of potential pathogens.

Adsorption of bacST4SA was tested against *E. faecium* HKLHS. This adsorption indicates the level of effectiveness of the bacteriocin, with a higher level of adsorption indicating higher levels of effectiveness. Incubation of cells with bacST4SA indicated that pancreatic juice enhances the adsorption of the bacteriocin to the cells. Bile also showed a 75% adsorption to the cells, indicating that bacST4SA will be effective in targeting the cells. Cell lysis studies showed significant reduction in growth when bacST4SA was added to growth media. The absorption of bacteriocins to sensitive target cells have been described for pediocin N5p produced by *Pediococcus pentosaceus* (Manca de Nadra *et al*., 1998), buchnerin LB produced by *Lactobacillus buchneri* (Yildirim *et al*., 2002) and plantaricin 423 produced by *Lactobacillus plantarum* 423 (Todorov and Dicks, 2006).

The number of genes that are up-regulated during certain stress responses vary and is very complex, therefore it is difficult to predict which genes will be affected during which stress conditions. Similar results were obtained in real-time PCR studies with *Oenococcus oeni* in stress conditions (Beltramo *et al*., 2006). Beltramo and co-workers established that a number of genes should be studied in order to establish significant changes in gene expression. Little information is available for the genome of *E. mundtii* ST4SA which also makes it difficult to select possible target genes for study. Further studies should focus on sequencing the genome of *E. mundtii* and then to test these genes during various stress responses. Further molecular studies focusing on these stress conditions should be done incorporating a number of genes. To the best of our knowledge this is the first study on the expression of the bacteriocin gene in lactic acid bacteria in stress conditions.

Concluded from these results, *E. mundtii* ST4SA will survive passage through the stomach and small intestine and be able to colonize the lower duodenum, where higher pH levels prevail. Bacteriocin production is constant in these conditions and will inhibit pathogenic organisms in the gut, which is beneficial to the host preventing disease. Further studies focusing on the genetic make-up of *E. mundtii* ST4SA genome should be

done to identify genes. This study serves as a basis for further studies to focus on gene expression of lactic acid bacteria in the gut, as this is the first study of its kind that we are aware of. These gene expression studies in lactic acid bacteria will aid to develop better, safer and more effective probiotic supplements.

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Addendum A

This addendum refers to the article "**Effect of gastro-intestinal conditions on *Enterococcus mundtii* ST4SA and production of bacteriocin ST4SA, as recorded by real-time PCR"** on page 70.

Table 1. Real-time PCR results of mundticin gene in simulated intestinal conditions

Mundticin gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct Mundticin	Ct 23 S	Mundticin-23S	Sample-calibrator	Relative to calibrator
Control 12h	22.61	17.44	5.17	0.00	**1.00**
	23.04	17.62	5.42		
	27.07	20.01	7.06		
			5.88		
Control 24h	22.89	20.19	2.70	-0.07	**1.05**
	24.69	16.9	7.79		
	27.26	20.2	7.06		
	25.1	19.38	5.72		
			5.82		
Bile 0.5% 12h	22.09	15.82	6.27	0.49	**0.71**
	23.46	17.37	6.09		
	27.2	19.89	7.31		
	23.74	17.93	5.81		
			6.37		
Bile 0.5% 24h	21.8	18.46	3.34	-0.93	**1.91**
	24.86	19.89	4.97		
	26.21	20.12	6.09		
	25.29	19.89	5.40		
			4.95		
Pancreatic 3% 12h	25.26	16.52	8.74	0.72	**0.61**
	24.74	20.62	4.12		
	26.16	19.21	6.95		
			6.60		
Pancreatic 3% 24h	24.51	20.74	3.77	-0.06	**1.04**
	25.79	19.19	6.60		
	27.76	20.36	7.40		
	25.31	19.8	5.51		
			5.82		
pH 5.5 12h	22.08	20.06	2.02	-1.52	**2.86**
	23.63	18.53	5.10		
	24.66	20.9	3.76		
	25.74	19.15	6.59		
			4.37		
pH 5.5 24h	23.67	20.35	3.32	0.03	**1.02**
	25.25	18.93	6.32		
	27.1	19.9	7.20		
	25.86	19.03	6.83		
			5.92		

Table 2. Real-time PCR results of GroES gene in simulated intestinal conditions

GroES gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct GroES	Ct 23S	GroES-23S	Sample-calibrator	Relative to calibrator
Control 12h	15.55	17.44	-1.89	0.00	**1.00**
	19.56	17.62	1.94		
			0.00		
			0.02		
Control 24h	18.65	16.9	1.75	0.15	**0.90**
	18.95	20.2	-1.25		
	19.56	19.38	0.18		
			0.23		
Bile 0.5% 12h			0.00	0.99	**0.50**
	19	17.37	1.63		
	19.67	19.89	-0.22		
	20.54	17.93	2.61		
			1.01		
Bile 0.5% 24h	19.32	19.89	-0.57	-0.77	**1.71**
	18.87	20.12	-1.25		
	18.68	19.89	-1.21		
			-1.01		
Pancreatic 3% 12h	19.93	16.52	3.41	0.82	**0.57**
	19.59	20.62	-1.03		
	19.34	19.21	0.13		
			0.84		
Pancreatic 3% 24h			0.00	-0.11	**1.08**
	19.91	19.19	0.72		
	19.36	20.36	-1.00		
	19.72	19.8	-0.08		
			-0.09		
pH 5.5 12h	18.32	18.53	-0.21	-0.36	**1.29**
	19.11	20.9	-1.79		
	19.76	19.15	0.61		
			-0.46		
pH 5.5 24h	16.52	20.35	-3.83	-1.13	**2.19**
	16.83	18.93	-2.10		
	20.06	19.9	0.16		
	20.35	19.03	1.32		
			-1.11		

Table 3. Real-time PCR results of RecA gene in simulated intestinal conditions

RecA gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct RecA	Ct 23S	RecA -23S	sample-calibrator	Relative to calibrator
Control 12h	16.25	17.44	-1.19	0.00	**1.00**
	15.97	17.62	-1.65		
	16.06	20.01	-3.95		
			-2.26		
Control 24h	16.82	20.19	-3.37	0.44	**0.74**
	16.71	16.9	-0.19		
	16.48	20.2	-3.72		
			0.00		
			-1.82		
Bile 0.5% 12h	16.7	15.82	0.88	1.24	**0.42**
	16.06	17.37	-1.31		
	16.7	19.89	-3.19		
	17.46	17.93	-0.47		
			-1.02		
Bile 0.5% 24h	16.68	18.46	-1.78	-0.64	**1.56**
	16.66	19.89	-3.23		
	16.79	20.12	-3.33		
	16.6	19.89	-3.29		
			-2.91		
Pancreatic 3% 12h	16.79			-0.45	**1.37**
	16.1	16.52	-0.42		
	16.02	20.62	-4.60		
	16.08	19.21	-3.13		
			-2.72		
Pancreatic 3% 24h	17.08	20.74	-3.66	0.70	**0.62**
	18.77	19.19	-0.42		
			0.00		
	17.63	19.8	-2.17		
			-1.56		
pH 5.5 12h	16.73	20.06	-3.33	-0.97	**1.96**
	15.98	18.53	-2.55		
	16.57	20.9	-4.33		
	16.41	19.15	-2.74		
			-3.24		
pH 5.5 24h	17.83	20.35	-2.52	0.12	**0.92**
	17.09	18.93	-1.84		
	16.56	19.9	-3.34		
	18.17	19.03	-0.86		
			-2.14		

Table 4. Real-time PCR results of mundticin gene in extreme intestinal conditions

Mundticin gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct mundticin	Ct 23S rRNA	mundticin-23S rRNA	sample-calibrator	Relative to calibrator
Control 12h	24.84	16.99	7.85	0.00	**1.00**
	18.52	16.14	2.38		
			5.12		
Skim milk +bile 12h	25.63	17.69	7.94	-0.43	**1.34**
	20.21	18.77	1.44		
			4.69		
pH 3 12h	26.81	18.51	8.30	-0.90	**1.86**
	20.81	20.67	0.14		
			4.22		
Bile +pancreatic 12h	25.2	20.14	5.06	-0.31	**1.24**
	21.72	17.17	4.55		
			4.81		
kontrol 24h	26.96	16.73	10.23	2.05	**0.24**
	19.78	15.68	4.10		
			7.17		
skim milk +bile 24h	25.18	17.76	7.42	-0.35	**1.28**
	19.98	17.87	2.11		
			4.77		
pH 3 24h	25.06	20.33	4.73	-3.31	**9.88**
	19.65	20.76	-1.11		
			1.81		
bile +pancreatic 24h	25.52	15.42	10.10	0.80	**0.57**
	18.74	17.01	1.73		
			5.92		

Table 5. Real-time PCR results of RecA gene in extreme intestinal conditions

RecA gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	**Ct RecA**	**Ct 23S rRNA**	**RecA-23S rRNA**	**sample-calibrator**	**Relative to calibrator**
Control 12h	17.35	16.84	0.51	0.00	**1.00**
	16.81	16.14	0.67		
			0.59		
Skim milk +bile 12h	17.39	17.95	-0.56	-0.74	**1.67**
	16.83	18.77	-1.94		
			-0.15		
pH 3 12h	16.78	21.04	-4.26	-4.85	**28.84**
	16.83	20.67	-3.84		
			-4.26		
Bile +pancreatic 12h	16.1	17.97	-1.87	-1.64	**3.11**
	16.95	17.17	-0.22		
			-1.05		
kontrol 24h	16.65	16.92	-0.27	-0.16	**1.11**
	16.82	15.68	1.14		
			0.43		
skim milk +bile 24h	17.15	16.76	0.39	-0.82	**1.77**
	17.02	17.87	-0.85		
			-0.23		
pH 3 24h	17.31	20.77	-3.46	-4.21	**18.44**
	16.99	20.76	-3.77		
			-3.62		
bile +pancreatic 24h	16.74	15.97	0.77	-1.20	**2.29**
	16.76	18.74	-1.98		
			-0.61		

Table 6. Real-time PCR results of GroES gene in extreme intestinal conditions

GroES gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct GroES	Ct 23S rRNA	GroES-23S rRNA	sample-calibrator	Relative to calibrator
Control 12h	17.83	16.84	0.99	0.00	**1.00**
	14.25	16.14	-1.89		
			-0.45		
Skim milk +bile 12h	18.17	17.95	0.22	0.08	**0.95**
	17.81	18.77	-0.96		
			-0.37		
pH 3 12h	18.17	21.04	-2.87	-1.71	**3.27**
	19.22	20.67	-1.45		
			-2.16		
Bile +pancreatic 12h	17.73	17.97	-0.24	-0.17	**1.13**
	16.16	17.17	-1.01		
			-0.63		
kontrol 24h	17.3	16.92	0.38	0.94	**0.52**
	16.28	15.68	0.60		
			0.49		
skim milk +bile 24h	15.27	16.76	-1.49	-0.31	**1.24**
	17.84	17.87	-0.03		
			-0.76		
pH 3 24h	17.5	20.77	-3.27	-3.54	**11.63**
	16.05	20.76	-4.71		
			-3.99		
bile +pancreatic 24h	15.73	15.97	-0.24	-0.25	**1.19**
	17.57	18.74	-1.17		
			-0.70		

Table 7: Real time PCR results of mundticin gene in pig intestinal fluids

Mundticin gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct mundticin	Ct 23 S	mundticin-23S	sample-calibrator	Relative to calibrator
Control 12h	20.53	20.35	0.18	0.00	
	20.92	19.53	1.39		
	20.43	20.27	0.16		
	20.93	19.29	1.64		
			0.84		
Control 24 h	20.80	18.16	2.64	1.01	**0.50**
	21.87	20.48	1.39		
	20.84	18.59	2.25		
	21.80	20.70	1.10		
			1.85		
Stomach control 12h	21.58	20.59	0.99	0.15	**0.90**
			0.99		
Stomach control 24h	20.48	20.52	-0.04	-0.88	**1.84**
			-0.04		
Stomach 12h	21.79	20.29	1.50	1.08	**0.47**
	21.30	19.47	1.83		
	21.66	19.23	2.43		
			1.92		
Stomach 24h	21.23	19.09	2.14	0.25	**0.84**
	19.15	20.30	-1.15		
	22.01	19.72	2.29		
			1.09		
Intestine control 12h	20.73	20.08	0.65	-0.19	**1.14**
			0.65		
Intestine control 24h	21.49	20.12	1.37	0.53	**0.69**
			1.37		
Intestine 12h	20.97	19.83	1.14	-0.31	**1.24**
	20.75	20.71	0.04		
	20.69	20.27	0.42		
			0.53		
Intestine 24h	20.81	20.71	0.10	0.84	**0.56**
	20.63	20.11	0.52		
	21.60	20.15	1.45		
			0.69		

Table 8. Real time PCR results of RecA gene in pig intestinal fluids

RecA gene			ΔCt	ΔΔCt	2^(-ΔΔCt)
Sample	Ct RecA	Ct 23 S	RecA-23S	sample-calibrator	Relative to calibrator
Control 12h	16.54	20.53	-3.99		**1.00**
	15.78	20.01	-4.23		
	16.18	20.77	-4.59		
	16.81	20.23	-3.42		
			-4.06		
Control 24 h	16.16	18.73	-2.57	0.77	**0.59**
	16.00	18.94	-2.94		
	16.63	20.98	-4.35		
			-3.29		
Stomach control 12h	16.06	21.02	-4.96	-0.90	**1.87**
Stomach control 24h	16.18	20.85	-4.67	-0.61	**1.53**
Stomach 12h	15.68	20.56	-4.88	0.22	**0.86**
	16.59	19.88	-3.29		
	16.19	19.55	-3.36		
			-3.84		
Stomach 24h	16.69	19.79	-3.10	0.18	**0.88**
	16.54	20.95	-4.41		
	15.95	20.08	-4.13		
			-3.88		
Intestine control 12h	16.48	20.10	-3.62	0.44	**0.74**
			-3.62		
Intestine control 24h	15.57	20.41	-4.84	-0.78	**1.72**
			-4.84		
Intestine 12h	16.29	20.20	-3.91	-1.16	**2.24**
	15.54	23.23	-7.69		
	16.07	20.12	-4.05		
			-5.22		
Intestine 24h	15.36	20.79	-5.43	-0.10	**1.07**
	17.13	20.47	-3.34		
	16.37	20.09	-3.72		
			-4.16		