

The ability of antimicrobial peptides to migrate across the gastrointestinal epithelial and vascular endothelial barriers

by

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Declaration

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Summary

Antibiotic resistance has become a major threat to humankind, necessitating research and development of alternative antimicrobial compounds. Many bacteria, including lactic acid bacteria, produce small antimicrobial peptides, referred to as bacteriocins. These peptides are generally not toxic, are active at low concentrations and have a narrow spectrum of antimicrobial activity. However, they usually have low *in vivo* stability due to degradation by proteolytic enzymes. Drug delivery systems are thus required to transport bacteriocins to the site of infection. Probiotic bacteria are an attractive delivery system, since these bacteria normally colonise the gastrointestinal tract and produce bacteriocins.

Numerous studies have been done on the probiotic Entiro™ which consists of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA. The bacteriocins produced by these strains, plantaricin 423 and bacST4SA, respectively, are active against a variety of pathogens and are potential alternatives to antibiotics. However, it is unknown whether these bacteriocins are able to migrate across the gastrointestinal epithelium and vascular endothelium in order to enter the bloodstream.

The aim of this study was to evaluate the stability, cytotoxicity and permeability of plantaricin 423 and bacST4SA *in vitro* and evaluate the potential use of these peptides as an alternative to antibiotics. The well-known lantibiotic, Nisin A, produced by *Lactococcus lactis* subsp *lactis*, was used as control and *Listeria monocytogenes* EGDe as target (sensitive) organism.

Migration of the lantibiotic, nisin A and class IIa bacteriocins, plantaricin 423 and bacST4SA across simulated models of the vascular endothelial and gastrointestinal epithelial barriers was studied by growing human umbilical vein endothelial cells (HUVEC)- and human colonic adenocarcinoma (Caco-2) cells on transmigration inserts and adding fluorescently labelled nisin A, plantaricin 423 and bacST4SA to the inserts. All three peptides diffused across HUVECs and Caco2 cells. Only 21% nisin A, 11% plantaricin 423 and 12% bacST4SA remained attached to Caco-2 cells and only 6% nisin A and 3% bacST4SA attached to the HUVECs, and plantaricin 423 did not attach. The viability of both cell types remained unchanged when exposed to 50 μ M nisin A, 50 μ M plantaricin 423 and 50 μ M bacST4SA, respectively. Furthermore, little extracellular lactate dehydrogenase (LDH) activity was recorded when cells were exposed to 100 μ M of each peptide, suggesting that the peptides are not cytotoxic. The three peptides retained 60% of their antimicrobial activity when 25 μ M of

each were exposed to 80% human plasma for 24 h. However, at higher concentrations (50 μ M) 68% of the original antimicrobial activity was recorded and at 100 μ M the peptides retained 79% of their activity. This is the first report of nisin A, plantaricin 423 and bacST4SA migrating across simulated gastrointestinal- and vascular barriers. *In vivo* studies are required to confirm these findings and determine the effect these peptides may have in the treatment of systemic infections.

Opsomming

Antibiotika weerstandigheid het 'n groot bedreiging vir die mensdom geword en daarom is daar 'n behoefte aan navorsing en ontwikkeling van alternatiewe antimikrobiese verbindings. Die meerderheid van bakterieë, insluitend melksuurbakterieë, produseer klein antimikrobiese peptiede, bekend as bakteriosiene. Hierdie peptiede is oor die algemeen nie toksies nie, is aktief teen lae konsentrasies en het 'n noue spektrum van antimikrobiese aktiwiteit. Hulle het egter gewoonlik 'n lae *in vivo* stabiliteit as gevolg van afbraak deur proteolitiese ensieme. Effektiewe vervoersisteme vir geneesmiddels is dus nodig om bakteriosiene na die plek van infeksie te vervoer. Probiotiese bakterieë is 'n aantreklike voervoersisteem, aangesien hierdie bakterieë gewoonlik die spysverteringskanaal koloniseer en bakteriosiene produseer.

Talle studies is gedoen op die probiotika, Entiro™ wat bestaan uit *Lactobacillus plantarum* 423 en *Enterococcus mundtii* ST4SA. Die bakteriosiene, plantarisien 423 en bakteriosien ST4SA (bakST4SA), wat deur hierdie stamme geproduseer word, is aktief teen 'n verskeidenheid patogene en is potensiële alternatiewe vir antibiotika. Dit is egter onbekend of hierdie bakteriosiene oor die spysverteringskanaal en vaskulêre endoteel kan migreer om die bloedstroom te bereik.

Die doel van hierdie studie was om die stabiliteit, sitotoksisiteit en deurlaatbaarheid van plantarisien 423 en bakST4SA *in vitro* te evalueer om vas te stel of hierdie peptiede as alternatief vir antibiotika kan dien. Die bekende lantibiotikum, nisin A, geproduseer deur *Lactococcus lactis* subsp *lactis*, is gebruik as die kontrole bakteriosien en *Listeria monocytogenes* EGDe as teiken (sensitiewe) organisme.

Migrasie van die lantibiotika, nisin A- en klas IIa-bakteriosiene, plantarisien 423 en bakST4SA oor gesimuleerde modelle van die vaskulêre endoteel en spysverterings versperrings was bestudeer deur endoteelselle vanaf menslike naelstring are (HUVECs) en koloniale adenokarsinoom (Caco-2) selle op transmigrasie houers te groei en fluoresserende nisin A, plantarisien 423 en bakST4SA by die houers in te voeg. Al drie peptiede het gediffundeer oor HUVECs en Caco2-selle. Slegs 21% nisin A, 11% plantarisien 423 en 12% bakST4SA het gebonde gebly aan Caco-2 selle. Slegs 6% nisin A en 3% bakST4SA het gebonde gebly aan die HUVECs. Plantarisien 423 het nie geheg nie. Die lewensvatbaarheid van beide seltipes bly onveranderd wanneer dit blootgestel word aan 50 µM nisin A, 50 µM plantarisien 423 en 50 µM bakST4SA, onderskeidelik. Verder is baie min ekstrasellulêre

laktaat dehidrogenase (LDH)-aktiwiteit aangeteken wanneer selle aan 100 μM van elke peptied blootgestel is, wat daarop dui dat die peptiede nie sitotoksies is nie. Die drie peptiede behou 60% van hul antimikrobiële aktiwiteit wanneer 25 μM van elk vir 24 uur aan 80% menslike plasma blootgestel is. Daar is egter by hoër konsentrasies (50 μM), 68% van die oorspronklike antimikrobiële aktiwiteit aangeteken en by 100 μM het die peptiede 79% van hul aktiwiteit behou. Dit is die eerste verslag wat rapporteer op die vermoë van nisin A, plantarisien 423 en bakST4SA om oor gesimuleerde spysverteringskanale en vaskulêre grense te migreer. *In vivo* studies word vereis om hierdie bevindinge te bevestig en die effek te bepaal wat hierdie peptiede moontlik kan hê in die behandeling van sistemiese infeksies

Bibliographical Sketch

Leané Dreyer was born in Johannesburg, Gauteng on the 24th of January 1993. She matriculated in 2011 at Hermanus High School, Western Cape. She enrolled for a B.Sc. degree in Molecular Biology and Biotechnology (2012) at the University of Stellenbosch and obtained the degree in 2014. In 2015 she obtained her B.Sc. (Hons) in Microbiology at the University of Stellenbosch. She enrolled as a M.Sc. student in Microbiology at the University of Stellenbosch in 2016.

Preface

Each chapter of this thesis is introduced separately and has been written according to the instructions of the Journal of Applied and Environmental Microbiology.

The literature review (Chapter 2) gives a broad overview of the antibiotic resistance crisis and bacteriocins as alternatives to antibiotics. The advantages and disadvantages of bacteriocins are discussed, with a focus on nisin A, plantaricin 423 and bacteriocin ST4SA. The importance of bacteriocins to maintain stability in *in vivo* environments and migrate across the gastrointestinal epithelium and vascular endothelium to be used as a therapeutic agent was researched and discussed. The literature review will be prepared for publishing.

The manuscript “Migration of nisin A, plantaricin 423 and bacST4SA across gastrointestinal epithelial and vascular endothelial cells, as determined with simulated models” will be prepared for publishing and is presented in Chapter 3.

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CHAPTER 1

INTRODUCTION

Antibiotic resistance has become a major threat to humankind, necessitating the discovery and development of alternative antimicrobial compounds (1). Many bacteria, including lactic acid bacteria (LAB), produce small antimicrobial peptides (AMPs), referred to as bacteriocins (2). These peptides are generally not toxic, are active at low concentrations and have a narrow spectrum of antimicrobial activity. However, they usually have low *in vivo* stability, especially in a complex environment such as the lumen, lamina propria or blood (3). Treatment of infected areas is thus only possible by using specifically designed drug delivery systems. Probiotic bacteria are an attractive delivery system, since these bacteria normally colonise the gastrointestinal tract (GIT) (4, 5). Bacteriocins produced in the GIT might diffuse through the gut-blood barrier (GBB) and reach infected areas.

This study focuses on two strains, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, present in the probiotic Entiro™. Both strains adhere to the mucus and epithelial cells in the GIT, thereby preventing the adhesion of pathogens to the same receptor sites (6). The bacteriocins produced by these strains, plantaricin 423 and bacteriocin ST4SA (bacST4SA), belong to class IIa and form pores in the cell membrane of target cells (7). Both bacteriocins are small (3935 Da and 4288 Da, respectively) and they have a good chance of migrating through the GBB. They are both active against various pathogens such as *Staphylococcus aureus*, *Clostridium sporogenes* and *Listeria monocytogenes*. *Listeria monocytogenes* is an opportunistic pathogen that causes listeriosis (food poisoning) and is considered a large risk to immune-compromised (AIDS) patients (8).

The development of a new antimicrobial compound takes many years and requires several *in vitro* and *in vivo* studies (9). The first phase of drug development usually involves experiments on human cell lines. Apart from using fewer experimental animals, studies on tissue culture are less expensive and results are obtained in a shorter period. Valuable information is obtained regarding the cytotoxicity, efficacy, permeability and transport of the tested compound (10). Results obtained from *in vitro* tissue culture studies, provide valuable insight to knowledge gained from *in vivo* and other *in vitro* studies.

The aim of this study was to evaluate the stability, cytotoxicity and permeability of plantaricin 423 and bacST4SA *in vitro* and evaluate the potential use of these peptides as an alternative to

antibiotics. The well-known lantibiotic, Nisin A, produced by *Lactococcus lactis* subsp *lactis*, was used as control and *Listeria monocytogenes* EGDe as target (sensitive) organism.

The objectives of this study were:

- to isolate and purify plantaricin 423 and bacST4SA from cultures of the wild-type strains;
- to determine the minimum inhibitory (MIC) concentration of each bacteriocin by using *L. monocytogenes* EGDe as target;
- to determine the stability of the bacteriocins in the presence of human blood plasma; and
- to label the peptides with a fluorescent marker and determine their ability to diffuse across endothelial- and epithelial cells, using simulated models of the gastrointestinal epithelial and vascular endothelial barriers.

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CHAPTER 2

LITERATURE REVIEW

Antibiotic resistance

The discovery of penicillin in 1928 by Alexander Fleming was a life-changing event in the history of medicine (1). Before the discovery of penicillin, there was no effective treatment for infections such as rheumatic fever, gonorrhoea or pneumonia. Hospitals were filled with patients suffering from sepsis contracted from simple cuts or scratches. The likelihood of dying prematurely due to infectious diseases in the early 19th century was as high as 40% (2). Penicillin and many other antibiotics discovered in later years were seen as powerful medicines and have saved millions of lives from life-threatening infectious diseases. At the time, scientists assumed that infectious diseases would no longer be a threat to society.

Unfortunately, shortly after World War II the advances of the prior decade were threatened by bacteria that became resistant to penicillin (3). The hopes of many people were shattered as they came to the realization that this “miracle drug” was no longer effective. In response to the crisis, new beta-lactam antibiotics were discovered and developed. For a short while the discovery of novel antibiotics restored medical practitioners’ confidence in antibiotics. However, the first methicillin-resistant strain of *Staphylococcus aureus* (MRSA) was identified the same decade. Most of the antibiotics currently in use were developed between the 1960s and 1980s. However, within a few months of use, bacteria resistant to each of the newly developed antibiotics were isolated. Resistance to antibiotics reached disastrous levels, with some strains being resistant to all classes of antibiotics. Some strains of *Acinetobacter baumannii*, are resistant to carbapenem antibiotics, considered to be the “last resort antibiotics” (4). Several strains of *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Helicobacter pylori* and *Streptococcus pneumoniae* are amongst the most resistant to antibiotics. In September 2016, a woman from Nevada died after becoming infected with an antibiotic-resistant strain of *Klebsiella pneumoniae* (5). She contracted bone infection after breaking her leg and died from septic shock. The strain was resistant to all 26 of the available antibiotics in the United States, including, colistin. Cases such as this will continue to occur and increase as more antibiotic resistant bacteria emerge.

Antibiotics are amongst the most commonly prescribed drugs (6). As many as 50% of the prescribed antibiotics are either not required, or are not effective in treating the infection. Despite this, doctors continue to prescribe antibiotics. In the USA, more than five prescriptions

are written each year for every six patients (7). The unnecessary prescription of antibiotics has led to the selection of bacteria resistant to several antibiotics (8). Genes encoding resistance to antibiotics is shared amongst pathogens, often across species borders, through horizontal gene transfer. To add to the problem, only few new antibiotics have been discovered over the last decade (9). If antibiotic resistance continues to spread, our integrative, highly technological world may find itself back in the dark ages of medicine. The World Health Organisation (WHO) has already declared antibiotic resistance a global crisis, worse than the AIDS epidemic. Antimicrobial resistance to tuberculosis, hospital acquired infections and common bacterial diseases is increasing mortality rates drastically (10). At least two million individuals in the United States (US) contract serious antibiotic-resistant bacterial infections each year (11). Approximately 23 000 people die each year due to infections caused by antibiotic-resistant bacteria.

Initial research showed that a continued increase in antibiotic resistance may lead to the death of 10 million people by 2050. Should this happen, the Gross Domestic Product (GDP) may decrease by as much as 3.5%, costing the world up to 100 trillion USD (11). Countries with high incidences of HIV and TB will, without doubt, suffer the most as resistance to antibiotics increase (11). South Africa may thus be heading for a major disaster.

Despite the frightening statistics set above, it does not capture the full picture of what a world without antimicrobials would be like (11). Prophylactic antibiotics are given to most patients after surgery to decrease the risk of bacterial infections. If antibiotics become useless, surgery would become far more dangerous. Many simple surgical procedures might be too menacing to attempt. Chemotherapy often suppresses patients' immune systems. Therefore, without antibiotics it would be impossible to prevent infection during cancer treatments. The same applies to AIDS and TB patients who also have weakened immune systems. An increase in antimicrobial resistance will also have an alarming effect on the safety of childbirth, resulting in high numbers of maternal and infant mortality. From a more direct perspective, less people would be willing to travel and trade. This will have a severe impact on all economies, particularly countries that depend on foreign investment, global trade and tourism.

Resistance to antibiotics is not just a health issue, but also a major economic problem (12). Ultimately, the cost of dealing with antimicrobial resistance is much less than dealing with potential consequences. This crisis requires immediate attention and although the next step is uncertain, it is clear that there is an urgent need for research and development of new target-

specific antibacterial compounds active against a broad range of pathogens. Several alternatives to antibiotics have been investigated. These include plant-derived metabolites, bacteriophages, RNA therapeutics, probiotics and antimicrobial peptides (AMPs) (9).

Antimicrobial peptides

Antimicrobial peptides (AMPs) may be a viable alternative to antibiotics (13). The first AMP to be fully described was Gramicidin, which was discovered in 1939 by the French microbiologist, René Dubos (8). Gramicidin, produced by *Bacillus brevis*, is active against Gram-positive bacteria. Since this discovery, a number of AMPs produced by insects, plants, humans and bacteria, have been described (10). Most AMPs are positively charged, smaller than 10 kDa and usually consist of hydrophobic and hydrophilic residues. Therefore, AMPs are able to dissolve in aqueous environments and enter lipid membranes (13).

The structure and positive charge of AMPs play a significant role in their mode of action (13). The positive charge allows AMPs to be attracted by negatively charged components, such as lipids in membranes of bacteria, fungi, viruses and protozoa. The amphipathic structure of AMPs permits penetration through membranes. Antimicrobial peptides are favourable candidates for novel therapeutic agents, since they are highly diverse in structure, extremely specific, widely distributed in nature and have low toxicity towards eukaryotes (8, 10). Moreover, AMPs are mainly active against specific bacterial groups, making them less harmful to microbiota in the human gastrointestinal tract (GIT). These peptides can be administered alone or in combination with other antimicrobial agents to treat infections (10). They also neutralize endotoxins produced by pathogens, or act as immune-stimulatory agents (14). Bacteriocins, a subgroup of peptides, may be used as alternatives to antibiotics.

Bacteriocins

Bacteriocins as alternative to antibiotics

Bacteriocins are ribosomally synthesized, post-translationally modified antimicrobial peptides that have the ability to inhibit the growth of closely related bacterial species (12). Virtually all bacterial species can produce at least one bacteriocin. Consequently, there is a vast diversity in the structure and mode of action of bacteriocins. The majority of bacteriocins that have been identified and characterized are produced by Gram-positive bacteria, particularly lactic acid bacteria (LAB) (15). Lactic acid bacteria have GRAS (generally recognized as safe) status that

renders them attractive bacteriocin producers. Most bacteriocins produced by LAB are smaller than 10 kDa, heat stable and act by permeabilizing the cell membrane of the target bacterium (16).

Bacteriocins produced by Gram-positive bacteria are divided into class I (modified bacteriocins) and class II (non-modified bacteriocins) (17, 18). Subclass Ia represents lantibiotics, defined as membrane-active peptides with thioether-containing amino acids such as, lanthionine (Lan) and β -methyllanthionine (MeLan). These amino acid residues are formed by a two-step post-translational process, catalysed by the enzymes, cyclase, dehydratase and synthetase. The Lan and MeLan residues form a crosslink that results in the formation of cyclic structures. Certain lantibiotics are composed of two peptides and may contain other post-translationally modified amino acids that may affect the structure and properties. Subclass Ib bacteriocins consist of labyrinthopeptins (18). Labyrinthopeptins contain labionins, i.e. modified carbacyclic amino acid residues that form cyclic structures. Subclass Ic bacteriocins are known as sactibiotics. Sactibiotics are characterized by sulphur and carbon molecules forming cross links. Class II bacteriocins are defined as non-lantibiotic, membrane active, heat-stable peptides, divided into three subclasses (18). Subclass IIa contains the anti-listeria, pediocin-like peptides, subclass IIb consist of two peptides and subclass IIc the thiol-activated peptides. Class III bacteriocins are heat-labile and commonly display enzymatic activity. Class IV are complex proteins.

Bacteriocins are often membrane permeabilizing, positively charged and hydrophobic or amphiphilic, but usually display different modes of action (15). A single bacteriocin can have more than one target site, depending on the primary structure. Bacteriocins function primarily at the cell envelope or within the cell by affecting the expression of genes and the production of proteins (9). Many lantibiotics, such as nisin, act by binding to lipid II during which they either inhibit peptidoglycan synthesis or form pores in the cell membrane (15). Bacteriocins in class IIa bind to susceptible cells by using components of the mannose phosphotransferase system as receptors and form pores in the cell membrane. Bacteriocins can also kill target cells by inhibiting DNA-, RNA- or protein-synthesis.

Bacteriocins have many beneficial properties which make them viable alternatives to antibiotics (9). These include their potency and high specific activity against pathogens. Lantibiotics and thiopeptides are generally more active against Gram-positive strains. Lantibiotics such as nisin, gallidermin and mutacin are active against *Streptococcus*

pneumoniae, vancomycin-resistant enterococci (VRE), *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA) and several mycobacteria. Another benefit of bacteriocins is their low oral toxicity towards humans (9). Several studies report the lack of toxicity of bacteriocins. Although cytotoxicity has been reported in certain studies, it is rare. Broad- and narrow spectrum bacteriocins have been described. Many bacteriocins display broad-spectrum antimicrobial activity. This is an attractive trait to target infection of unknown cause. However, broad-spectrum bacteriocins can kill mutualistic microbiota. Therefore, bacteriocins with narrow-spectrum antimicrobial activity might be more beneficial under certain circumstances.

This review addresses the lantibiotic nisin A, and the class IIa bacteriocins, plantaricin 423 and bacteriocin ST4SA. Properties of these bacteriocins render them possible alternatives to antibiotics.

Nisin A

Nisin A, produced by *Lactococcus lactis* subsp. *lactis*, is composed of 34 amino acids and contains the post-translationally modified amino acid residues, α,β -didehydroalanine, α,β -didehydrobutyrine, *m*-lanthionine and (2S,3S,6R)-3-methyl-lanthionine (28, 29). This lantibiotic is active against a variety of Gram-positive bacteria, including *Listeria*, *Staphylococcus*, *Bacillus* and *Clostridium* spp. (10). The peptide binds to lipid II in the bacterial cell wall and prevents cell wall biosynthesis. Another mode of action is pore formation in the cell membrane. Nisin binds to the pyrophosphate structures of lipid II using its A and B lanthionine rings on the (N)-terminal (Figure 1). By bending at the flexible region between the C and D rings, nisin inserts the carboxy (C)- terminal end into the phospholipid bilayer of the cell membrane. This causes the formation of aqueous transmembrane pores, leading to depolarization of the bacterial cytoplasmic membrane and the efflux of nucleotides, amino acids, ions and other cytoplasmic metabolites, resulting in cell death (20).

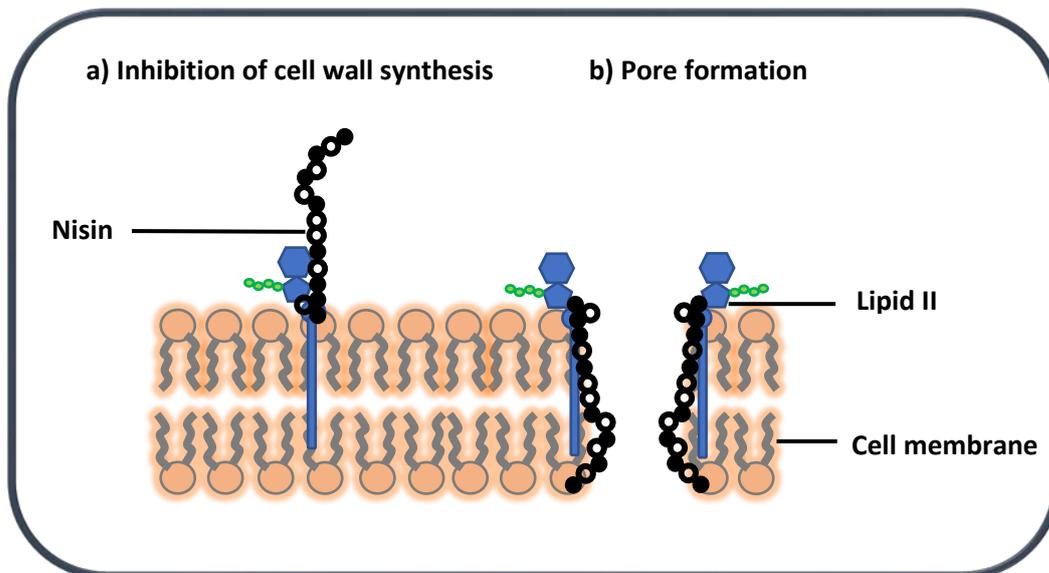


Figure 1 Binding of nisin to lipid II. Nisin acts by (a) inhibiting cell wall synthesis or by (b) forming pores in the cell membrane. Adapted from (20).

Nisin has GRAS status and has therefore been used as a food preservative for many years (21). This lantibiotic gained significant recognition due to its high potency, broad spectrum of activity, low cytotoxicity and low likelihood of sensitive bacteria developing resistance. Numerous studies described the efficacy of nisin as an antimicrobial drug and it can, therefore, be regarded a viable alternative to antibiotics.

Plantaricin 423

Plantaricin 423 is a class IIa bacteriocin produced by *Lactobacillus plantarum* 423, isolated from sorghum beer (22). *Lactobacillus plantarum* 423 is commonly found in fermented foods and occurs naturally in the human GIT (23). This strain has many probiotic properties and is included in the probiotic, Entiro™, in combination with *Enterococcus mundtii* ST4SA. Extensive *in vitro* and *in vivo* studies have shown that *L. plantarum* 423 withstands harsh conditions of the GIT and attaches to the small intestine using specific adhesion molecules (23). This prevents gastrointestinal pathogens such as *Listeria monocytogenes* from attaching to this area of the GIT (24, 25). In a study done by Van Zyl et al. (23) *in vivo* bioluminescence was used to prove that colonization of mice with *L. plantarum* 423 and *E. mundtii* ST4SA prevented systemic *L. monocytogenes* EGDe infection. Thus, probiotic lactic acid bacteria may offer an alternative to conventional antibiotics in the treatment of *Listeria* infections. However, in a study done by Botes et al. (24) adhesion of *L. monocytogenes* ScottA to Caco-2 cells was not prevented by *L. plantarum* 423 and *E. mundtii* ST4SA. Instead the cell-free supernatants

of these strains, containing the antimicrobial peptides, plantaricin 423 and bacteriocin ST4SA (bacST4SA), prevented the invasion of *L. monocytogenes* ScottA into Caco-2 cells.

Plantaricin 423 is active against a variety of Gram-positive bacteria, including many opportunistic pathogens such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Clostridium sporogenes* and *Streptococcus thermophilus* (26). The peptide forms pores in the cytoplasmic membrane, leading to dissipation of the proton motive force and cell death. Plantaricin 423 binds to target cell membranes by means of electrostatic interactions between its positively charged amino acids and negatively charged phospholipids in the bacterial membrane (Figure 2). Although activity is inhibited by proteinase K, pepsin, papain, α -chymotrypsin and trypsin, the peptide remains active after 30 min at 100°C. Plantaricin 423 is small (3935 Da), which increases the likelihood of the peptide diffusing across the gut-epithelial barrier to prevent gastrointestinal infections.

Bacteriocin ST4SA

Bacteriocin ST4SA (bacST4SA), belongs to class IIa and it is produced by *Enterococcus mundtii* ST4SA (27). Strain ST4SA was isolated from soybeans and is regarded as safe. No detrimental effects were recorded when tested on mice (28). No abnormalities in blood profiles or organ functions were recorded during a safety study on humans (classified information). Strain ST4SA prefers a less acidic environment and anaerobic conditions, and adheres to the lower part of the gastrointestinal tract. *Enterococcus mundtii* ST4SA also has the ability to adhere to Caco-2 cells under conditions simulating those of the GIT, as reported by Botes et al. (24). The antimicrobial peptide produced is 4288 Da in size and it is active against Gram-positive pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pneumoniae* (27). Bacteriocin ST4SA is also active against Gram-negative bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. This is an unusual phenomenon, since most bacteriocins are only active against Gram-positive species (29). Similarly to plantaricin 423, bacST4SA acts by dissipation of the proton motive force (Figure 2) and may play an important role in the competitive exclusion of pathogens in the gastrointestinal tract (30).

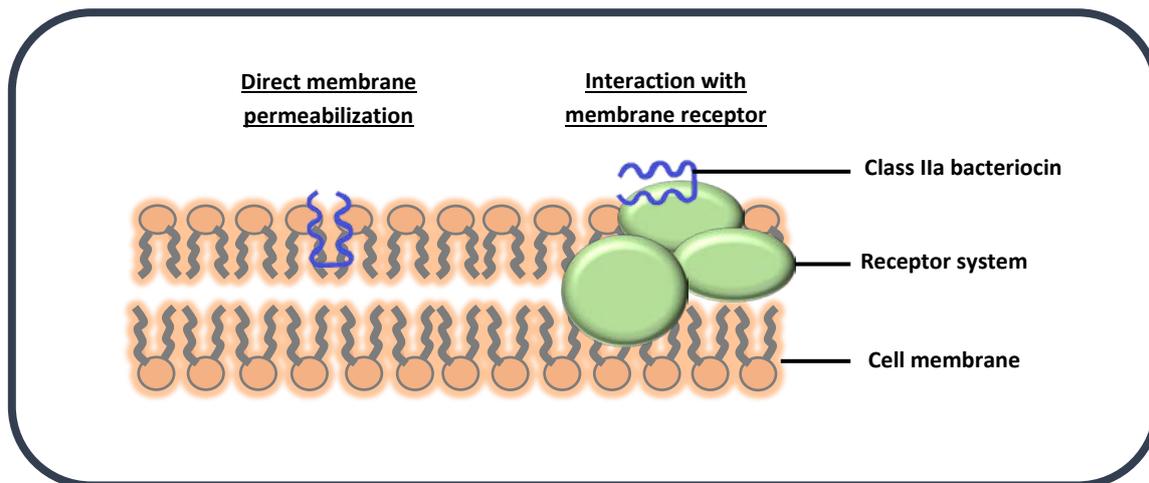


Figure 2 Illustration of the mode of action of class IIa bacteriocins such as plantaricin 423 and bacST4SA. Class IIa bacteriocins form ion-selective pores in the cell membrane by binding to a pore forming receptor such as the Mannose phosphotransferase system (Man-PTS) resulting in dissipation of the proton motive force and depletion of intracellular ATP. Adapted from (31).

Disadvantages of bacteriocins and possible solutions

Although bacteriocins have many properties which suggest that they are viable alternatives to antibiotics, a number of limitations need to be addressed (12). It should be noted that the emergence of resistant bacteria is a possibility (32). In addition, bacteriocin toxicity has been reported towards eukaryotes. Furthermore, the production, stability, administration and delivery of bacteriocins are also major obstacles that should be kept in mind when using bacteriocins for clinical use. These limitations require extensive research.

Toxicity

Data on the effect of bacteriocins on the host in terms of toxicity and immune response are limited (33). However, from the limited data available, minimal toxic effects against the host have been reported. One of the foremost examples in the lantibiotic, nisin A, which has been used as a food preservative for many years, without having any detrimental effects to consumers (34). An *in vivo* safety evaluation was done by Sahoo et al. (35) to determine the toxicity of bacteriocin TSU4 and ensure its safety in industrial applications. Bacteriocin TSU4 was administered to Male BALB/c mice for toxicity tests. No mortality or infections were observed during the experimental period. Additionally, there was no major increase in antibody

count during immunogenicity tests. However, in certain cases toxicity has been reported. In a study done by Gupta et al. (36) the toxic effect of a mixture of AMPs isolated from *L. plantarum* LR/14 was evaluated on *Drosophila melanogaster*. Significant toxicity and a delay in the life cycle of the fly were observed when it was exposed to 10 mg/ml of the peptide mixture. Since limited data is available on the toxicity of bacteriocins, more research is required to determine the dosage and timing of bacteriocin administration.

Previous studies have shown that some eukaryotic cells are more sensitive towards certain bacteriocins than others. Vaucher et al. (37) investigated the *in vitro* cytotoxicity of the antimicrobial peptide, P40 (produced by *Bacillus licheniformis* P40) against VERO cells. P40 had much higher hemolytic activity compared to nisin. Testing in a variety of models and detailed pharmacokinetic studies are therefore required before bacteriocins can be administered to humans. The determination of the cytotoxicity of antimicrobial peptides is a crucial step to permit their safe use.

Resistance

Resistance to bacteriocins has occurred. It is crucial to evaluate the risk of resistance development once a new drug has been described and proven to be effective and safe (9). Additionally, it is important to evaluate the frequency at which an organism can develop resistance to a given bacteriocin (38). The mechanisms involved in bacteriocin resistance can be divided into a) acquired resistance and b) innate resistance (38, 39).

Innate resistance may result from different mechanisms such as immunity mimicry, bacteriocin degradation, bacterial cell-envelope changes and resistance associated with growth conditions (38). During immunity mimicry, non-bacteriocin-producing strains carry genes that are homologous to bacteriocin immunity systems. Expression of these genes confers protection against the associated bacteriocin. Certain bacteria can also produce proteases that degrade bacteriocins. Some *Bacillus* spp. such as *Bacillus cereus* and *Paenibacillus polymyxa* produce nisinase during sporulation, which degrades nisin (40). Additionally, mutations in genes that code for proteins required for the structure of the cell envelope can lead to changes in the charge and structure of the cell envelope and this can also result in resistance development towards certain bacteriocins. Another mechanism that can lead to innate resistance is associated with the growth conditions of bacteria. In a study done by Jydegaard et al. (41), bacteriocin inactivation by *L. monocytogenes* 412 was studied with comparison to its growth phase. Cells

in their stationary phase of growth exhibited higher resistance rates to nisin and pediocin than cells in their exponential phase. This can be attributed to the fact that stationary phase bacteria are more resistant or adaptable to stress conditions such as high or low osmotic concentrations, acidic conditions or heat shock (42). Moreover, during the stationary phase no cell wall synthesis occurs, resulting in the absence of lipid II. Thus, bacteriocins that act by binding to lipid II, such as nisin, cannot bind to the cell wall.

Unlike innate resistance, the properties associated with acquired resistance are only found in certain strains of each bacterial species (38). The mechanisms responsible for resistance vary greatly amongst different strains and species. Acquired resistance results from gene mutations or horizontal gene transfer via transformation, conjugation or transduction (38). These mutations or gene alterations result in changes in the cell wall, cell membrane, receptors or transport systems, which consequently leads to resistance.

To improve the effectiveness of individual bacteriocins and to prevent the emergence of resistance, bacteriocins could be used in combination with other antimicrobials or membrane-active substances (12, 38). The use of bacteriocins with different modes of action may also reduce the dosage of bacteriocins. Moreover, since resistance from one bacteriocin may extend to another, especially of the same class, it would be preferable to use bacteriocins of different classes or subclasses. Thus, preliminary studies should be done to determine the most effective combinations of bacteriocins by examining the possibility of cross-resistance before integrating them. Additionally, bioengineering bacteriocins may also lead to a diminished rate of resistance development and also contribute towards better production (18).

Production

The purification of bacteriocins from cell culture is a time-consuming and expensive process (43). Heterologous expression is a viable alternative for the mass production of bacteriocins and can deliver large quantities of the desired bacteriocin (9). Unfortunately, in most cases the peptides eventually kill the producing cell (44). However, an advanced understanding of the production machinery of bacteriocins has shown that it may be possible to overcome these problems (45). The design of an efficient expression system depends on various aspects such as cell growth, location of the final recombinant peptide, the level of expression and choice of genes, plasmids and regulatory factors (46).

Escherichia coli is used as the primary prokaryotic host for cloning and expression since its genome has been extensively characterized (46). Additionally, many tools are available for its manipulation. However, this organism is not suitable for every application due to potential toxicity of the recombinant proteins, and their stability, translation and structural characteristics. Moreover, *E. coli* may lack the required secretion proteins in its cell wall since it is a Gram-negative organism. Other bacteria may offer advantages as host for heterologous expression. Certain LAB species show tremendous advances in expression and gene stabilization. In a study done by Chikindas et al. (47), production and secretion of pediocin PA-1 was achieved in *Pediococcus pentosaceus* PPE1.2 that had been transformed with a plasmid containing the *ped* operon under control of the lactococcal promoter P32. The amount of pediocin produced was 4-fold higher than that of the natural producer.

Additionally, heterologous production of bacteriocins is further advanced by adding signalling peptides that are recognised by secretory pathways. McCornick et al. (48) transformed *Carnobacterium divergens* LV13 with pJKM14, a plasmid containing the carnobacteriocin B2 immunity gene and a fusion between the sequence encoding the signal peptide of divergicin A and mature carnobacteriocin B2. The transformation resulted in coproduction of divergicin A and carnobacteriocin B2. In another study heterologous production of enterocin B (produced by certain strains of *Enterococcus faecium*) in *C. piscicola* LV17A was achieved by transforming the host with a plasmid containing enterocin B structural (*entB*) and immunity (*eniB*) genes (49). Many extensively studied systems may serve as useful models to produce LAB bacteriocins.

Administration, stability and delivery

Conventional antibiotics are administered orally, subcutaneously, intravenously or intramuscularly (12). Parenteral invasive administration is required for bacteriocins since they have a peptidic nature (50). Oral administration is only suitable for local applications since bacteriocins are susceptible to proteases, heat and other stresses. Bacteriocins are also known to have a very short plasma half-life.

Once bacteriocins have been administered, they have to be delivered to the site of infection, which has also been a major challenge (12). The physiochemical properties of bacteriocins are an obstacle when they are applied in a chemically complex environment. Since they are positively charged and hydrophobic or amphiphilic, they might not reach the targets efficiently

because of unspecific adherence to negatively charged or lipophilic surfaces or molecules. Many bacteriocins bind strongly to blood cells and plasma proteins, lowering the availability of the peptide (51). Additionally, the interaction of the peptides with blood components can change their effectiveness against a certain bacterial strain. Peptides that are administered orally may not be easily absorbed if they are larger than 3 kDa in size and smaller peptides may be denatured by digestive proteases (12). To solve this problem, techniques are required with which to efficiently deliver the peptides to the bacterial targets.

A detailed understanding of their mechanisms of action and peptide engineering may be the solutions to the issues related to the administration, stability and delivery of bacteriocins. Another viable alternative would be to use a protective vector that transports the peptide to the specific site. Ugurlu et al. (52) used specialized tablets to deliver nisin to specific parts of the gut. Nanoparticle systems, hydrogel beads, microspheres and matrix tablets are other examples of natural polymer-based colon drug delivery systems (53). All the available approaches have limitations and advantages and require further research. Some bacteriocins benefit from the fact that, in addition to being administered by standard methods, they have the potential to be produced at the site of infection by probiotic bacteria (54). Probiotics protect the bacteriocins against acids in the stomach as well as proteases and other factors present in the GIT and can deliver them to the target site by serving as a vector system. Probiotics are a possible solution for most of the delivery based problems mentioned above, since antimicrobial activity is thought to be an important means for probiotics to competitively exclude or inhibit invading bacteria and can therefore be used in the treatment of gastrointestinal infections.

Intestinal Microbiota

The gastrointestinal tract is the most complex ecosystem in the human body, containing more than 10^{14} microorganisms (55). These microbes play a vital role in establishing the intestinal immune system and improve nutrient and energy uptake. The majority of intestinal bacteria that have been isolated are mainly from the phyla of Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria (55). However, these isolated bacteria represent less than 10% of the total intestinal microbiota. Furthermore, the intestinal microbial composition of everyone is unique. It is estimated that approximately 1000 microbial species reside in the human intestinal tract with only about 15% that are shared among individuals (56). Many factors contribute towards the composition of an individual's microbiota, these include route of birth, feeding pattern during childhood, antibiotic treatment, diet, disease, medication, consumption of alcohol, etc.

Studies have proven that certain metabolites that are secreted by intestinal bacteria may cause diseases and that alterations of intestinal microbiota are linked to certain infections, inflammatory bowel diseases (IBD), obesity, diabetes, cardiovascular disease, mental disease, auto-immune disease, cancer, etc (55, 57).

The Gut-Blood barrier

The GBB is an intricate system, containing multiple layers as illustrated in Figure 3 (57). The barrier plays an important role in maintaining homeostasis between the blood stream and gastrointestinal tract. It regulates the absorption of water, electrolytes and nutrients from the gut lumen, into the bloodstream (58). The intestinal barrier also serves as a protective barrier by preventing pathogenic microorganisms and luminal toxins from entering the blood stream (59). It contains a mucus layer, an epithelial cell lining and a vascular endothelium layer.

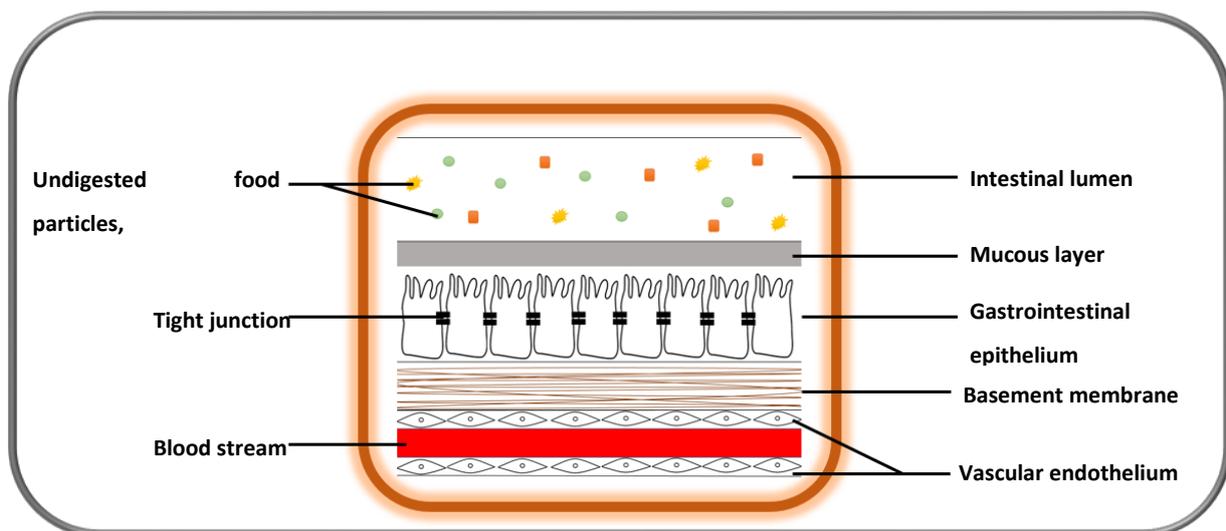


Figure 3 The GBB consists of a mucus layer, a monolayer of epithelial cells and a monolayer of endothelial cells that line blood vessels. This barrier protects the host by preventing passage of harmful compounds or pathogens from the gut lumen to the bloodstream. Adapted from (57).

Mucus layer

The mucus layer (Figure 3) forms a protective coat on the epithelium (59). This layer protects villi from physical friction caused by luminal content, chemical toxins and adhesion of bacteria. It also forms an important diffusion barrier, restricting the movement of certain molecules or

pathogens. Disruption of the intestinal mucous layer or suppression of mucous production has shown to lead to hyperpermeability.

Mucus consists of large, highly glycosylated proteins called mucins (60). Mucins are concentrated into mucin domains which are built on a protein core. All mucins have domains which give them specific properties. Transmembrane mucins have a domain that allow them to attach to the epithelial cell membrane and acts as a diffusion barrier in the GIT and gel-forming mucins form mucus that protects and lubricates the GIT.

Epithelial layer

The epithelial layer (Figure 3) is a single layer of epithelial cells that line the gut lumen (61). Intestinal epithelial cells (IEC) play an important role in the absorption of nutrients and certain specialized IECs contribute towards immune defence and secrete hormones. These include enteroendocrine cells which release hormones involved in the regulation of digestion and goblet cells which produce mucins that play an important role in the non-specific immune response.

The epithelium mediates selective permeability by transcellular and paracellular pathways (Figure 4) (61). Lipophilic and small hydrophilic molecules can pass the barrier transcellularly, while larger hydrophilic molecules pass the barrier paracellularly. During transcellular permeability, solutes are transported through the epithelial cells. This is regulated by selective transporters for amino acids, short chain fatty acids, electrolytes and sugars. During paracellular permeability, solutes are transported in spaces between epithelial cells. This is regulated by intercellular complexes present at the apical-lateral membrane junction. Amino acids and vitamins are transferred by means of active transport. The epithelium consists of a monolayer of IECs that are connected by desmosomes, tight junctions and adherens junctions. Tight junctions and adherens junctions use transcellular proteins to connect to the actin cytoskeleton. The cytoskeleton is crucial for paracellular transport.

Spaces between epithelial cells are sealed by tight junctions (TJs), desmosomes and adherent junction (AJ) proteins (Figure 4) (61). The junction proteins are distributed across the gastrointestinal membrane and the number of proteins varies between the small intestine, large intestine, and between villi and crypts. The desmosomes and adherent junctions link the cells

together and the tight junctions control water and ion permeability and the absorption of proteins and bacterial antigens.

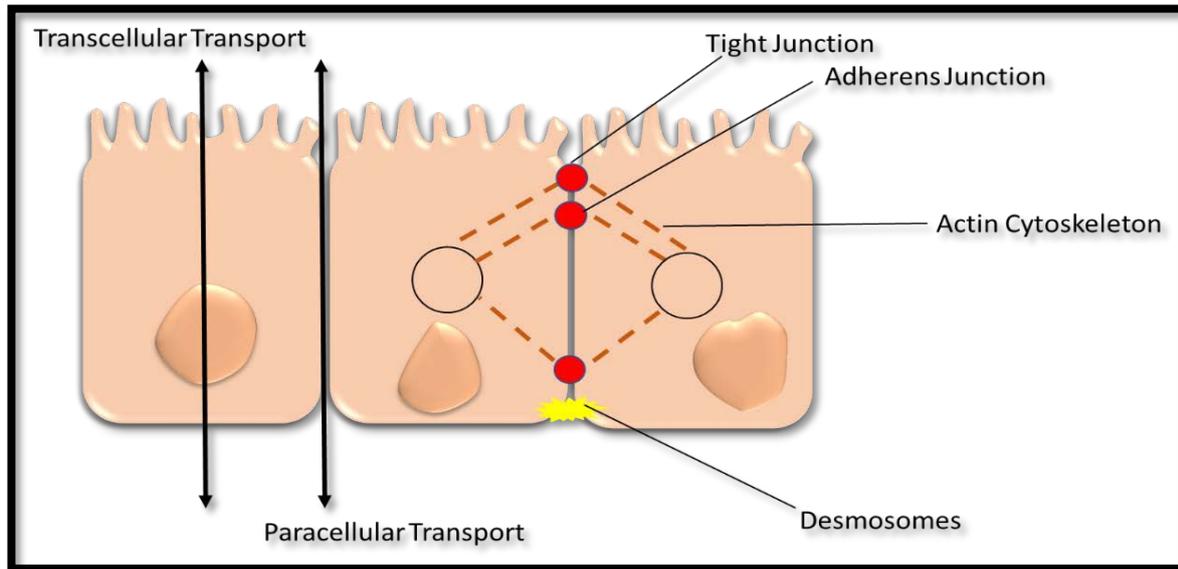


Figure 4 Transcellular and paracellular transport and epithelial junctional complexes. Adapted from (60).

Tight junctions consist of transmembrane proteins such as claudin, tricullulin and occludin that connect to the cytoskeleton of adjacent cells, connecting the cells together (62). This forms a barrier to paracellular diffusion of solutes and fluids. Desmosomes are connected to keratin filaments and adherens also attach to the cytoskeleton intracellularly by means of transcellular proteins (61). The cytoskeleton is a structure of protein filaments that expands through the cytosol of eukaryotic cells and makes contact points on the outer surface of the cell by means of junction proteins (59, 62). The cytoskeleton is essential for the paracellular pathway and for maintaining structure and functionality. It is thus a crucial structure for intestinal barrier function.

Endothelium layer

Endothelial cells (ECs) line the interior surface of blood vessels and lymphatic vessels and forms the endothelium (63). The endothelium is extremely important as it forms a selective barrier for the movement of molecules between blood and tissue. Endothelial cells are connected to each other by tight junctions, adherens junctions and gap junctions. Other cell types such as fibroblasts and pericytes contribute towards the maintenance of the endothelium where they form a vascular unit (64).

Spadoni et al. (64) hypothesized the existence of an additional barrier in the gastrointestinal tract, known as the gut-vascular barrier that restricts the size of molecules that can pass through. To evaluate the presence of this barrier they injected mice with different molecular sizes of fluorescein isothiocyanate (FITC)-dextran and examined the intestine for any dye leakage. They observed that a molecule of 4 kD had the ability to move through the endothelial barrier, whereas a molecule of 70 kD, could not.

The endothelial barrier plays an important role in intestinal barrier function (59). In a study done by Sun et al. (65) different detergents were administered to rats to evaluate the response of the intestinal barrier. Endothelial and epithelial barrier integrity was examined for leakage. They showed that an increase in epithelial barrier dysfunction is directly proportional to detergent dosage. Higher doses induced an increase in endothelial barrier permeability.

In vitro studies using cultured endothelial cells have shown that endothelial adhesion molecules and the production of cytokines can be expressed when the cells are exposed to bacterial endotoxin (LPS) or pro-inflammatory cytokines (65). During inflammation, an increase in cytokine levels and growth factors in the blood cause ECs to undergo remarkable changes, resulting in injury to the intestinal barrier. (66).

The effect of the gut microbiota on the GBB

As mentioned above, numerous factors can influence the integrity and structure of the GBB (58). Many studies have demonstrated the impact of the gut microbiota on the function of the GBB. Pathogens and probiotics can modify the function of tight junctions directly or by inducing an immune response (58). This leads to the increased permeability of the GBB, making it easier for lipopolysaccharide (LPS) to penetrate the barrier. The LPS molecule is present in the outer membrane of Gram-negative bacteria and is one of the strongest stimuli of immune response. This leads to a cycle causing further permeability. The intestinal microbiota of healthy individuals cannot access the liver and can only reach the spleen if the mesenteric lymph nodes (MLNs) are excised (64). Therefore, the microbiota is excluded from the bloodstream since the MLNs create a barrier, preventing the systemic circulation of the microbiota. However, if the GBB becomes damaged and starts to leak, microbes may cross this barrier and enter the blood stream, resulting in bacteraemia. Additionally, microbial pathogens have evolved to adhere, invade and disrupt the GBB (67). Certain pathogens can disrupt

intracellular junctions by interacting with cell receptors. *Listeria monocytogenes* is an opportunistic pathogen that can cross the intestinal barrier.

Listeria monocytogenes

Clinical Features

Listeriosis has been recognised as a food-borne infection, mainly caused by the pathogenic Gram-positive bacterium, *Listeria monocytogenes* (68). Listeriosis primarily occurs in newborns, the elderly and immune-compromised individuals, especially AIDS patients. The risk of contracting listeriosis is 300 to 1000 times higher for AIDS patients.

Despite the low incidence rate of this disease, it has a high mortality rate, ranging between 15% and 30% among patients despite early antibiotic treatment (69, 70). This makes *L. monocytogenes* one of the most deadly food-borne pathogens in the world (71). The foods most often implicated include dairy products, sausages, fish and ready-to-eat dishes that are consumed without cooking (69). *Listeria monocytogenes* has the ability to tolerate acidic conditions, high salt concentrations and low temperatures, making it a serious threat for the food processing industry (69).

Pathophysiology

Listeria monocytogenes infection can occur in two basic forms: perinatal listeriosis and listeriosis in adult patients (69). Perinatal listeriosis occurs when *L. monocytogenes* invades the foetus via the placenta leading to intra-amniotic infection, also known as chorioamnionitis (69). This infection usually results in abortion, or the birth of a baby with granulomatosis infantiseptica, a clinical syndrome characterized by the presence of micro-abscesses spread over the body. Listerial infection in adults commonly leads to listerial meningitis, mainly affecting the central nervous system (CNS). Another recurrent form of listeriosis in adults is septicaemia or bacteraemia. Infection of healthy individuals also generally leads to gastroenteritis (71). Further there are other unusual forms of listeriosis such as arteritis, myocarditis, hepatitis, pneumonia, etc. *Listeria* has the astounding ability to cross three protective barriers in humans, the fetoplacental barrier, intestinal barrier and the blood-brain barrier (72).

Studies have shown a direct correlation between clinical episodes of listeriosis and gastrointestinal symptoms (69). Evidence suggests that gastroenteritis may be the main clinical indication of listeriosis and that *L. monocytogenes* can be seen as a possible infectious agent in cases of diarrheal disease in humans (69). Additionally, the fact that contaminated food is the major source of infection indicates that the gastrointestinal tract may be the main site of entry of the pathogen into the host.

Listeria monocytogenes is a facultative intracellular microorganism (73). It has the ability to enter the host through the intestinal mucosa by means of direct invasion or translocation (71). During direct invasion, *L. monocytogenes* permeates enterocytes lining the epithelium of microvilli, consequently infecting the intestinal cells. *Listeria monocytogenes* can also translocate across the M-cells of Peyer's patches. However, studies have shown that the latter mechanism is not very efficient.

Entry and colonization of host cells

In order for *L. monocytogenes* to cross the gastrointestinal tract, the pathogen needs to adhere to the surface of the epithelial cells (67). Many bacteria produce mucinases that enable them to attach to epithelial cells by hydrolysing the mucins in the mucosal barrier. However, *L. monocytogenes* does not produce mucinases, but instead produces several surface proteins that can bind to a specific type of mucin. The proteins, Internalin B (InlB), Internalin C (InlC) and Internalin J (InlJ) are responsible for this adherence step (71). These proteins are encoded by *inlB*, *inlC* and *inlJ* genes.

Once *Listeria* has attached to the epithelial cells, it crosses the intestinal barrier by invading enterocytes using Internalin A (InlA), encoded by the *inlA* gene (69, 71, 74). Internalin A binds to the protein, E-cadherin, present in the cell membrane of the host and induces phagocytosis of *L. monocytogenes* (71). The InlA-E-cadherin is species-specific, therefore InlA does not interact with mouse E-cadherin, making mice an unsuitable model for oral infection with *L. monocytogenes*. Consequently, there is a necessity for further models for human listeriosis.

After *L. monocytogenes* invades the host cell (Figure 5), it is surrounded by the membrane of the phagocytic vacuole (71). Different phospholipases cooperate with Listeriolysin O (LLO), a pore-forming hemolysin, to lyse the phagosome membrane and the pathogen is released into the cytoplasm where it starts to replicate (75).

Once *Listeria* is released from the phagosome, ActA is expressed (71, 74). This protein initiates the nucleation and polymerization of g-actin and f-actin filaments of the host. The polymerization action leads to the expulsion of the pathogen from the cytoplasm. Sometimes the pathogen is propelled into the cytoplasm of a neighbouring cell and the resulting pseudopods are then endocytosed by these cells, promoting cell-to-cell spread. The cycle repeats in the newly infected cells.

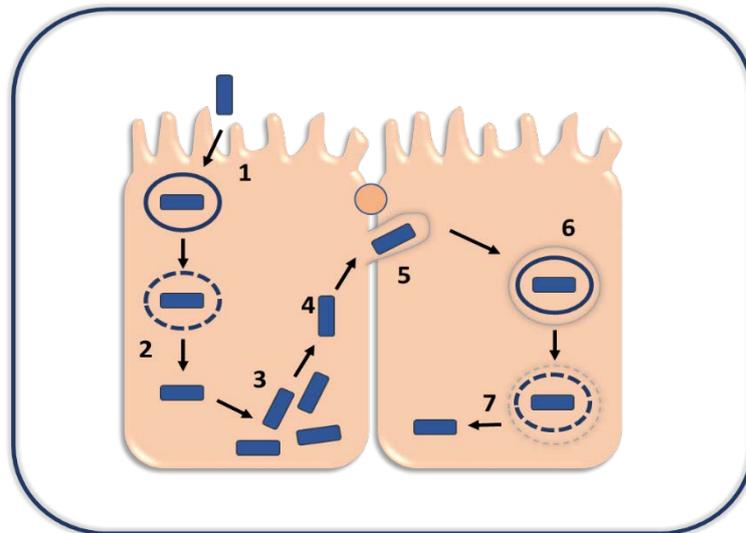


Figure 5 Phases in the intracellular life-cycle of *L. monocytogenes*. (1) Adherence and cell entry mediated by internalin proteins; (2) escape from phagolysosome; (3) replication in cytosol; (4) intracellular motility due to actin nucleation; (5) formation of bacterial protrusions called listeriapods; (6) engulfment of protrusions; (7) lysis of two-membrane vacuole. Adapted from (71, 74, 76).

Virulence

Listeria monocytogenes has many genes that contribute towards its virulence. The expression of most of these genes is regulated by the transcriptional regulator, PrfA, present in the *plcA-prfA* operon (Phosphoinositide phospholipase) (77). PrfA is activated once *Listeria* enters the cytoplasm of the host cell and then regulates the expression of several genes. These genes include *plcA* and *plcB*, both responsible for the production of phosphoinositide phospholipase C (PI-PLC), which helps *L.monocytogenes* escape from the phagosome (78); *hly*, which encodes pore-forming listeriolysin O; *mpl*, which encodes for a metalloprotease that contributes towards the encoding of other genes; *actA*, which encodes ActA, a surface protein

that enables actin to assemble and attach to the cell. Other virulence inducing genes that are not regulated by PrfA, include the aforementioned *inlA* and *inlB* genes.

Antibiotic treatment and resistance

Listeria monocytogenes is susceptible to most antibiotics active against Gram-positive bacteria (79, 80). Listeriosis is usually treated with benzylpenicillin or ampicillin combined with an aminoglycoside. Erythromycin, tetracycline or chloramphenicol are used as alternative antibiotics.

Listeria monocytogenes seldom develops resistance against antibiotics. However, recent studies have shown that the rate of resistance is rising (68). Most of the mechanisms involved in resistance include gene acquisition such as self-transferable plasmids capable of horizontal gene transfer (81). In a study done by Morvan et al. (68) the prevalence of resistance was determined for all *L. monocytogenes* strains isolated from humans between 1989 and 2007. A panel of 23 antibiotics was tested against 4668 *L. monocytogenes* strains. Among these strains, 61 were resistant to at least one antibiotic and two isolates were reported to be multi-drug resistant. Even though several other multi-drug resistant strains have been reported over the years, multi-drug resistance remains unusual in *L. monocytogenes*, but as with many other pathogens, the list of effective drugs will decrease (82). Therefore, there is a need for sustained surveillance of the susceptibility of *L. monocytogenes* to antibiotics.

In a study done by Champion et al. (83) the effect of nisin V was determined on murine models that have been infected with a lux-tagged *L. monocytogenes* strain. Bio-imaging and liver and spleen evaluation revealed that nisin V was effective with respect to controlling the infection.

Probiotics

The early days of probiotics

In 1908, the Russian zoologist, Elie Metchnikoff, observed that a large amount of people living in Bulgaria lived longer than 100 years (84). He soon realized that Bulgarians consumed large quantities of yogurt. He consequently isolated bacteria from yogurt and determined that they conferred health benefits.

As defined by the World Health Organization (WHO) probiotics are microorganisms which confer health benefits when ingested in adequate amounts (85). Lactic acid bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* are members of the intestinal microbiota and are most frequently used as probiotics due to their safety and easy handling (84).

Probiotics have numerous health benefits for the host (86). These beneficial microbes can influence the composition of the mutualistic microbiota, fight against toxins or adverse substances which originate from food, microbes or the host, they can produce bacteriocins that combat pathogens and they are able to modify the host epithelial and immune system.

The effect of probiotics on the GBB

Numerous studies using intestinal epithelial cells and mice have demonstrated that certain probiotics such as *Lactobacillus rhamnosus* GG, or the probiotic mix, VSL#3, interact with intestinal cells and maintain integrity of the GBB (86). Thus, many diseases can be prevented or treated by selecting the appropriate probiotic composition that can strengthen the GBB. Studies have shown that several *Lactobacillus* spp. induce gene-regulation pathways that lead to upregulation of IL-1 β , resulting in the transcription of genes involved in B-cell maturation and lymphogenesis, which contributes towards enhanced barrier stability and function. Strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus thermophilus* suppressed the expression of pro-inflammatory cytokines IL-6 and IL-7 and stimulated the expression of tight junction proteins, leading to enhanced barrier stability. Probiotics can protect the host through various mechanisms, which will be discussed below.

Competitive exclusion of pathogens

One major attribute of probiotics is their ability to bind to intestinal epithelial cells and prevent adhesion of pathogenic strains (86). Many probiotic strains, especially LAB have a variety of surface determinants that promote mucus adhesion. Once probiotic strains have adhered to intestinal epithelial cells they prevent colonization by pathogenic bacteria by blocking adhesion sites.

Probiotics may also competitively exclude pathogens by exhausting the available nutrients and leaving fewer nutrients for survival of pathogenic bacteria (86, 87). When health-promoting bacteria thrive in the GIT, they produce organic acids and fatty acids as the main products of their fermentative metabolism. This lowers the pH of the GIT, preventing many pathogens such

as *Salmonella* and *E. coli* from growing. Therefore, probiotic strains can change the physical environment of the GIT so that pathogens are unable to colonize and survive inside the host.

The effect of probiotics on the immune system

Probiotics can protect the host from pathogens by stimulating the immune system (86, 88). Substances produced by LAB exert immunomodulatory activity by regulating the expression of toll-like receptors (TLRs), inhibiting inflammatory responses, activating the proliferation of lymphocytes and the production of antibodies, especially secretory IgA and activating dendritic cells (DCs) and natural killer (NK) cells (89). *Bifidobacterium* and *Lactobacillus* can effectively prevent the development of gastric mucosal lesions, alleviate allergies and provide defence against many pathogenic infections by enhancing innate and adaptive immunity.

Antimicrobial substances produced by probiotics

Most probiotic strains have the ability to produce antibacterial substances such as organic acids, hydrogen peroxide and bacteriocins (86). These substances inhibit the growth of pathogens by working individually or in combination. As mentioned previously, probiotics can be considered to be a delivery mechanism for bacteriocins. Most probiotic bacteria can produce bacteriocins *in vitro*. However, recent studies have shown that strains can also produce bacteriocins *in vivo* (84). A study done by Corr et al. (90) demonstrated the therapeutic effect of bacteriocin ABP118, produced by *Lactobacillus salivarius* UCC118 in mice infected with *L. monocytogenes*. Another study has shown that the bacteriocin, mutacin B-Ny266, produced by *Streptococcus mutans*, is able to inhibit multi-resistant pathogens in a mouse model (91). These AMPs produced by probiotic bacterial strains contribute towards host protection by acting as: (i) colonizing peptides, allowing the probiotic strain to compete with host microbiota; (ii) killer peptides, which eliminate pathogens or (iii) signal peptides, resulting in the recruitment of other bacteria in the immune system or GIT (43).

Probiotics are potential candidates for delivery and production of therapeutic agents, such as bacteriocins, within the GIT. For this study, focus will be on two probiotic strains, *L. plantarum* 423 and *E. mundtii* ST4SA and the bacteriocins that are naturally produced by these strains, plantaricin 423 and bacST4SA.

Lactobacillus plantarum 423 and *Enterococcus mundtii* ST4SA

As mentioned previously *L. plantarum* 423 and *E. mundtii* ST4SA are combined to form the well-known probiotic, Entiro™. Both *L. plantarum* 423 and *E. mundtii* ST4SA, contain genes that encode adhesion proteins which help them bind to the mucus and epithelial layers of the gastrointestinal tract (23). Adhesion prevents pathogens from binding and causing disease. One of the most unique properties of Entiro™ is its ability to produce powerful antimicrobial peptides, plantaricin 423 and bacST4SA (described above) that kill gastrointestinal pathogens. These bacteriocins have the potential to be developed as alternatives to antibiotics.

Drug development

Before a new drug can be developed, one should determine which requirements are necessary for the process. The mission of the Food and Drug Administrations (FDA) centre for drug evaluation and research (CDER) is to ensure that drugs marketed are safe and effective. Thousands of newly discovered drugs are potential candidates for development as a medical treatment (85). However, after pharmacological studies are done, only a small number are suitable for further testing.

From discovering a potential drug to actually having a commercial medication requires a long, expensive and complicated process (92). The development of a commercial drug can take as long as 13 years (10). On average, only 1 in 5000 candidate compounds will reach the market. Most failures during early development are due to toxicity, and the majority of failures during late-stage development are due to a lack of efficacy. Due to these complications, only about 20 new drugs are approved each year. The first step in the drug development process entails the discovery of a potential drug (85) (Figure 6). Once a potential compound has been identified, experiments are conducted to gather information on the absorption, distribution, metabolism and excretion of the drug. This may be referred to as the ADME-process (84, 92). Additionally, the interaction with other drugs, potential benefits, mechanism of action, best route of administration, toxicity, effectiveness and difference in efficacy between different groups of people are also determined during the development process. The second step in the drug development process is preclinical research. Before a new drug can be tested in humans, experiments must be conducted *in vitro* and *in vivo* to determine the dosing and toxicity levels. After preclinical testing, results are examined and researchers decide whether human tests should commence. During clinical research, trials are conducted in different phases to examine

the toxicity, dose and side effects of the drug when administered to humans. The results are examined and if the drug meets the criteria, the clinical research is approved. Once the drug development company has evidence from the early tests, preclinical and clinical research that the drug is both safe and effective, the company can apply to market the drug. The data is submitted to the FDA review team and a decision is made. If the drug is approved, it will be marketed and sold as prescription or over-the-counter medication. The last step of the drug development process involves post-market safety monitoring by the FDA. Any problems associated with the safety and effectiveness of the drug after it has been approved, will be monitored and any issues will be reported. The drug might even be retracted from the market.

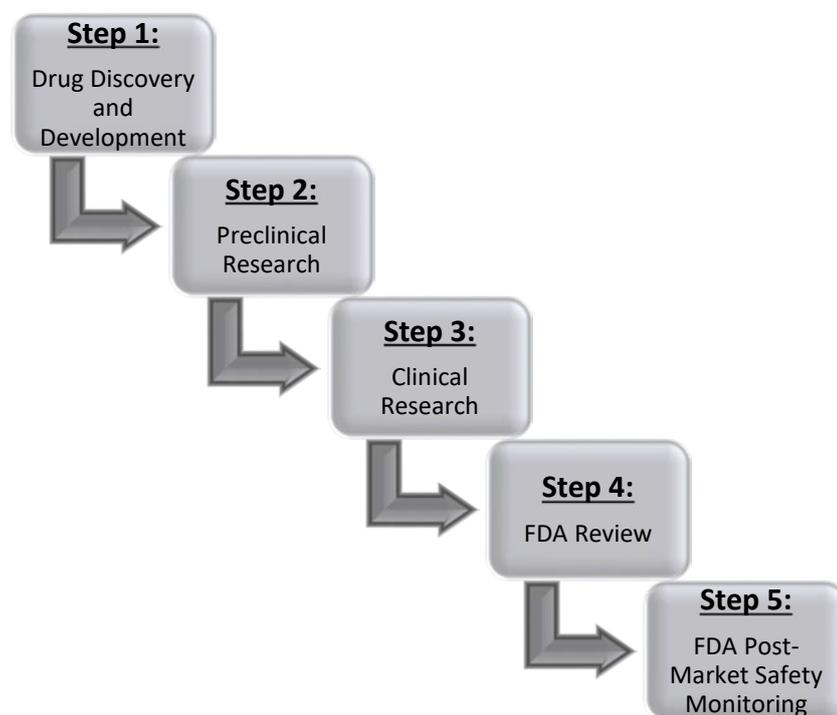


Figure 6 Steps in the drug development process (84).

At the start of the 20th century, drugs were not developed based on research regarding the safety of drugs (94). Instead, the FDA focused on the purity and physical and chemical characteristics of drugs. Drugs were mainly developed from plant extracts. Substances such as morphine and cocaine, which are subjected to restrictions today, were freely sold. However, with recent advances in technology, the pharmaceutical industry has made exceptional progress regarding the development of new medications (94).

Various assays using cell cultures of human or animal origin are now available (95). The two-dimensional (2-D) cell culture is an important tool for drug discovery. Through these assays it

is possible to observe the toxicity and efficacy of substances by accessing cell death statistics, immune response, protein expression, etc.

Using tissue culture as tool for studying pathophysiology and pharmacology

Human colonic adenocarcinoma (Caco-2) cells

The popularity of using epithelial cell lines in drug development studies has increased drastically (96). Caco-2 cells are the most commonly used cell line for drug transport studies since it is similar to the gastrointestinal epithelium (96). These cells originate from human colonic adenocarcinoma. They are favourable since they are of human origin and the oral route is the most important route of drug administration and the clinical success of a drug may depend on the degree at which it is absorbed in the GIT. These cells can therefore be used to simulate the GIT during drug development to access drug cytotoxicity, permeability and effectiveness. Furthermore, Caco-2 cells have also been used to study epithelial barrier function, gastrointestinal pathogens, infection and disease (97).

Drug transport studies are easy to perform on permeable supports such as transmigration inserts. To mimic the gastrointestinal epithelium, Caco-2 cells are cultured on these transmigration inserts until they reach confluency and differentiate (98) (Figure 7). Transmigration inserts are usually made from polyester, polycarbonate or polyethylene terephthalate. The latter is claimed to have non-specific protein-binding properties. The inserts can be transparent or translucent. Transparent inserts are preferred during microscopy studies.

These Caco-2 monolayers are reliable for studies on the absorption of drugs after oral intake in humans (96, 97). Numerous studies have compared absorption data in humans with Caco-2 permeability and have found high correlation, especially if compounds are transported by means of the paracellular pathway. Valuable results and new information can be obtained through these simple *in vitro* models. Most of the recently available information regarding drug transport mechanisms has been acquired from epithelial cell monolayers.

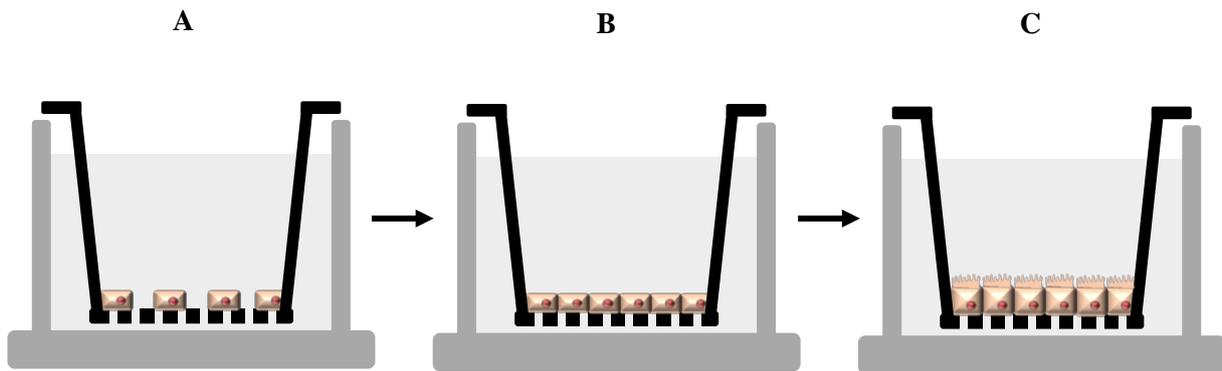


Figure 7 The differentiation of Caco-2 cells on transmigration inserts. The cells are seeded onto transmigration inserts (A). After the cells reach confluence (B), they start to differentiate spontaneously. The cells are fully differentiated after an incubation period of around 25 days (C). Adapted from (98).

Human umbilical vein endothelial cells (HUVECs)

The vascular endothelium serves a variety of important functions. Endothelial cell monolayers have frequently been used to determine the effect of certain pathophysiological stimuli or stresses that accompany gastrointestinal tract diseases or disorders (99).

Although most microvascular studies are done on *in vivo* murine models, rodents are known to have many differences to human with respect to vascular biology (100). Recent advances in technology have allowed researchers to harvest endothelial cells from several organ systems of different species, including humans (63, 99). The most common method used for isolating endothelial cells is to remove the cells from large blood vessels using specific enzymes. Human umbilical vein endothelial cells (HUVECs) have been enzymatically removed from human umbilical veins and are most often used for *in vitro* inflammation studies.

Endothelial cell monolayers have been used to study microvascular responses associated with certain pathogenic infections associated with the GIT, since the endothelium forms an important layer in the GBB (99). Pathogens or toxic molecules must cross the vascular endothelium to enter the blood stream to invade the host. In contrast, antimicrobials such as antibiotics and bacteriocins, need to cross this barrier to have a beneficial effect on the host during treatment of a disease or infection.

With reference to the above, we can conclude that it is critical to assess the interaction of drugs with intestinal epithelial cells and vascular endothelial cells to evaluate their selective toxicity before they can be considered for delivery to infectious sites.

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CHAPTER 3

Migration of nisin A, plantaricin 423 and bacteriocin ST4SA across gastrointestinal epithelial and vascular endothelial cells, using *in vitro* simulations

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ABSTRACT The antibiotic resistance crisis has resulted in an urgent need for the development of new antimicrobial compounds that can be used in clinical settings. Bacteriocins have many properties which suggests they are viable alternatives to antibiotics. However, little research has been done regarding the pharmacodynamics and *in situ* delivery of bacteriocins. Migration of the lantibiotic, nisin A and class IIa bacteriocins, plantaricin 423 and bacST4SA across vascular endothelial and gastrointestinal epithelial cells was studied using *in vitro* simulations. Monolayers of human umbilical vein endothelial cells (HUVECs) and human colonic adenocarcinoma (Caco-2) cells were seeded onto transmigration inserts placed in a 24-well tissue culture plate with growth medium and exposed to fluorescently labelled nisin A, plantaricin 423 and bacST4SA. All three peptides diffused across HUVECs and Caco2 cells. A small percentage of the peptides (21% nisin A, 11% plantaricin 423 and 12% bacST4SA) remained attached to Caco-2 cells. Only 6% nisin A and 3% bacST4SA attached to the HUVECs, while plantaricin 423 did not attach. The viability of both cell types remained unchanged when exposed to 50 μ M nisin A, 50 μ M plantaricin 423 and 50 μ M bacST4SA, respectively, as determined with XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphenyl)-2H-Tetrazolium-5-Carboxanilide) and neutral red assays, whereas a slight decrease in cell viability was observed when the cell lines were treated with 100 μ M peptide. Furthermore, nisin A, a FDA food preservative, displayed significantly higher levels of cytotoxicity compared to plantaricin 423 and bacST4SA in both cell lines ($P < 0.05$). The three peptides retained 60% of their antimicrobial activity when individually exposed to 80% human plasma for 24 h at a concentration of 25 μ M, but with increasing concentrations, this loss of antimicrobial activity became less pronounced (68% and 79% activity retained at 50 μ M and 100 μ M respectively). The stability of the peptides in the presence of human blood plasma increased as exposure time increased from 24 h to 48 h to 72 h, as expected. This is the first report of nisin A, plantaricin 423 and bacST4SA migrating across simulated gastrointestinal- and vascular barriers. *In vivo* studies are required to confirm these findings and determine the effect these peptides may have in the treatment of systemic infections.

Keywords Caco-2 cells, HUVECs, XTT assay, neutral red assay, LDH release assay, human plasma, cytotoxicity

INTRODUCTION

Several studies have shown the tremendous health benefits of probiotics, defined by the FAO/WHO as living microorganisms which convey health benefits on the host when administered in adequate amounts (1, 2). Lactic acid bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* are most frequently used as probiotics (3). Many of these strains produce antimicrobial compounds, including bacteriocins, that may assist in the control of gastrointestinal infections (4). Studies using human intestinal epithelial cells and mice have suggested that probiotics may also play a role in maintaining the integrity of the gut-blood barrier (GBB) (5). However, little is known about the migration of bacteriocins across gastrointestinal epithelial and vascular endothelial cell barriers.

The probiotic properties of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA have been well studied (6–13). *Lactobacillus plantarum* 423 adheres to the small intestine and lower part of the gastrointestinal tract (GIT) and *E. mundtii* ST4SA to the lower part of the GIT (8). This prevents gastrointestinal pathogens such as *L. monocytogenes* from attaching to the GIT (6, 13). Van Zyl and co-workers (9) used *in vivo* bioluminescence to show that colonization of mice with *L. plantarum* 423 and *E. mundtii* ST4SA prevents systemic *L. monocytogenes* EGDe infection. These bacteria can also adhere to Caco-2 cells under conditions simulating the gastrointestinal tract as determined by Botes et al. (6). However, the adhesion of *Listeria monocytogenes* ScottA to Caco-2 cells was not prevented by *L. plantarum* 423 and *E. mundtii* ST4SA. Instead, the cell-free supernatants of these strains, containing the antimicrobial peptides, plantaricin 423 and bacST4SA, prevented the invasion of *L. monocytogenes* ScottA into Caco-2 cells (6).

Lactobacillus plantarum 423 produces plantaricin 423, a class IIa bacteriocin, which is active against a variety of Gram-positive bacteria, including many opportunistic pathogens such as *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium sporogenes* (14, 15). *Enterococcus mundtii* ST4SA produces bacteriocin ST4SA (bacST4SA), which is also a class IIa bacteriocin (16). The peptide is active against Gram-positive pathogens such as *Staphylococcus aureus*, *E. faecalis* and *Streptococcus pneumoniae* and Gram-negative bacteria such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (12, 16). Plantaricin 423 and bacST4SA bind to cell membranes by means of electrostatic interactions between positively charged amino acids and negatively charged phospholipids in the bacterial membrane (7, 14, 15). Pores are formed in the cytoplasmic membrane, resulting in dissipation of the proton

motive force and cell death. This may play an important role in the competitive exclusion of pathogens in the gastrointestinal tract.

Nisin A, produced by *Lactococcus lactis* subsp. *lactis* is active against a variety of Gram-positive bacteria, including *Listeria*, *Staphylococcus*, *Bacillus* and *Clostridium* spp. (17). Many studies (17, 18) have been done on nisin A making it a viable control in bacteriocin studies. It is commercially available as Nisaplin® which has been used as a food preservative for years and has GRAS (generally regarded as safe) status (17, 19, 20). This lantibiotic binds to lipid II in the bacterial cell wall and prevents cell wall biosynthesis and/or forms pores in the cell membrane, leading to depolarization of the bacterial cytoplasmic membrane and the efflux of cell components, all of which result in cell death (21).

Experiments conducted on mice have shown that bacteriocins may have beneficial therapeutic properties *in vivo*. Corr et al. (22) demonstrated that the probiotic strain, *Lactobacillus salivarius* UCC118, produces a bacteriocin (Abp118) *in vivo* that protects mice against infection with *L. monocytogenes* EGDe. Nisin F suppressed the growth of *S. aureus* in the peritoneal cavity for at least 15 min (23). However, bioluminescence revealed the re-emergence of *S. aureus* after 44 h, suggesting that nisin F was degraded by proteolytic enzymes (23). In another study, Brand and co-workers (24) showed that intraperitoneal administration is an optimal route for treatment with bacteriocins. An immediate decrease in infection was observed after nisin F was injected into mice infected with *L. monocytogenes* EGDe and *S. aureus* (24). Moreover, nisin F may have a stabilizing effect on the microbiota, since the bacterial population in the GIT of mice remained relatively unchanged after nisin F was administered intraperitoneally (25). In another study, nisin stimulated the activity of interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor (TNF) in mice (26). However, the immune response triggered by nisin was too low to result in a detectable immune reaction (26). Jabés and colleagues (27) evaluated the *in vivo* efficacy of nisin NAI-107 in animal models with severe infection induced by methicillin-resistant *S. aureus* (MRSA), glycopeptide-intermediate *S. aureus* (GISA), penicillin-intermediate *S. pneumoniae* (PISP) or vancomycin-resistant *Enterococci* (VRE). Results demonstrated that intravenously administered NAI-107 was comparable or superior to traditional reference compounds used. Furthermore, NAI-107 was more effective when administered intravenously than subcutaneously. This suggested limited bioavailability by the latter route due to protein binding or degradation by proteases.

Bacteriocins with bactericidal properties, such as those mentioned above, may serve as an alternative to antibiotics and could improve the outcome of a number of bacterial infections, including those caused by multidrug-resistant strains (28). Bacteriocins are bactericidal at low (nanomolar or micromolar) concentrations and are not toxic to humans when administered at MIC (minimum inhibitory concentration) levels (29, 30). Furthermore, since bacteriocins are ribosomally encoded, genes involved in the production of the mature (active) peptide are easily identified and cloned into a different host, or the producer strain may be genetically engineered to produce an increased yield of native bacteriocin (31, 32).

Drugs administered orally not only need to withstand harsh conditions of the GIT, but also need to cross the highly selective gastrointestinal epithelial and vascular endothelial cells of the GBB (gut-blood barrier), to enter the blood stream. Most bacteriocins are degraded by proteolytic enzymes in the GIT and are unstable in chemically complex environments such as blood because they are degraded by proteases and bind to blood cells and plasma proteins (32–34). The proteolytic stabilities of therapeutic compounds vary among serum, plasma and fresh blood, because the composition of proteases differs between these blood components and because different proteases act at different cleavage sites (35). In blood coagulation, serine proteases are mostly involved, thus cleaving C-terminal lysine or arginine. These proteases also degrade peptides that contain basic residues for binding to targets via ion interactions.

Bacteriocins can be encapsulated in protective nanoparticles or macrophages to overcome *in vivo* degradation (36, 37). Another option is to use probiotic bacteria (encapsulated if needed) as delivery vehicles to constantly secrete bacteriocins in the GIT (38). With recent developments in nanotechnology (37, 39, 40), macrophage modification (36) and the latest knowledge gained on the expression of genes coding for bacteriocin production and secretion (14, 31, 41–43), target-specific delivery of bacteriocins could soon become a reality. However, little research has been done on the pharmacodynamics of bacteriocins (44, 45) and we do not know if these peptides are able to migrate across gut epithelial or vascular endothelial barriers.

Mechanisms associated with barrier selectivity are not fully understood. Molecular size and physiochemical properties of substances may play a crucial role (46). The hydrophobicity of bacteriocins suggests they may interact with epithelial cells in the GIT, but this could also lead to undesired cytotoxicity. Moreover, because of the small size of bacteriocins, they may have the ability to migrate across barriers paracellularly. Human colonic adenocarcinoma (Caco-2) cells are most commonly used in drug transport studies since the oral route is the most

important drug administration route and the degree of intestinal absorption may determine the clinical success or failure of a therapeutic agent (47). Human umbilical vein endothelial cells (HUVECs) are enzymatically removed from human umbilical veins and are commonly used in *in vitro* studies, especially in inflammation studies.

In this study, HUVEC and Caco-2 cell monolayers were used to simulate the epithelial and vascular endothelial barriers in the GBB to study the migration of nisin A, plantaricin 423 and bacST4SA across these barriers. We also report on the cytotoxic effect these peptides have on the two cell lines. Furthermore, bacteriocin stability was monitored in human plasma.

MATERIALS

Bacterial growth media, trifluoroacetic acid (TFA), XAD-16 beads, ammonium sulphate, acetonitrile and isopropanol were from Merck (Darmstadt, Germany). SepPak C18 columns were from Waters (Massachusetts, USA). Nisaplin was from Danisco (Copenhagen, Denmark). The bicinchoninic acid (BCA) protein assay was from Pierce Biotechnology (Massachusetts, USA). NHS-fluorescein was from Thermo Fisher Scientific (Massachusetts, USA). Trypsin and trypan blue were from Whitehead Scientific (Cape Town, SA). Dulbecco's modified Eagle's medium (DMEM) was from Merck (Darmstadt, Germany) and complete endothelial growth medium (EGM) was from Lonza (Basel, Switzerland).

METHODS

Peptide production and purification

Single colonies of *L. plantarum* 423 and *E. mundtii* ST4SA were inoculated into 10 ml MRS broth, incubated for 18 h at 37°C and transferred to 2 L MRS broth. After 18 h of incubation at 37°C, cells were harvested (11 000 x g for 20 min at 4°C). The pH of the cell-free supernatant was adjusted to 7.0 and heat-treated at 80°C for 10 min to inactivate proteases and precipitate heat labile proteins. Bacteriocins were precipitated from the cell-free supernatants with 70% saturated ammonium sulphate, as described by Todorov et al. (48) and placed on an orbital shaker (200 rpm) at 8°C for 48 h for precipitation to take place. The precipitate was collected by centrifugation (20 000 x g, 1 h) and resuspended in sterile phosphate buffered saline (PBS; pH 7.0). The suspension was heat treated at 80°C for 15 min to bring peptides into solution and then freeze-dried. The freeze-dried peptides were dissolved in sterile PBS and added to 40 g

XAD-16 beads that have been pre-activated with 80% (v/v) isopropanol containing 0.1% (v/v) trifluoroacetic acid (TFA). After 24 h on an orbital shaker, the beads were collected and washed with 30% (v/v) ethanol for 15 min. Hydrophobic peptides were eluted from the beads with 70% (v/v) isopropanol containing 0.1% (v/v) TFA. The beads were left in suspension for 18 h on an orbital shaker (100 rpm) at 8°C. The peptides were separated from the beads by filtering through a 0.45 µm cellulose acetate filter and the isopropanol evaporated with rotary evaporation (RotaVapor®, Buchi). Peptides were further purified using Sep-Pak C18 reverse phase columns and eluted using 60% (v/v) isopropanol containing 0.1% (v/v) TFA. Isopropanol was removed with rotary evaporation, active fractions were freeze-dried and antimicrobial activity was tested by using the agar well diffusion assay (49). Commercially available Nisaplin® was dissolved in 25% acetonitrile (v/v) containing 0.1% (v/v) TFA and centrifuged to remove undissolved particles. The supernatant was loaded onto a Sep-Pak C18 reverse phase column and eluted with 40% (v/v) acetonitrile containing 0.1% (v/v) TFA. Active fractions were freeze-dried and tested for activity as mentioned previously (49).

Peptides were further purified by fast protein liquid chromatography (FPLC) (ÄKTA purifier; GE Healthcare Life Sciences, Mississauga, Canada). Samples were dissolved in 10% (v/v) acetonitrile containing 0.1% (v/v) TFA and added to a HiScale Source 15 RRC column (HIVD SepSci, South Africa). Plantaricin 423 and bactST4SA were eluted with an increasing gradient of 10% (v/v) to 55% (v/v) acetonitrile containing 0.1% (v/v) TFA. Peaks were detected measuring absorbance at 254 nm. Fractions from each peak were collected and tested for activity as described elsewhere (49). Active samples were freeze-dried, dissolved in 10% (v/v) acetonitrile and loaded onto a Discovery BIO Wide Pore C18 HPLC (high performance liquid chromatography) column (10 µm, 250 x 10 mm; Sigma-Aldrich, Missouri, US). Plantaricin 423 and bacST4SA were eluted in an acetonitrile gradient of 25% (v/v) to 60% (v/v) acetonitrile containing 0.1% (v/v) TFA and nisin in an increasing gradient of 10% (v/v) to 60% (v/v) acetonitrile containing 0.1% (v/v) TFA. Peaks were collected and tested for activity as described elsewhere. Active peptides were freeze-dried, re-suspended in sterile ultrapure water containing 0.1% (v/v) TFA and analysed by liquid chromatography-mass spectrometry (LC-MS; Central Analytical Facility, Stellenbosch, South Africa). A Waters Quadrupole Time-of-Flight Synapt G2 (Waters Corporation, Milford, USA) was used for the LC-MS.

Determination of minimum inhibitory concentration (MIC)

The bicinchoninic albumin assay (BCA) was used to determine the concentration of the peptides. The MIC of the peptides against *Listeria monocytogenes* EGDe was determined using a microtiter plate. Briefly, an overnight culture of the microorganism was inoculated into *L. monocytogenes* EGDe enrichment broth (LEB) and incubated overnight at 37°C. The culture was inoculated into fresh LEB and incubated until an OD₆₀₀ of 0.1 was reached. A series of peptide concentrations was prepared and 50 µl of each added per well, followed by the addition of 150 µl culture. The controls were ultrapure water containing 0.1% (v/v) TFA with 150 µl culture or media. Microtiter plates were incubated at 37 °C and OD₆₀₀ readings were taken at T₀, T₅ and T₁₈ (hours). The MIC was determined as the lowest peptide concentration required to inhibit the growth of *L. monocytogenes* EGDe after 18 h.

Plasma stability assay

Human blood was collected in anticoagulant tubes (Greiner Bio-One, Kremsmünster, Austria), centrifuged at 500 x g (10 min, 25 °C) and the plasma collected. Different plasma concentrations (0%, 40% and 80%, v/v) were prepared by diluting with sterile ultrapure water containing 0.1% (v/v) TFA, and aliquoted into sterile microcentrifuge tubes. Nisin A, plantaricin 423 and bacST4SA, each suspended in sterile ultrapure water containing 0.1% (v/v) TFA, were added to the plasma to final concentrations of 25 µM, 50 µM and 100 µM, respectively. Sterile ultrapure water containing 0.1% (v/v) TFA was used as control. Incubation was at 37°C for 3 days and bacteriocin activity was tested by agar diffusion, using *L. monocytogenes* EGDe as target (49). *Listeria monocytogenes* EGDe was used as target organism. Digital images of the plates were taken after incubation and activity quantified by measuring the size of the zones using ImageJ software (version 1.5; <https://imagej.nih.gov/ij/>).

Cell culture

Human colonic adenocarcinoma (Caco-2) cells (South African Medical Research Council, Cape Town, SA) were used between passages 50-60.) and were grown in DMEM. Human umbilical vein endothelial cells (HUVECs; Lonza, Basel, Switzerland) were used between passages 5-15 and grown in complete EGM. Both cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂. Cell cultures were routinely sub-cultured

before reaching confluence. Cells were treated with trypsin and counted using a haemocytometer.

For viability and cytotoxicity studies, cells were seeded in 96-well flat-bottom culture plates (1×10^4 cells/well). To determine migration of nisin A, plantaricin 423 and bacST4SA across the gastrointestinal epithelial and vascular endothelial barriers, HUVECs and Caco-2 cells were grown on an 8 μ M pore size tissue culture insert (Qiagen, Hilden, Germany). All cell culture experiments were done in triplicate and have been repeated more than three times.

Viability and cytotoxicity assays

XTT Assay

The colorimetric XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphenyl)-2*H*-Tetrazolium-5-Carboxanilide) assay (Merck, USA) was used to assess cell viability based on metabolic activity. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of XTT, resulting in the formation of an orange formazan product which is measured spectrophotometrically. The HUVECs or Caco-2 cells were grown in 100 μ l of culture medium for 24 h at 37°C. Each of the bacteriocins (nisin A, plantaricin 423 and bacST4SA) were added to the culture at concentrations of 25 μ M, 50 μ M and 100 μ M. Sterile ultrapure water containing 0.1% (v/v) TFA was used as control. After 24 h of incubation at 37°C, the reaction mixture containing PMS (N-methyl-dibenzopyrazine-methylsulfate/phenazine methosulphate) and XTT solution, was added to each well and incubated for 4 h at 37°C. Absorbance readings were recorded at 450 nm, using a microtiter plate reader (EZ Read 400, Biochrom, Cambridge, UK). The percentage viability was calculated as $A_T/A_C \times 100$; where A_T is the absorbance of peptide-treated cells and A_C is the absorbance of control (untreated cells).

Neutral Red Assay

Cell viability was verified using the neutral red assay (Merck, Darmstadt, Germany). Neutral red dye is transported into the lysosome of viable cells. Thus, the amount of dye uptake is proportional to the number of viable cells. Cells were treated with different concentrations of the peptides as described for the XTT assay. Sterile ultrapure water containing 0.1% (v/v) TFA was used as control. After incubation at 37°C for 24 h, neutral red solution was added to the cells (10% of the culture volume). After 4 h of incubation at 37°C, the medium was removed

by aspiration and the cells were rinsed with neutral red fixative. The fixative was removed, followed by the addition of neutral red and the cells were gently stirred for 10 min on a gyratory shaker. Absorbance readings were recorded at 540 nm and 690 nm, respectively, using a microtiter plate reader. The percentage viability was calculated as the absorbance of treated cells divided by the absorbance of untreated cells ($A_T/A_C \times 100$).

LDH Release Assay

HUVECs and Caco-2 cells were exposed to three different concentrations (25 μ M, 50 μ M and 100 μ M) nisin A, plantaricin 423 and bacST4SA and the presence of extracellular LDH (Lactate Dehydrogenase) determined using the LDH release assay kit (Merck, Darmstadt, Germany). Sterile ultrapure water containing 0.1% (v/v) TFA was used as negative control (referred to as spontaneous LDH activity) and lysis buffer (10X) was used as positive control (referred to as maximum LDH activity). After incubating the cultures in the presence of the peptides for 24 h at 37°C, culture supernatants were pipetted into a 96-well plate. Reaction mixture was added to the supernatants and plates were incubated at 25°C for 30 min. Absorbance was measured at 490 nm and 680 nm, respectively. The percentage cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{(\text{Compound-treated LDH activity}) - (\text{Spontaneous LDH activity})}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100$$

Labelling peptides with NHS-fluorescein

Purified stock solutions of nisin A, plantaricin 423 and bacST4SA were prepared by dissolving the peptides in sterile ultrapure water containing 0.1% (v/v) TFA. Concentrations were determined using the BCA assay, as described elsewhere. Immediately before conjugating fluorescein to the peptides, a stock solution of NHS-fluorescein was prepared by dissolving 1 mg of reagent in 100 μ l DMF. The amount of NHS-fluorescein to be added to each peptide was calculated using the following equations:

$$\text{ml peptide} \times \frac{\text{mg peptide}}{\text{ml peptide}} \times \frac{\text{mmol peptide}}{\text{mg peptide}} \times \frac{15 \text{ mmol NHS}}{\text{mmol peptide}} = \text{mmol NHS}$$

$$\text{mmol NHS} \times \frac{473.4 \text{ mg (molecular weight of NHS)}}{\text{mmol NHS}} \times \frac{100 \text{ } \mu\text{l Solvent (DMF)}}{1 \text{ mg}} = \mu\text{l NHS}$$

The calculated concentration of NHS-fluorescein was added to the peptides and incubated in the dark on ice for 2h. Non-reacted NHS-fluorescein was removed by Sep-Pak C18 reverse phase column chromatography. Plantaricin 423 and bacST4SA were eluted with 60% isopropanol containing 0.1% (v/v) TFA and nisin A with 40% acetonitrile containing 0.1% (v/v) TFA. Rotary evaporation was used to remove the isopropanol. Peptides were freeze dried and resuspended in sterile ultrapure water containing 0.1% TFA. Concentrations of the labelled peptides were determined with the BCA assay and diluted to 50 μM . Labelled peptides were filtered through a 0.45 μm cellulose acetate membrane and spotted (20 μl) onto *Listeria* enrichment agar seeded with 1% *L. monocytogenes* EGDe (OD₆₀₀ 0.1). Plates were incubated overnight at 37°C and the fluorescent peptides visualised under a UVP 3UV Ultraviolet Lamp (254 nm; Thermo Fisher Scientific, Massachusetts, USA). Inhibition zones were measured using ImageJ software and compared to those of non-labelled peptide to determine the percentage of activity loss due to labelling.

Ability of bacteriocins to migrate across gastrointestinal epithelial and vascular endothelial cells

HUVECs and Caco-2 cells were seeded onto transmigration inserts (Whitehead Scientific, Cape Town, SA), as shown in Fig. 1, at a cell density of 1×10^5 cells/insert (200 μl). Inserts were carefully placed in a 24-well tissue culture plate containing 700 μl cell growth media. Cells were incubated at 37°C in a humidified atmosphere in the presence of 5% CO₂, until confluency was reached and a monolayer formed. Fluorescently labelled peptides (nisin A, plantaricin 423 and bacST4SA) were added, respectively, to the apical side of the inserts at a concentration of 50 μM . Transmigration inserts containing no monolayer, was used to determine the total fluorescence. Samples were incubated for 3 h while absorbance was

measured at 490 nm every 30-min using a microplate reader. Before each reading, inserts were placed in new microplates containing fresh media. The amount of migrated peptide was calculated as a percentage of total fluorescence in wells (without monolayer):

$$\% \text{ Migrated peptide} = \frac{\text{Absorbance in well (containing monolayer)}}{\text{Absorbance in control well (no monolayer)}} \times 100$$

After 3 h, the absorbance of the residual labelled peptide in the inserts (non-migrated peptide) was measured after transferring the non-migrated peptide to 24-well plates containing culture media to a final volume of 700 μ l. The percentage residual peptide was calculated as a percentage of total fluorescence:

$$\% \text{ Residual (non-migrated peptide)} = \frac{\text{Absorbance in insert}}{\text{Absorbance in control well (no monolayer)}} \times 100$$

The cells were washed with PBS and visualised in the IVIS® 100 *In Vivo* Imaging System (Caliper Life Sciences, Hopkinton, US) to detect any labelled peptide that have attached to the cells or have entered the cells. The percentage of peptide that attached to, or entered the cells was quantified by subtracting the sum of migrated peptide and retained peptide from the total fluorescence. To determine the cytotoxicity of NHS-fluorescein labelled peptide, a trypan blue exclusion test was performed. Briefly, cells treated for 3 h with labelled nisin A, plantaricin 423 or bacST4SA were harvested by centrifugation as described elsewhere and resuspended in 1 ml PBS. One-part cell suspension (100 μ l) was added to 100 μ l trypan blue (0.4%) and incubated for 3 min at room temperature. The number of unstained (viable) and stained (non-viable) cells was determined using a haemocytometer. The percentage viable cells were calculated by dividing the total number of viable cells per ml with the total number of cells per ml. The integrity of the monolayers was monitored by ensuring TEER (trans-epithelial electrical resistance) readings remained above 300 Ω throughout the experiment (Millicell-ERS volt-ohm meter, Merck-Millipore, Massachusetts, United States).

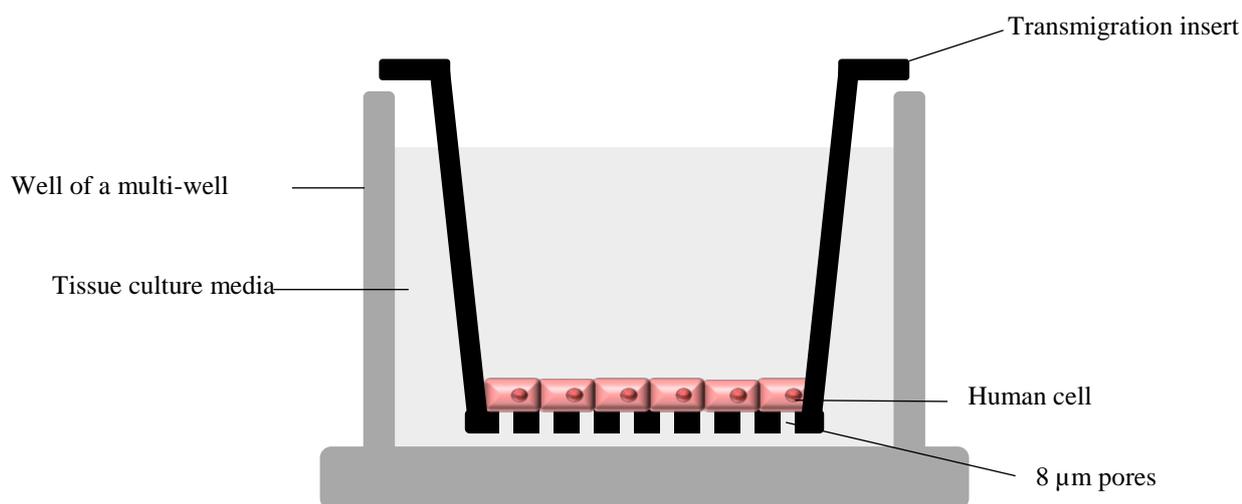


Figure 1 Simulated gastrointestinal epithelial / vascular endothelial barrier constructed with HUVECs and Caco-2 cells, respectively. Transmigration inserts on which the cell lines have been cultured were inserted into wells of a 24-well plate.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) followed by Dunnet's multiple comparison test to detect significant differences ($p < 0.05$). Data was analysed using GraphPad Prism software (version 5.0, USA).

RESULTS

MIC levels of nisin A, plantaricin 423 and bacST4SA

The MIC levels of nisin A, plantaricin 423 and bacST4SA were 8 μM , 12 μM and 10 μM , respectively, as determined against *L. monocytogenes* EGDe.

Plasma stability of the bacteriocins

The antimicrobial activity of nisin A, plantaricin 423 and bacST4SA, each tested at 25 μM , 50 μM and 100 μM , decreased slightly after 3 days of incubation in the absence of plasma (Figure 2). Of the three bacteriocins, bacST4SA was the most stable in the presence of human plasma at all three plasma concentrations (Figure 2). Nisin A was the least stable. At a concentration of 25 μM , all three bacteriocins lost a maximum of 40% of their antimicrobial activity after 24 h in the presence of 80% plasma (Table 1). However, when 50 μM and 100 μM of the bacteriocins were exposed to 80% plasma, a maximum of 21% to 32% of their activity was lost.

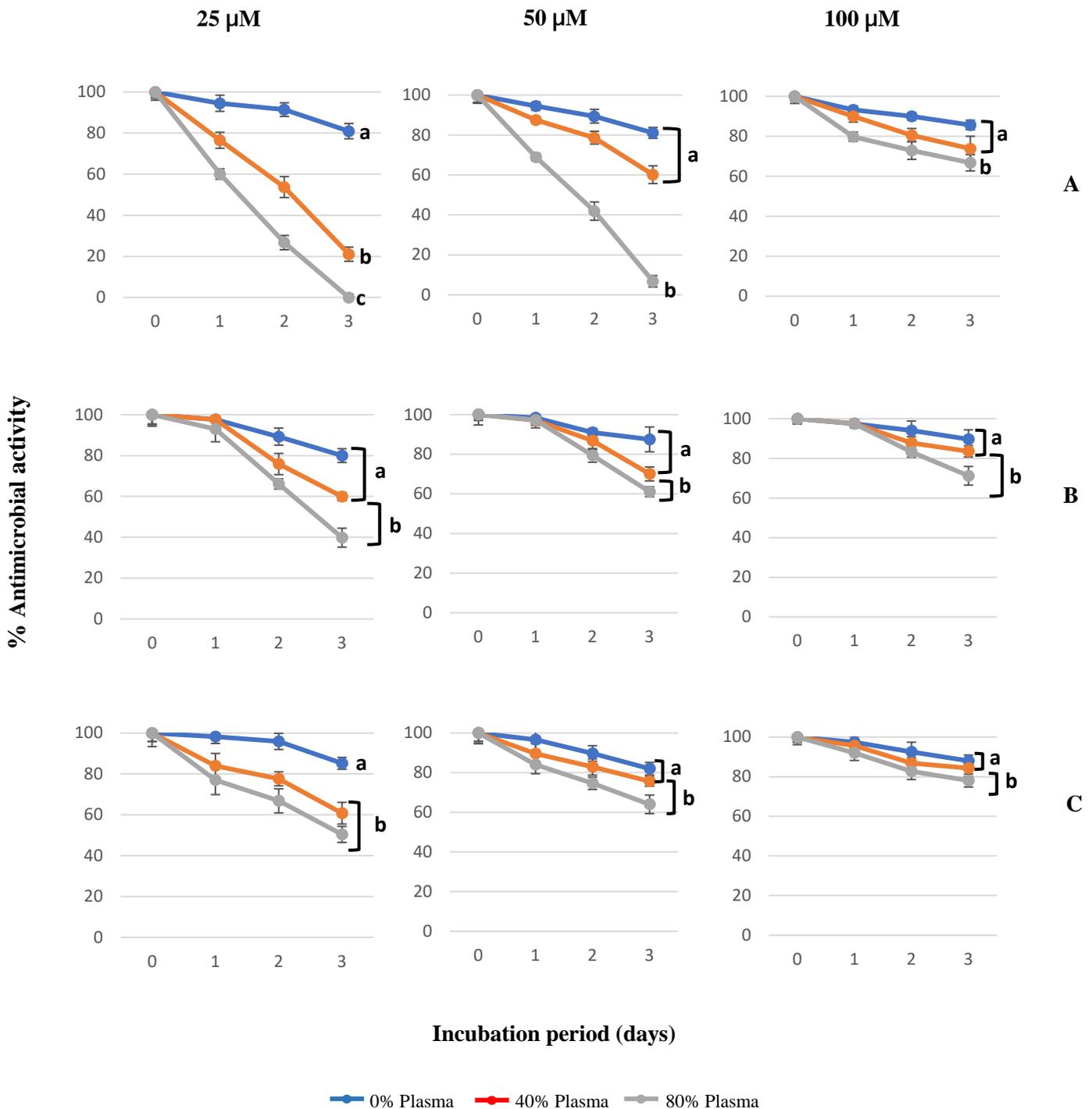


Figure 2 Effect of human blood plasma on the antimicrobial activity of nisin A, plantaricin 423 and bacST4SA over three days. Changes in the antimicrobial activity against *L. monocytogenes* EGDe were observed by spotting the peptides on agar plates and measuring the inhibition zones. A: 25 μM, 50 μM and 100 μM nisin A; B: 25 μM, 50 μM and 100 μM plantaricin 423; C: 25 μM, 50 μM and 100 μM bacST4SA. Antimicrobial activity of the peptides in the presence of 0% plasma (●), 40% plasma (●) and 80% plasma (●) are represented

as a percentage of the initial activity (100% at day 0). Curves without common letters (a-c) differ significantly ($p < 0.05$).

Table 1 Reduction of the antimicrobial activity of nisin A, plantaricin 423 and bacteriocin ST4SA due to 40% plasma and 80% plasma. The percentage decrease in antimicrobial activity was calculated for 25 μM , 50 μM and 100 μM of the peptides.

Plasma concentration	% Decrease in antimicrobial activity								
	Nisin A			Plantaricin 423			Bacteriocin ST4SA		
	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
40%	79	40	26	40	30	17	39	24	16
80%	100	93	33	60	39	29	50	36	22

Cell viability and cytotoxicity of bacteriocins

The cytotoxicity of nisin A, plantaricin 423 and bacST4SA, against HUVEC and Caco-2 cells is shown in Figures 3 to 5. Results obtained with XTT (Figure 3) and neutral red (Figure 4) assays showed no significant difference in percentage viability of both cell types after treatment with 25 μM and 50 μM of the three respective bacteriocins. However, 100 μM nisin A, plantaricin 423 and bacST4SA caused a slight reduction in HUVEC and Caco-2 cell viability. Based on the LDH release from the two cell lines, the relative cytotoxicity of the peptides was significantly when compared to the positive control at all three concentrations (Figure 5). However, the cytotoxicity levels of 100 μM nisin A, plantaricin 423 and bacST4SA were 41%, 21% and 12% against HUVECs and 29%, 17% and 14% against Caco-2 cells. Thus, nisin A displayed significantly higher levels of cytotoxicity compared to plantaricin 423 and bacST4SA. All three peptides were more toxic to HUVECs than Caco-2 cells as the overall relative viability of the cells was lower after treatments during the XTT and neutral red assays and the relative cytotoxicity as assessed by the LDH release assay increased in a peptide dose-dependent manner. Of the three peptides, nisin A was the most toxic to HUVECs and Caco-2 cells and bacST4SA was best tolerated.

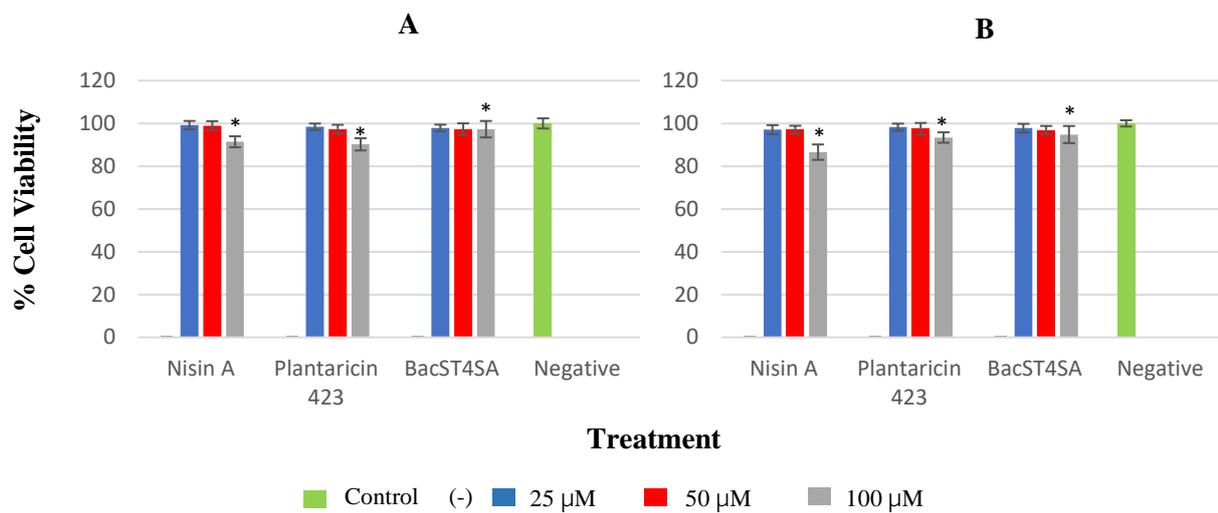


Figure 3 The effect of antimicrobial peptides, nisin A, plantaricin 423 and bacST4SA cell viability using an XTT assay. A: Cell viability of CACO-2. B: Cell viability of HUVECs. Cell viability after treatment with 25 µM (blue), 50 µM (red) and 100 µM (grey) peptide are represented as a percentage of total cell viability. Sterile ultrapure water containing 0.1% (v/v) TFA was used as negative control (green) and represents 100% cell viability. Significantly different values are indicated ($p < 0.05$) are indicated by *.

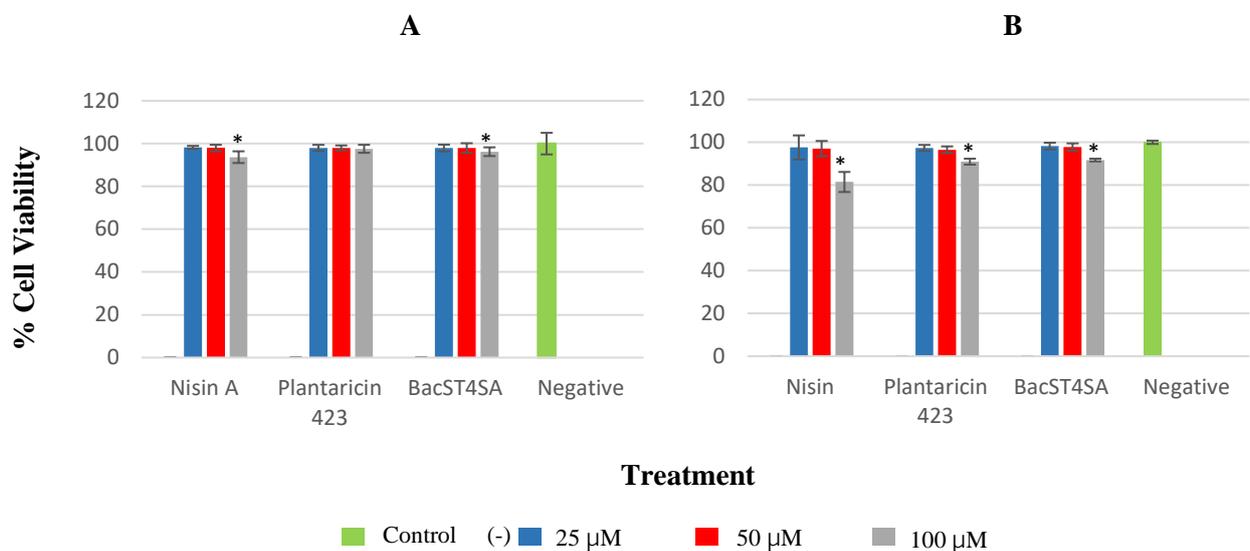


Figure 4 The effect of antimicrobial peptides, nisin A, plantaricin 423 and bacST4SA on cell viability using a neutral red assay. A: Cell viability of Caco-2. B: Cell viability of HUVECs. Cell viability after treatment with 25 µM (blue), 50 µM (red) and 100 µM (grey) peptide are represented as a percentage of total cell viability. Sterile ultrapure water containing 0.1% (v/v) TFA was used as negative control (green) and represents 100% cell viability. Significantly different values are indicated ($p < 0.05$) are indicated by *.

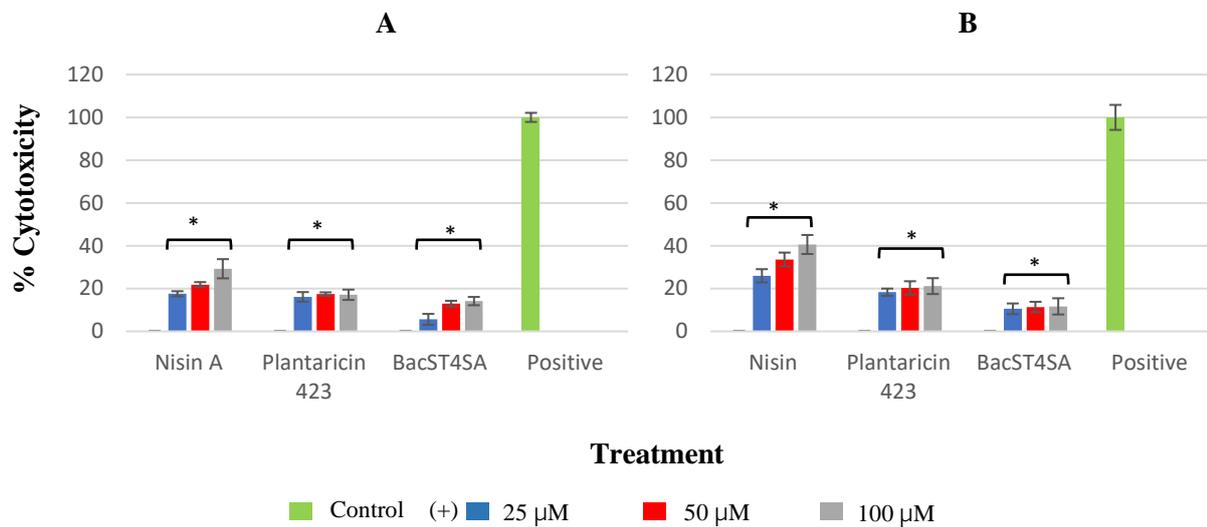


Figure 5 The effect of antimicrobial peptides, nisin A, plantaricin 423 and bacST4SA on plasma membrane integrity using an LDH release assay. A: Cytotoxicity towards Caco-2. B: Cytotoxicity towards HUVECs. Cytotoxicity after treatment with 25 µM (blue), 50 µM (red) and 100 µM (grey) peptide are represented as a percentage of maximum LDH release. Lysis buffer was used as positive control (green) and represents 100% cytotoxicity. Significantly different values ($p < 0.05$) are indicated by *.

Peptide labelling

Nisin A, plantaricin 423 and bacST4SA were successfully labelled with NHS-fluorescein, as shown by clear fluorescent zones on agar seeded with *L. monocytogenes* EGDe (Figure 6). The labelled peptides were, however, less active than the unlabelled peptides, as measured by a decrease in activity of 32% by nisin A, 24% by plantaricin 423 and 21% by bacST4SA (Figure 7).

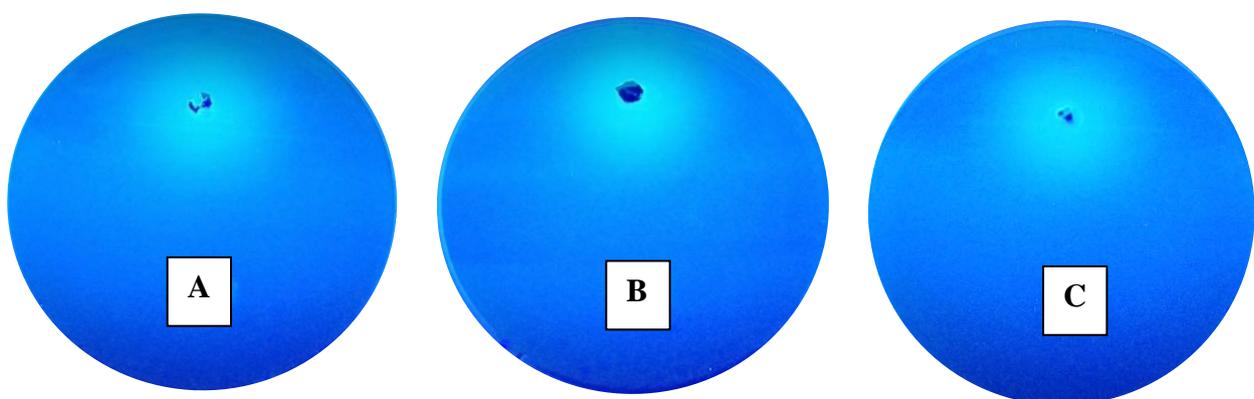


Figure 6 Clear fluorescent zones visualized with UV light at 254 nm. NHS-fluorescein labelled nisin A (A), plantaricin 423 (B) and bacST4SA (C).

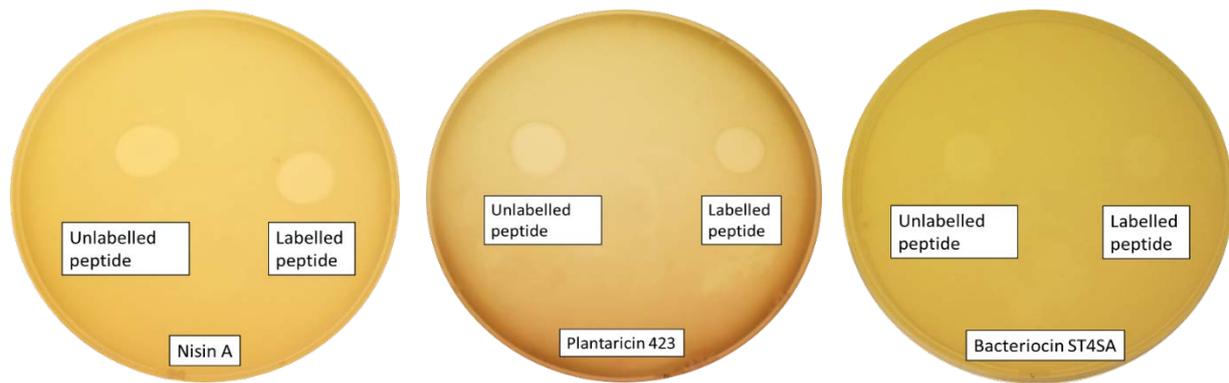


Figure 7 Inhibition zones of labelled and unlabelled nisin A, plantaricin 423 and bacST4SA after spotting against *L. monocytogenes* EGDe.

Ability of bacteriocins to migrate across gastrointestinal epithelial and vascular endothelial cells

Migration of nisin A, plantaricin 423 and bacST4SA across HUVEC and Caco-2 cells is shown in Figure 8. After 3 h of incubation, 75% of nisin A, 85% of plantaricin 423 and 84% of bacST4SA migrated across the Caco-2 cell monolayer and a total of 90% nisin A, 96% plantaricin 423 and 93% bacST4SA migrated across the HUVEC monolayer (Figure 8). Based on these results, nisin A, plantaricin 423 and bacST4SA migrated more easily through the endothelial layer (HUVECs) than the epithelial layer (Caco-2 cells). In the case of all three bacteriocins, almost full migration across the two cell types occurred within the first 90 min (Figure 8). A larger percentage of plantaricin 423 migrated across the cells. After 3 h of incubation, approximately 4% nisin A, plantaricin 423 and bacST4SA remained in the tissue culture inserts after being added to either the HUVEC or Caco-2 cell monolayers (Figure 9). In the control inserts (no monolayer), similar results were obtained (also $\pm 4\%$ residual peptide).

Low concentrations or no fluorescently labelled peptides attached to the HUVEC and Caco-2 cells after 3 h of incubation (Figure 10). Higher levels of fluorescence were visible in cells that have been treated with nisin A. No fluorescence was detected in HUVECs treated with plantaricin 423. The percentage attached and/or entered peptide was calculated; 21% nisin A, 11% plantaricin 423 and 12% bacST4SA remained attached to Caco-2 cells and/or entered them and 6% nisin A, 0% plantaricin 423 and 3% bacST4SA attached to the HUVECs. None of the labelled peptides were cytotoxic to any of the two cell lines, as indicated by trypan blue staining (data not shown).

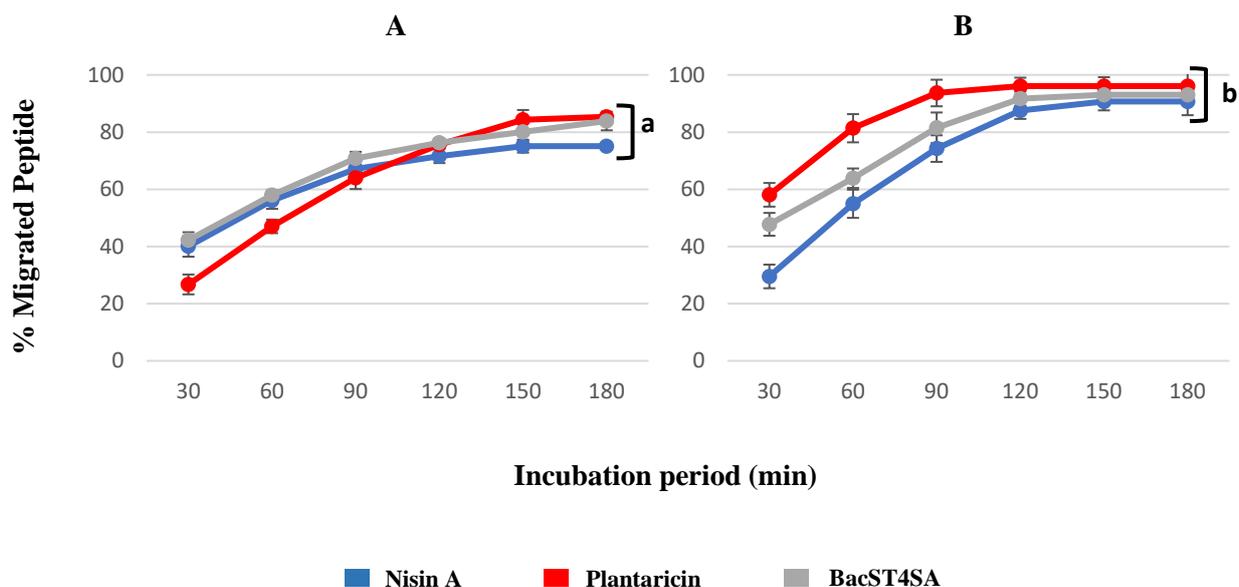


Figure 8 Ability of nisin A, plantaricin 423 and bacST4SA to migrate across a simulated barrier of gastrointestinal epithelial and vascular endothelial cells A: Migration of peptides across a Caco-2 cell monolayer. B: Migration of peptides across a HUVEC monolayer. Migrated nisin A (blue), plantaricin 423 (red) and bacST4SA (blue) is expressed as percentage of total fluorescence. Curves without common letters differ significantly ($p < 0.05$).

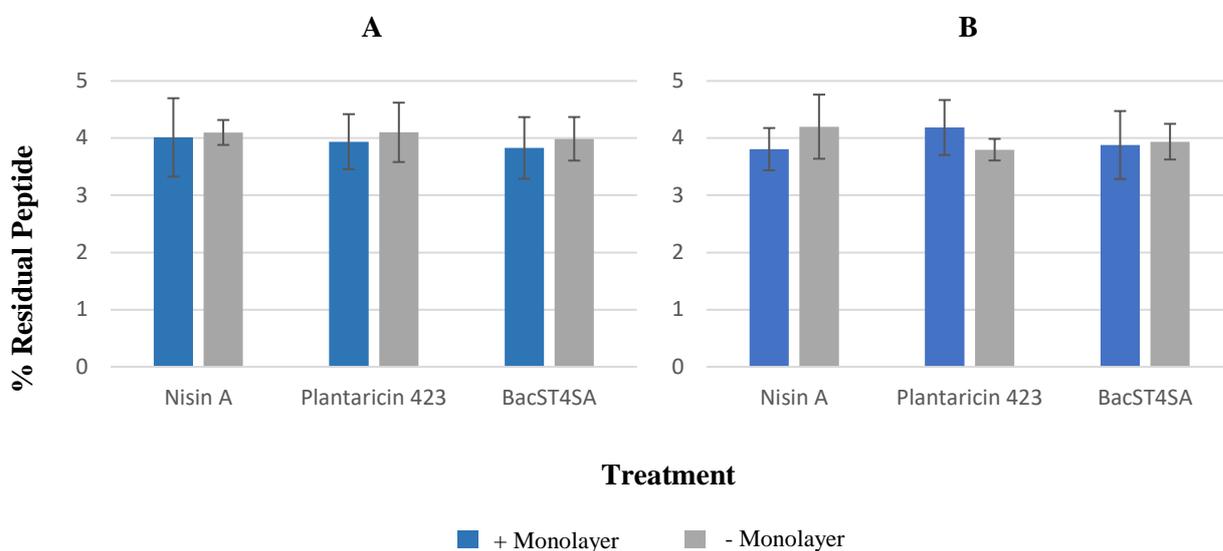


Figure 9 Percentage residual peptide that remained in the insert after 3 h of incubation. Peptides that did not migrate across the Caco-2 cell monolayer (A) and the HUVEC monolayer (B) are compared to the control (no monolayer) and expressed in percentage values. No significant differences were recorded.

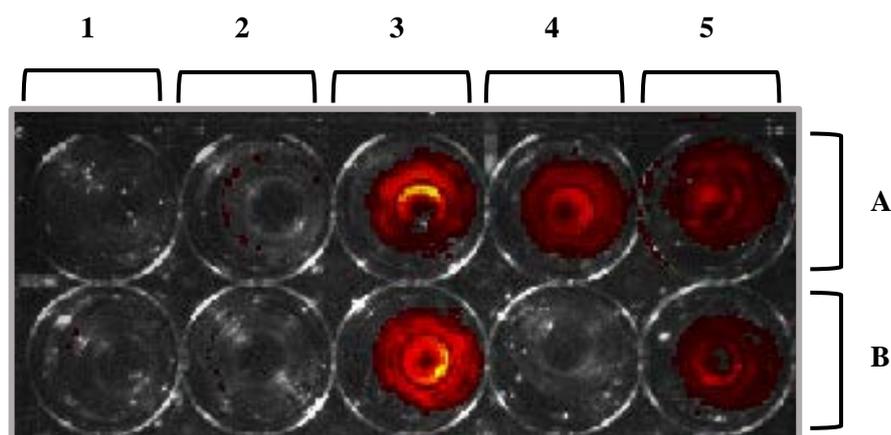


Figure 10 Fluorescent peptides that attached to the Caco-2 cells (A) and HUVECs (B) after 3 h of incubation. (1) No peptide or fluorescent marker; (2) NHS-fluorescein; (3) fluorescently labelled nisin A; (4) fluorescently labelled plantaricin 423; (5) fluorescently labelled bacST4SA.

DISCUSSION

It is crucial to assess the stability, safety and effectiveness of antimicrobial compounds to determine if they can be used as alternatives to antibiotics. Nisin A, plantaricin 423 and bacST4SA were more stable in human plasma when added at a concentration of 100 μM compared to 50 μM and 25 μM . Higher concentrations resulted in higher bioavailability of antimicrobials (50). Thus, a smaller percentage of the total amount of peptide administered was degraded by proteases or bound to proteins present in the blood. In a previous study (23), *Staphylococcus aureus* Xen 36-infected mice were treated with nisin F, which is similar to nisin A used in this study. Nisin F was administered intraperitoneally at a concentration of 640 AU (arbitrary units). Results indicated that nisin F suppressed the growth of *S. aureus* Xen 36 *in vivo* only for the first 15 min. The authors hypothesized that the re-emergence of *S. aureus* was due to degradation of nisin F by proteolytic enzymes. This may explain the loss in antimicrobial activity recorded for nisin A, plantaricin 423 and bacST4SA after prolonged exposure (1-3 days) to blood plasma. However, since these bacteriocins are membrane active and have hydrophobic natures, they may attach to cells or other proteins, limiting their availability (11, 15, 17). Most antibiotics are delivered to the site of infection within 3 h (29, 50). Thus, results from this study indicate that the peptides remain stable in blood plasma for a sufficient period. Protein binding, GIT conditions, food and proteases may affect the oral

administration of antimicrobials. However, *in vivo* experiments are required to determine the optimal dosage as high dosages may be toxic.

Previous studies have shown that certain AMP such as melittin, pediocin and colicin E6 have the ability to interact with mammalian proteins involved in metabolism, disrupt mitochondrial processes, cell structure and cause apoptosis (18, 51). It is therefore crucial to study the interaction of AMPs with mammalian cells to evaluate their potential toxicity before they can be considered for delivery to infectious sites. The hydrophobicity of nisin A, plantaricin 423 and bacST4SA suggests they may interact with epithelial cells in the GIT and cause undesired toxicity (51). In this study, nisin A, plantaricin 423 and bacST4SA did not cause a decrease in HUVEC or Caco-2 cell viability at concentrations up to 50 μM in the XTT and neutral red assays. Results from the LDH release assay indicate that nisin A, plantaricin 423 and bacST4SA are cytotoxic against Caco-2 cells or HUVECs at a concentration of 100 μM . However, nisin A, a FDA approved food preservative, displayed significantly higher levels of toxicity compared to plantaricin 423 and bacST4SA in both cell lines ($P < 0.05$). This increase in cytotoxicity could be associated with an increased hydrophobicity, however, the mechanisms involved in the different toxicity levels requires further study. The overall cytotoxicity of nisin A, plantaricin 423 and bacST4SA was greater against HUVECs compared to Caco-2 cells. In a study done by Murinda et al. (18), nisin, pediocin and selected colicins showed different cytotoxicity levels amongst colonic cells and Vero (kidney) cells. These differences may be due to the eukaryotic cell type used. However, other explanations include, potential presence of impurities in the AMP preparations used, loss of activity after purification, different exposure times, solvents or media used (51). The differences in cytotoxicity levels between HUVECs and Caco-2 cells are likely associated with different cell lines used as the peptides were pure and all solvents removed. Cytotoxicity studies carried out by Maher et al. (51) showed that $\sim 80 \mu\text{M}$ nisin A did not have a significant cytotoxic effect on Caco-2 cells. This current study expands on this report by demonstrating that the cytotoxicity of nisin A, plantaricin 423 and bacST4SA increased as concentrations increased. Future *in vivo* studies on animals would provide more information regarding the maximum tolerated doses as well as cell type-specific responses to the peptides.

The gastrointestinal barrier is highly selective and prevents the passage of toxic compounds of the luminal microbiota whilst allowing the absorption of nutrients from the gut lumen (52). The molecular size and physiochemical properties of substances play a significant role in this

selectivity. Moreover, the vascular endothelial barrier may play a vital role in maintaining the function and structure of the intestinal barrier. Orally administered therapeutic agents need to maintain stability throughout the gastrointestinal tract, cross the intestinal mucous layer, the epithelial barrier and the vascular endothelial barrier to reach the blood stream so that they can be transported to the site of infection. Oral drug delivery vectors such as probiotics can be used to transport AMPs to infectious sites (51). The delivery of nisin A to the site of gastrointestinal colonic infections has been investigated and is currently under patent (53). In this study, a simulation of the gastrointestinal epithelial and vascular endothelial barrier was used to determine if bacteriocins can cross these barriers. Since bacteriocins are small they may diffuse across these barriers by means of the paracellular pathway. However, during passive diffusion, a concentration equilibrium might be expected on either side of the transmigration insert, while the data from this study suggests the peptides traversed it. This may be explained by the fact that the transmigration inserts were placed into fresh media prior to each determination, resulting in multiple dialysis steps.

Results from this study correspond to this hypothesis. The AMPs, nisin A, plantaricin 423 and bacST4SA can migrate across a simulation of the epithelial and endothelial layers. Moreover, a small amount of the peptides can attach to or enter epithelial (Caco-2) cells and nisin A and bacST4SA can attach to or enter endothelial cells (HUVECs). This study does not determine whether the bacteriocins attach or enter the mammalian cells, but it may indicate antigenicity of the peptide simulating phagocytosis, pinocytosis or another method of intracellular transport. Nisin A acts by binding to lipid II in the cell membrane of bacteria. Since similar lipids are present in the eukaryotic cell membrane, nisin A may have the ability to bind to these lipids, resulting in cytotoxicity. However, further research is required to determine exactly how (active or passive transport) these peptides are transported across epithelial -and endothelial cells.

Plantaricin 423 is most effective at migrating across these barriers but is least effective at attaching to the cells. In contrast, nisin A is the least effective at crossing the barriers but a larger amount of the peptide attached to the cells. The difference in ability of the peptides to attach to mammalian cells can be due to differences in hydrophobicity, amino acid composition, mode of action or size. The exact mechanisms involved in eukaryotic cell attachment requires further investigation. A small percentage of labelled peptide remained in the tissue culture inserts (~4%) after an incubation period of 3 h. Since this percentage can be

compared to residual peptide in control inserts (no monolayer) one can conclude that a small amount of peptide attaches to the insert. This could be due to the hydrophobicity of the insert.

In conclusion, current data illustrated that nisin A, plantaricin 423 and bacST4SA have no significant cytotoxicity at effective antimicrobial doses, that the peptides remain stable in blood plasma at the concentrations tested, and that they have the capacity to readily migrate across epithelial membranes for systemic delivery *in vivo*. These promising results should be followed up by *in vivo* studies to determine potential tissue-specific responses to these peptides. Advanced visualisation techniques such as microfluidic and confocal microscopy could shed more light on potential occurrence of cell internalisation of peptides, as well as membrane transport mechanisms involved.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

The continued success of humankind relies on the ability to treat and prevent diseases (1). Unfortunately, the inappropriate and unnecessary use of antibiotics has led to the development of antibiotic-resistant bacteria (2). Approximately 2 million people in the United States are infected with resistant bacteria each year, resulting in a minimum of 23 000 deaths annually (3). The burden of infectious diseases is substantial in developing countries, such as South Africa, due to limited access to clean water and sanitation, malnutrition, ineffective treatment and the HIV/AIDS -and TB epidemics (4). Thus, alternatives to antibiotics are urgently needed (1).

Bacteriocins may be a viable alternative to conventional antibiotics, since they are highly potent against pathogens, as determined with *in vitro* and *in vivo* studies (5–7). Bacteriocins are generally non-toxic (8–10) and strains may be genetically modified to produce broad -and narrow-spectrum peptides. One of the major disadvantages of bacteriocins is their susceptibility to proteolytic enzymes (11). Proteases in the GIT and blood may degrade orally and parenterally administered bacteriocins. While some bacteriocins may be stable enough to reach the site of infection, *in situ* bacteriocin production by probiotics is a viable alternative.

The gut microbiota promotes human health by improving nutrient uptake, excluding pathogens and modulating the immune system (1). Regardless of the method used in administering antibiotics, the gut microbiome is always affected. An altered gut microbiome leads to infection by intestinal pathogens and diseases such as irritable bowel syndrome, become more prevalent. Probiotics maintain or improve the commensal gut microbiota and prevent the colonization by pathogens. Once bacteriocins have been produced in the GIT by probiotics, they need to cross the highly selective gastrointestinal epithelial and vascular endothelial barriers to enter the blood stream and be transported to the site of infection (12, 13). However, little research has been done on the migration of bacteriocins across these barriers.

In this study, nisin A, plantaricin 423 and bacST4SA migrated across simulations of the gastrointestinal epithelial and vascular endothelial barriers. Even though a small percentage of the peptides attached to the cells (HUVECs and Caco-2 cells), they did not cause a decrease in cell viability at concentrations up to 50 μ M. Nisin A, a FDA food preservative showed significantly higher levels of cytotoxicity compared to plantaricin 423 and bacST4SA. Nisin A has the lowest MIC towards *L. monocytogenes* EGDe, which might explain the fact that it is

the most toxic towards HUVECs and Caco-2 cells, compared to plantaricin 423 and bacST4SA. Moreover, the mode of action of nisin A, a lantibiotic, is different towards plantaricin 423 and bacST4SA, both classII bacteriocins.

The mammalian phospholipid cell membrane illustrates combinations of hydrophobic and hydrophilic interactions (14). The hydrophilic fatty acid chains of the phospholipids are clustered away from contact with water, maximizing hydrophobic interactions. The hydrophobic groups at the exterior surface of the bilayer interact with exterior hydrophobic molecules and thus allow the passive diffusion of these molecules across the membrane (14, 15). In contrast, hydrophilic molecules cannot be transported by means of passive diffusion, but instead require interaction with transport molecules. Thus, nisin A, plantaricin 423 and bacST4SA most likely interact with the cells and diffuse over the cell membranes by means of the paracellular pathway because of their hydrophobicity and small size. Nisin A is the least hydrophobic of the three peptides, with only 8 hydrophobic residues (16). It is also the smallest of the three peptides (3354 Da). This corresponds to the migration ability of nisin A, as a lower concentration diffused across the cell barriers, compared to the larger plantaricin 423 and bacST4SA peptides. Plantaricin 423 consists of 9 hydrophobic residues (17) and bacST4SA has 14 (18). A higher concentration of plantaricin 423 diffused across the barriers, compared to bacST4SA. This may be due to the much larger size of bacST4SA (4288 Da) compared to plantaricin 423 (3935 Da), but the exact mechanism associated with cell attachment and migration requires further research. It is also crucial to assess the migration ability of bacteriocins in the presence of the mucus layer. The mucus layer forms a protective barrier, preventing the diffusion of certain pathogens and molecules (19). Studies have shown that most probiotic bacteria attach to the mucus layer (20–23), but it is unknown whether bacteriocins produced by these probiotics, attach to the layer as well. This may prevent the diffusion of bacteriocins into the bloodstream, but further studies are required to determine this.

This is the first report where NHS-fluorescein labelled bacteriocins were used to determine the migration ability of bacteriocins across HUVEC and Caco-2 cell monolayers. This is a viable system that can be used in other drug transportation studies using different bacteriocins. Moreover, since NHS-fluorescein is not cytotoxic towards these human cell lines, it might also have no toxic effects on animal models, suggesting that this labelling system could potentially be used in *in vivo* experiments to track therapeutic peptides. These promising results should be following up by *in vivo* studies to determine potential tissue-specific responses to these

peptides. Advanced visualisation techniques such as microfluidic and confocal microscopy could shed more light on potential occurrence of cell internalisation of peptides, as well as membrane transport mechanisms involved.

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