ALINE DIAS PAIVA

MECANISMO DE AÇÃO E EFEITOS IMUNOESTIMULATÓRIOS DE BOVICINA HC5, UMA BACTERIOCINA PRODUZIDA POR *Streptococcus bovis* HC5

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2011

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T
P149m
2011

Paiva, Aline Dias, 1983-
Mecanismo de ação e efeitos imunoestimulatórios de bovicina HC5, uma bacteriocina produzida por *Streptococcus bovis* HC5 / Aline Dias Paiva. – Viçosa, MG, 2011.
xxix, 159f. : il. (algumas col.) ; 29cm.

Orientador: Hilário Cuquetto Mantovani.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bactérias anaeróbicas. 2. Bacteriocinas. 3. Agentes anti-infecciosos. 4. Imunologia molecular. I. Universidade Federal de Viçosa. II. Título.

CDD 22. ed. 579.355

ALINE DIAS PAIVA

Mecanismo de ação e efeitos imunoestimulatórios de bovicina HC5, uma bacteriocina produzida por *Streptococcus bovis* HC5

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

APROVADA: 28 de julho de 2011

Prof. Sérgio Oliveira de Paula
(Coorientador)

Profª. Maria Cristina Baracat Pereira
(Coorientadora)

Profª. Regina Maria N. Drummond

Profª. Maria Aparecida S. Moreira

Prof. Hilário Cuquetto Mantovani
(Orientador)

BIOGRAFIA

ALINE DIAS PAIVA, filha de Hélio de Assis Paiva e Odília Dias Paiva, nasceu em 08 de Junho de 1983, em Juiz de Fora - Minas Gerais.

Iniciou o curso de Nutrição, na Universidade Federal de Viçosa, em Março de 2001, pelo qual graduou-se Nutricionista em Julho de 2005.

Em Agosto de 2005 ingressou no curso de Mestrado em Microbiologia Agrícola, na Universidade Federal de Viçosa, defendendo a dissertação em Junho de 2007. Em Agosto deste mesmo ano, ingressou no curso de Doutorado em Microbiologia Agrícola, na Universidade Federal de Viçosa, defendendo a tese em Julho de 2011.

SUMMARY

LIST OF FIGURES

CHAPTER 1

CHAPTER 4

CHAPTER 5

LIST OF TABLES

LIST OF ABBREVIATIONS

NRP = nonribosomal-synthesized peptide

AMP = ribosomal-synthesized peptide

LPS = lipopolysaccharide

PG = phosphatidylglycerol

PS = phosphatidylserine

CL = cardiolipin

BLIS = bacteriocin-like inhibitory substance

VRE = vancomycin resistant enterococci

MRSA = methicillin-resistant *Staphylococcus aureus*

Ser = serine

Thr = threonines

Trp = tryptophan

Dha = dehydroalanine

Dhb = dehydrobutyrine

LAB = lactic acid bacteria

GRAS = generally recognized as safe

GlcNAc = *N*-acetylglucosamine acid

MurNAc = *N*-acetylmuramic acid

IL = interleukin

DOPC = 1,2-dioleoyl-*sn*-glycero-3-phosphocoline

DOPG = 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol

DMoPC = 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocoline

DLPC = 1,2-dilauroyl-*sn*-glycero-3-phosphocoline

CF = carboxyfluorescein

HPTS = 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt

TFA = trifluoroacetic acid

RP-HPLC = reverse-phase high-performance liquid chromatography

MIC = minimum inhibitory concentrations

Mg = magnesium

OD = optical density

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

$DiSC_2(5)$ = 3,3'-diethylthiadicarbocyanine iodide

LUVs = large unilamellar vesicles

MES = 2-(N-morpholino)ethanesulfonic acid

ITC = isothermal titration calorimetry

Ka = apparent binding constant

GUVs = giant unilamellar vesicles

NBD = nitrobenzoxadiazole

E/M = monomer and excimer signal ratio

TEMPO-PC = 1,2-dioleoyl-*sn*-glycero-3-TEMPO-phosphocoline

5DOX-PC = 1-palmitoyl-2-stearoyl(5-DOXYL)-*sn*-glycero-3-phosphocoline

12DOX-PC = 1-palmitoyl-2-stearoyl(12-DOXYL)-*sn*-glycero-3-phosphocoline

3-LII = water-soluble Lipid II

CD = circular dichroism

K_{SV} = Stern-Volmer quenching constant

Q_{ef} = quenching efficiency

A.U. = arbitrary units.

β-LG = β-lactoglobulin

FPLC = fast protein liquid chromatography

HE = hematoxylin and eosin

AB = Alcian Blue

PAS = periodic acid-Schiff

real-time PCR = real-time polymerase chain reaction

cDNA = complementary DNA

mRNA = messenger RNA

Ct = cycle threshold method

S = sense primer

AS = antisense primer

PBS = phosphate buffer saline

NC = negative control group

Bov = bovicin HC5 group

PC = positive control group.

OVA = ovalbumin

SD = standard deviation

L = lumen

EP = simple cuboidal epithelium

BB = brush border

V = villum

LP = lamina propria

LC = Lieberkühn crypt

E = edema

V = blood vessel

Sm = submucosa

MC = muscle layer

IC = inner circular muscle layer

OL = outer longitudinal muscle layer

MT = mucosal thickness

T_H1 = helper T lymphocytes subgroup 1

T_H2 = helper T lymphocytes subgroup 2

Treg = regulator T lymphocytes

IgA = immunoglobulin A

IgE = immunoglobulin A

CFU = colony-forming units

V = mice treated with viable *S. bovis* HC5 cells

HK = mice treated with heat-killed *S. bovis* HC5 cells.

PAMP = pathogen-associated molecular pattern

PRR = pattern-recognition receptor

TLR = toll-like receptor

GALT = gut associated lymphoid tissue

APC = antigen-presenting cell

STAT = signal transducer and activator of transcription

RESUMO

PAIVA, Aline Dias, D.Sc. Universidade Federal de Viçosa, julho de 2011. **Mecanismo de ação e efeitos imunoestimulatórios de bovicina HC5, uma bacteriocina produzida por *Streptococcus bovis* HC5.** Orientador: Hilário Cuquetto Mantovani. Coorientadores: Sérgio Oliveira de Paula e Maria Cristina Baracat Pereira.

Bovicina HC5 é um lantibiótico do tipo A produzido por *Streptococcus bovis* HC5, uma bactéria gram-positiva anaeróbia, isolada do rúmen de bovinos. Bovicina HC5 é um peptídeo de 2449 Da, anfifílico e positivamente carregado, com amplo espectro de atividade e que contém um padrão de anéis de lantionina semelhante ao encontrado em nisina, a bacteriocina mais conhecida. Resultados prévios indicaram que bovicina HC5 promove a liberação de potássio intracelular e a depleção de ATP intracelular nas células sensíveis, sendo que essa atividade é aumentada em condições de pH ácido. Entretanto, a atividade antimicrobiana de bovicina HC5 persiste mesmo em valores de pH alcalinos, o que sugere que bovicina HC5 possa ter outro mecanismo de ação juntamente com a depleção de potássio e ATP. Ainda que bovicina HC5 tenha sido caracterizada como uma bacteriocina efetiva e de amplo espectro antimicrobiano, a segurança para sua aplicação *in vivo* permanece limitada em função de poucas informações relacionadas ao mecanismo de ação aos efeitos imunoestimulatórios de bovicina HC5. Além disso, embora algumas estirpes de *S. bovis* sejam genética e fisiologicamente distintas de isolados patogênicos envolvidos em infecções em humanos, os efeitos da administração de *S. bovis* HC5 a modelos animais ainda não foram estudados. Neste trabalho, o mecanismo de ação de bovicina HC5 foi elucidado, tendo como foco o papel do Lipídeo II, sendo também avaliados os efeitos morfológicos e imunoestimulatórios de bovicina HC5 e *S. bovis* HC5 após administração oral a camundongos BALB/c. Utilizando membranas modelo artificiais de diferentes composições lipídicas e ensaios de permeabilidade foi possível determinar que o

Lipídeo II, um importante precursor da síntese da parede celular em bactérias, é utilizado como alvo específico de bovicina HC5 em células bacterianas sensíveis. A atividade de formação de poros de bovicina HC5 foi claramente dependente da espessura da membrana e pode ser detectada somente em membranas finas. Bovicina HC5 foi capaz de recrutar moléculas de Lipídeo II em estruturas semelhantes a poros, e de sequestrar moléculas de Lipídeo II em domínios, inibindo a síntese da parede celular bacteriana. Em seguida, a interação entre bovicina HC5 e Lipídeo II foi examinada utilizando espectroscopia de fluorescência do triptofano e dicroísmo circular. A presença de Lipídeo II alterou a orientação de bovicina HC5 em membranas lipídicas, de paralelo para perpendicular em relação à superfície da membrana, e a estrutura secundária de bovicina HC5 foi também significativamente alterada após interação com Lipídeo II. A interação de bovicina HC5 com Lipídeo II foi altamente estável, ocorrendo mesmo em pH 2,0. De acordo com esses resultados, bovicina HC5 possui mecanismo de ação semelhante ao da nisina, embora algumas diferenças em relação à capacidade de formação de poros e interação com Lipídeo II tenham sido demonstradas. Os efeitos da administração oral de bovicina HC5 e *S. bovis* HC5 (células viáveis e mortas após tratamento térmico) a sistemas modelos animais foram também avaliados. A administração oral de bovicina HC5 por 58 dias a camundongos BALB/c resultou em reduzido ganho de peso e alterações no intestino delgado, embora nenhuma mudança fisiológica tenha sido detectada no intestino. Um aumento da expressão relativa de TGF-β, INF-γ e IL-12 no intestino delgado foi observado após administração de bovicina HC5. Importantes alterações histológicas e morfométricas foram detectadas no intestino dos animais tratados com *S. bovis* HC5. A produção de citocinas no intestino também foi alterada após administração oral de *S. bovis* HC5, e o padrão de expressão de citocinas diferiu entre células vivas e mortas após tratamento térmico. Um aumento na expressão relativa de IL-12 e INF-γ foi observado em animais que receberam células viáveis de *S. bovis* HC5, enquanto um aumento na expressão relativa de IL-5, IL-13 e TNF-α foi detectado em animais tratados com células de *S. bovis* HC5 mortas após tratamento térmico. Esses resultados indicam que a administração oral de bovicina HC5 causou alterações morfológicas no intestino delgado de camundongos BALB/c, sendo capaz de estimular o sistema imune em nível local. A administração oral de *S. bovis* HC5 resultou em alterações histológicas e morfométricas no intestino, havendo diferenças distintas na atividade imunoestimulatória de células vivas e mortas de *S. bovis* HC5.

ABSTRACT

PAIVA, Aline Dias, D.Sc. Universidade Federal de Viçosa, July, 2011. **Mode of action and immunostimulatory effects of bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5**. Advisor: Hilário Cuquetto Mantovani. Co-Advisors: Sérgio Oliveira de Paula and Maria Cristina Baracat-Pereira.

Bovicin HC5 is a Type A lantibiotic produced by *Streptococcus bovis* HC5, an anaerobic gram-positive bacterium isolated from the bovine rumen. Bovicin HC5 is a positively charged amphiphilic peptide of 2449 Da, with a broad spectrum of activity, and contains a similar pattern of lanthionine rings found in nisin, the most well known bacteriocin. Previous results indicated that bovicin HC5 promotes the release of intracellular potassium and the depletion of intracellular ATP from target cells, and this activity is enhanced in acidic pH. However, the antimicrobial activity of bovicin HC5 persists even at more alkaline pH values, which suggests that bovicin HC5 could have another mechanism of action besides the depletion of potassium and ATP. Even though bovicin HC5 has been characterized as an effective and broad-spectrum bacteriocin, the safety for application *in vivo* is still limited by the lack of information regarding the mechanism of action and immunostimulatory effects of bovicin HC5. Moreover, although bovine *S. bovis* strains are genetically and physiologically distinct from pathogenic isolates implicated in human infections, the effects of the administration of *S. bovis* HC5 to animal models are yet to be studied. In this work, we elucidated the mechanism of action of bovicin HC5, focusing on the role of Lipid II, and evaluated the morphologic and immunostimulatory effects of bovicin HC5 and *S. bovis* HC5 after oral administration to BALB/c mice. Using artificial model membranes of different phospholipid compositions and permeability assays, we determined that Lipid II, the essential bacterial cell wall precursor, is the specific target of bovicin HC5 in sensitive bacterial cells. The pore-forming activity of bovicin HC5 was clearly dependent on the

membrane thickness, and could be detected only in thin membranes. Bovicin HC5 was able to recruit Lipid II molecules as a pore-like structure and sequestered Lipid II into domains, inhibiting the bacterial cell wall biosynthesis. The interaction between bovicin HC5 and Lipid II was further examined using tryptophan fluorescence and circular dichroism spectroscopy. The presence of Lipid II changed the orientation of bovicin HC5 in lipid membranes from parallel to perpendicular with respect to the membrane surface, and the secondary structure of bovicin HC5 was significantly changed upon binding to Lipid II. The interaction of bovicin HC5 with Lipid II was highly stable, occurring even at pH 2.0. According to these results, bovicin HC5 has a primary mode of action similar to nisin, although some differences regarding the pore formation and interaction with Lipid II were demonstrated. The effects of the oral administration of bovicin HC5 and *S. bovis* HC5 (viable and heat-killed cells) to an animal model system were also evaluated. The oral administration of bovicin HC5 for 58 days to BALB/c mice resulted in low weight gain and some impairment of small intestine, although no physiological changes have been detected in small intestine. An increase of TGF-β, INF-γ and IL-12 relative expression in the small intestine occurred upon administration of bovicin HC5. Regarding the effects caused by *S. bovis* HC5, important histological and morphometric alterations were observed in the intestine of the treated animals. The cytokine production was altered in the intestine after oral administration of *S. bovis* HC5 and differed between live and heat-killed cells. An increase in the relative expression of IL-12 and INF-γ was observed in animals that received viable *S. bovis* HC5 cells, while an increase in IL-5, IL-13 and TNF-α relative expression was detected in mice treated with heat-killed S. bovis HC5 cells. These results indicate that oral administration of bovicin HC5 caused morphological alterations in the small intestine of BALB/c mice, and was able to stimulate the host immune system, at local level. The oral administration of *S. bovis* HC5 resulted in histological and morphometric alterations in the intestine, and there was a distinguishable difference in the immunostimulatory activity of live and heat-killed *S. bovis* HC5 cells.

CHAPTER 1

Literature Review

1.1. Introduction

Small biological molecules (<10 kDa), including nonribosomal-synthesized peptides (NRPs) and ribosomal-synthesized peptides (AMPs), play an important role in the innate immune response against microbial infections, thus ensuring first-line defenses to many species, from plants to animals. AMPs are a diverse group of molecules and include plant thionins and defensins, insect defensins and cecropins, amphibian magainins and temporins, defensins and cathelicidins from higher vertebrates, as well as fungal defensin-like peptides, cyanobactins and bacteriocins (Hancock and Sahl, 2006). Most of the AMPs share common biophysical properties that appear to be important to antimicrobial activity, such as cationicity, amphipathicity and hydrophobicity (Yount *et al.*, 2006; Sang and Blecha, 2008).

The net positive charge is essential for the initial electrostatic attraction to the anionic components at cell envelopes and phospholipid membranes of fungi and bacteria. On fungal surfaces, the negative charge is often due to phosphomannans or related constituents (Salzman *et al.*, 2004), while the negatively charged components of bacterial cell surfaces include lipopolysaccharide (LPS) of gram-negative bacteria and the teichoic and teichuronic acids of gram-positive bacteria. Moreover, bacterial membranes are enriched in acid phospholipids, such as phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL), which confer a net negative charge to the membrane and account for more than 90 % of the membrane lipids in gram-positive bacteria. One additional parameter that may contribute to electrostatic attraction between cationic peptides and their bacterial targets is the increased (about 50 %

greater) membrane potential ($\Delta\psi$) across the bacterial membranes compared to most eukaryotic cells (Hancock and Rozek, 2002).

The amphipatic structure, with clusters of hydrophobic and hydrophilic residues within the tertiary structure of the peptide, correlates with antimicrobial efficacy and peptide toxicity, and most antimicrobial peptides are inherently amphipathic or become amphipathic in anisotropic environments (Yount *et al.*, 2006). The proportion of hydrophobic residues in antimicrobial peptides is around 50 %, a feature that is important for antimicrobial activity as it governs the extent to which the peptide may partition into lipid bilayers. Although hydrophobicity and amphipathicity are required for effective permeabilization of the bacterial membrane, optimal balance between these two characteristics is essential to peptide activity, since highly amphipathic or hydrophobic molecules also tend to disrupt mammalian cells (Zelezetsky *et al.*, 2005).

Antimicrobial peptides produced by micro-organisms have been a popular topic of research and many ribosomal- and nonribosomal-synthesized peptides have been reported over the years (Pavlova and Severinov, 2006; Sang and Blecha, 2008). The peptides ribosomally synthesized by species of Bacteria and certain strains of the Archaea domain are named bacteriocins. Bacteriocins are active even in small concentrations, exhibit bactericidal or bacteriostatic activity, and are active against both human and veterinary pathogens, being potentially useful for the food industry and for medical and veterinary applications (Cleveland *et al*., 2001; Drosinos *et al.*, 2006).

Bacteriocins differ in size, microbial target, mode of action, secretion and immunity mechanisms. Several uncharacterized substances produced by bacteria and with bacteriocin-like activity have also been identified and are referred to as bacteriocin-like inhibitory substances (BLIS) (Tagg and Ragland, 1991).

Bacteriocins serve to effectively inhibit competing micro-organisms in natural habitats, conferring ecological advantages in complex bacterial communities (Balakrishnan *et al.*, 2002). Alternatively, bacteriocins are used to influence the dynamics of bacterial populations, to inhibit the invasion of other strains or to cause the death of neighboring cells, ensuring the survival and perpetuation of the bacteriocin producing cells (Burkard *et al.*, 2007). Additional roles have been proposed for gram-positive bacteriocins, mediating quorum sensing (Gobbetti *et al*., 2007) and acting as communication signals in bacterial consortia, such as biofilms (Gillor, 2007).

The spectrum of activity of bacteriocins can be narrow and confined to closely related species, or it can be relatively broad, inhibiting the growth of a much wider range of micro-organisms, including food-borne pathogens and spoilage micro-

organisms, such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium tyrobutyricum* (Cotter *et al*., 2005; Gálvez *et al.*, 2008). In general, the main mechanisms of action for bacteriocins are pore formation in the target cell membrane, inhibition of cell wall synthesis and inhibition of enzyme activities (RNAse or DNAse) (Cleveland *et al*., 2001).

Bacteriocins produced by gram-positive bacteria differ from those synthesized by gram-negative bacteria in ecological and evolutionary aspects. In gram-positive bacteria, the biosynthesis of bacteriocins is self-regulated and it is not a lethal event. The spectrum of antimicrobial activity is broader, the release of the peptide is controlled by specific regulatory mechanisms and the clusters of genes are generally in the chromosome, encoding proteins with structural roles and proteins involved in post-translational modifications, regulation, immunity and transport through the membrane. In gram-negative bacteria, the production of bacteriocins is a lethal event that involves cell lysis and the release of the peptide is controlled by common regulatory mechanisms, such as the SOS regulon (Riley and Wertz, 2002).

1.2. Bacteriocins produced by gram-positive bacteria

Gram-positive bacteria produce bacteriocins that are as abundant as and even more diverse than those produced by gram-negative bacteria. It has been reported that bacteriocins from gram-positive bacteria only kill other gram-positive bacteria (with varied spectrum of activity) and have little inhibitory activity toward gram-negative bacteria, fungi or virus (Klaenhammer, 1993; Riley and Wertz, 2002).

However, some bacteriocins also inhibit certain gram-negative bacteria, including *Klebsiella pneumoniae*, *Pseudomonas* spp., and *Campylobacter jejuni* (Todorov and Dicks, 2006). These bacteriocins include bifidin I, produced by *Bifidobacterium infantis* BCRC 14602; thermophilin 81, produced by *Streptococcus thermophilus*; plantaricin 35 d, produced by *Lactobacillus plantarum*; lacticin NK24, produced by *Lactococcus lactis* NK24; and bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K (Ivanova *et al.*, 1998; Messi *et al.*, 2001; Todorov, 2009; Cheikhyoussef *et al.*, 2009).

Bacteriocins with potent activity against antibiotic-resistant bacterial strains, such as vancomycin resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) have also been reported (Sit and Vederas, 2008). Recently, bacteriocins produced by *L. plantarum* exhibited anti-fungal activity against

Absidia spp., *Aspergillus niger*, *Epicoccum nigrum* and *Penicillium* sp. (Todorov, 2010). Studies about the bioactivity of bacteriocins against virus are still scarce, but some of them have been recently characterized as potent antiviral peptides, such as bacteriocin ST5Ha, a pediocin-like bacteriocin produced by *Enterococcus faecium*, that is active against herpes simplex virus type 1 strain F, an important human pathogen (Todorov *et al.*, 2010).

Some species of bacteriocin-producing gram-positive bacteria are pathogenic, including *Listeria monocytogenes*, *Enterococcus* sp. and *Clostridium perfringens* (Rood and Cole, 1991). For these bacteria, bacteriocin loci are found in association with IS sequences or near cell wall-associated serine protease genes. According to Dupuy *et al.* (2006), it is possible that some common molecular mechanism is involved in the regulation of synthesis of toxins and bacteriocins, such as sigma factors in *Clostridium* (sigma factors are sequence-specific, DNA-binding subunits of RNA polymerase, that ensure the recognition of appropriate promoter sites (Helmann and Moran, 2002)).

The genes coding for bacteriocin production are mostly organized in operon clusters, which can be localized in the chromosome or in mobile elements (e.g. plasmids and transposable elements). Gene expression is often coordinated, and the operons harbor genes for biosynthesis, regulation, self-immunity, and secretion (Klaenhammer, 1993; Rossi *et al*., 2008).

Like other ribosomally derived antibiotic scaffolds, the bacteriocins produced by gram-positive bacteria are initially synthesized as biologically inactive precursor peptides; the N-terminal leader sequences are cleaved by proteases to yield the active peptide. The leader peptide is an essential recognition element for the post-translational tailoring enzymes and is also necessary for immunity and export signaling (Nolan and Walsh, 2009). Additionally, gram-positive bacteria have protective mechanisms against self-produced bacteriocins. Immunity is achieved by specific immunity proteins and/or by regulatory proteins that control bacteriocin synthesis or transport (Willey and van der Donk, 2007; Draper *et al*., 2008).

Some bacteriocins can remain adsorbed to the cell-surface of the producer cells, avoiding being released to the extracellular medium, a phenomenon that has been reported for mutacin 1140, pediocin AcH, nisin, sakacin A, leuconocin Lcm and lacticin 3147 (Todorov, 2010). In the case of plantaricin C19, produced by *L. plantarum* C19, maximal adsorption to the producer cells occurred between pH 5.0 and 7.0, with a complete loss of adsorption at pH 1.5 and 2.0 (Atrih *et al.*, 2001).

Usually, the production of bacteriocins by gram-positive bacteria shows secondary metabolite kinetics and its activity is detected at the end of exponential phase and early stationary phase of growth (Todorov, 2010). Normally, the bacteriocin title decreases at the stationary phase and continues to decrease with prolonged incubation, probably due to proteolytic degradation, protein aggregation, adsorption to the cell-surface or feedback regulation (Ondaa *et al.*, 2003; Todorov, 2010). For some strains, bacteriocin production appears to be temperature dependent, and according to Dufour *et al*. (2007), the regulation of bacteriocin expression is not cell cycle dependent, per se, but is influenced by the density of the cell culture.

Some bacteriocins are stable in the range of pH 2.0 to 8.0, suggesting that activity might not be affected by changes in pH during growth. However, constant changes in medium pH and composition may lead to changes in activity levels of bacteriocins. Not all bacteriocins are heat-stable, but thermostability at 100 ºC has been reported for some peptides and may be explained by their low molecular mass or by differences in peptide structure (Todorov, 2010).

Many factors influence the efficacy of bacteriocins towards target cell. Such factors include the 1) structure and concentration of bacteriocins; 2) the physiological status and cell wall structure of the target cells; 3) the composition and membrane potential of the target cell; 4) the presence of specific proteases at or nearby the target cells envelope; 5) the chemical composition of the environment; 6) the amount of target cells and/or planktonic cells able to adsorb the bacteriocin and 7) the absence or the presence of mutations in some cellular components, as the specific receptors for bacteriocins (Eijsink *et al.*, 2002).

Based on their primary structure, molecular mass, heat stability and functional similarity, bacteriocins produced by gram-positive bacteria can be classified in four major groups (Cotter *et al*., 2005):

- Class I, or lanthionine-containing bacteriocins, or lantibiotics: includes small peptides (< 5 kDa; 18–39 residues), post-translationally modified to their bioactive forms by multi-enzyme complexes. First of all, serines (Ser) and threonines (Thr) present in precursor peptides are dehydrated by LanB dehydratases, to give dehydroalanines (Dha) and dehydrobutyrines (Dhb), respectively; LanC cyclases then catalyze the subsequent intramolecular Michael addition of cysteine residues onto these dehydro amino acids, resulting on lanthionine type thioether crosslinks (intramolecular covalent bridges), from

which lantibiotics derive their name (Pag and Sahl, 2002). In some cases, one enzyme (bi-functional LanM modifying enzymes) carries out both dehydration and cyclization steps (Xie *et al.*, 2004). The thioether linkages are stable to hydrolysis and are also critical for lantibiotic physiological function (van Kraaij *et al.*, 2000).

- Class II, or non-lanthionine-containing bacteriocins, or non-lantibiotics: includes small (< 10 kDa) membrane-active peptides, with limited or no extensive post-translational modifications. Non-lantibiotics are still divided into three subgroups: Class IIa is composed by pediocin-like anti-*Listeria* peptides, with their conserved disulfide bond and N-terminus characterized by a consensus YGNGVXC motif (this consensus region may cause the bactericidal effect directly, and is considered to be involved in the action against *Listeria* strains; however, many other bacteriocins that lack this structural homology with class IIa peptides are still active against *Listeria monocytogenes*); Class IIb bacteriocins require a combination of two polypeptides for full antimicrobial activity (e.g. enterocin L50), while class IIc are other bacteriocins (e.g. acidocin B); Class IId has been proposed by Gray *et al.* (2006a) and consists of bacteriocins that are sec-dependent, such as thuricin 17 (Gray *et al.*, 2006b) and bacthuricin F4 (Kamoun *et al.*, 2005).

- Class III, or bacteriolysins: comprises large, heat-labile proteins (> 30 kDa) that catalyze the hydrolysis of bacterial cell walls resulting in autolysis of targeted bacteria (e.g. helveticin J and lactacin B) (Drider *et al.*, 2006; Dobson *et al*, 2007).

- Class IV: an additional proposed class, which comprises complex bacteriocins that requires lipid or carbohydrate moieties for activity. Little is known about the structure and function of this class (e.g. leuconocin S and lactocin 27) (Vermeiren *et al*., 2006).

Additionally, two other schemes of lantibiotic classification exist. According to the earliest scheme proposed by Jung (1991), the lantibiotics can be classified in type A and type B. The type A lantibiotics includes elongated, amphipathic, flexible and pore-forming lantibiotics (e.g. nisin, bovicin HC5, subtilin, epidermin, gallidermin, Pep5),

while type B lantibiotics includes the rigid and globular peptides, that act by inhibition of enzymes (e.g. mersacidin and actagardine, duramycin and its analogues, cinnamycin and ancovenin).

As already mentioned, the gene clusters possess either two (LanB, LanC) or one (LanM) modification enzyme, and this difference on biosynthetic pathway was used to propose a new classification scheme: class I lantibiotics, which are modified by LanB and LanC (nisin and epidermin); class II lantibiotics, bacteriocins with a GG cleavage site in their leader peptide and that are modified by LanM enzymes (lacticin and its analogous, several two-peptide lantibiotics); class III lantibiotics, peptides that have no or little antibacterial action and perform other functions, often related to morphogenesis (SapT, SapB and their orthologues) (Willey and van der Donk, 2007).

Recently, Goto *et al.* (2010) reported the discovery of a new family of lanthionine synthetases, termed LanL, in the mycelial soil bacterium *Streptomyces venezuelae*. Moreover, they have shown that putative lantibiotic biosynthetic gene clusters are widespread in nature and not restricted to gram-positive bacteria, as long believed, being found in gram-negative bacteria, such as the proteobacterium *Myxococcus xanthus*, and in cyanobacteria, such as *Nostoc punctiforme* and *Prochlorococcus*.

Nevertheless, many lantibiotic-like gene clusters direct the production of peptides that do not have antibiotic activity, but that may have other, often unknown, functions as signaling molecules or morphogenetic peptides (Lia *et al.*, 2010). Goto and co-workers (2010) suggested the name lanthipeptides for lanthionine-containing peptides that are related to lantibiotics by structure and biosynthetic routes, but that do not have any antimicrobial activity.

Among the bacteriocins produced by gram-positive bacteria, the most promising for practical applications are those produced by lactic acid bacteria (LAB). Several genera of LAB have been described as bacteriocin producing bacteria, including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Bifidobacterium*. Strains showing antimicrobial activity against different target bacteria have been isolated from different food matrices such as fermented dairy products, vegetables, fruits, meat and fish and also from the human and animal gastrointestinal tract (Todorov *et al.*, 2010).

The most well known bacteriocin is nisin, a heat-stable antibacterial peptide produced by *Lactococcus lactis* subsp. *lactis*, and belonging to the Class I bacteriocin. It

is a small (3.5 kDa), 34 amino acid, cationic, hydrophobic peptide with five characteristic (beta-methyl) lanthionine rings. It affects primarily vegetative cells and prevents the outgrowth of spores of gram-positive bacteria. Until now, five natural nisin variants (A, Z, Q, U, and F) have been identified (de Kwaadsteniet *et al.*, 2008) and nisin provides a paradigm for studies of lantibiotic structure, biosynthesis and mode of action of antimicrobial peptides (Nolan and Walsh, 2009).

Micro-organisms susceptible to nisin include strains of lactic acid bacteria, *Bacillus*, *Clostridium*, *Staphylococcus*, *Listeria* and *Streptococcus* genera (Cleveland *et al.*, 2001), *Actinomyces*, *Corynebacterium*, *Gardnerella*, *Mycobacterium*, *Campylobacter*, *Haemophilus*, *Helicobacter*, and *Neisseria*. In order to increase the antimicrobial activity against gram-negative bacteria, yeasts, or moulds, nisin is often used in combination with other synergistic preservation methods, including pH reduction and addition of salt in high concentration (Rayman *et al.*, 1983), preheating of the product (Boziaris *et al.*, 1998), addition of chelating agents (e.g. EDTA) (Branen and Davidson, 2004) and detergents (e.g. Tween 80) (Joerger, 2003).

In 1969, nisin received international acceptance as food additive by the Codex Alimentarius Commision (JECFA, 1969). In 1988, the US Food and Drug Administration conferred GRAS (Generally Recognized As Safe) status to nisin for use in certain food applications (FDA, 1988). The Science Committee on Food evaluated the safety of nisin in 1990 and allocated its safety intake in 0.13 mg/kg body weight/day (SCF, 1992). In 1995, nisin (code E234) was authorized for food preservation in the European Union by Directive 95/2/EC on food additives other than colours and sweeteners. In 2001, FDA affirmed nisin as GRAS for use as an anti-microbial agent on cooked meat and poultry products when used at a maximum level of 250 ppm of nisin in the finished products (FDA, 2001).

So far, nisin is the only bacteriocin approved for use in over 50 countries as food preservative, including USA, European Union, Australia and New Zealand (Delves-Broughton, 2005). Nisin has been used in dairy products, canned foods (vegetables, soups), hot baked products (crumpets) and pasteurized liquid eggs (Delves-Broughton *et al.*, 1996). The approval of nisin for food usage is a logical consequence of the fact that nisin is produced by *L. lactis*, a food grade lactic acid bacterium that has a long history of use as a component of starter cultures in dairy industry.

Nisin is readily inactivated by trypsin, pancreatin and alpha-chymotrypsin (Jarvis and Mahoney, 1969), it is not detected in human saliva 10 min after the consumption of 0.005 mg nisin/kg (Claypool *et al.*, 1966) and the intestinal microbiota

of human flora-associated rats is not affected after consumption of nisin (Bernbom *et al.*, 2006), suggesting that ingested nisin is completely digested to amino acids prior to absorption. According to Reddy *et al.* (2004), nisin does not possess any subchronic or chronic toxicity, reproductive/developmental toxicity, genotoxicity and carcinogenicity, and can be safely used.

Nisin is not the only bacteriocin produced by *L. lactis*. Other lantibiotics produced by the same genera include the single peptide lacticin 481 and the two-component system lacticin 3147 (de Vuyst and Leroy, 2007). Non-lantibiotic bacteriocins from *L. lactis* include: pediocin-like bacteriocins (class IIa) such as lactococcin MMFII; two-peptide component bacteriocins (class IIb) such as lactococcin G and M; thiol-activated bacteriocins (class IIc) such as lactococcin B; and heat-labile, lactococcus-specific bacteriocins (class IId) such as lactococcin A (diplococcin) and lactococcin 972 (Oppegard *et al.*, 2007).

1.3. Mode of action of bacteriocins produced by gram-positive bacteria

In general, type A lantibiotics are active in the nanomolar concentration range and kill bacteria by permeabilizing the plasma membrane, leading to the collapse of ion gradients across the cell membrane. Efficient permeabilization is the result of pore formation, using a specific receptor. The pore formation process of type A lantibiotics has been studied in detail using various physiological and artificial membrane system.

The bacteriocin nisin has at least five different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions. These multiple activities result in the high potential of nisin towards sensitive cells. In model membranes systems, micromolar concentrations of nisin are usually required to cause perturbation effects. In contrast, many gram-positive bacteria have MIC values in the nanomolar concentration range (Pag and Sahl, 2002).

An explanation for this discrepancy was provided when Lipid II, a hydrophobic carrier for peptidoglycan monomers, was identified as a high-affinity receptor that enabled pore formation at nanomolar concentrations of nisin (Brötz *et al.* 1998; Breukink *et al.*, 1999). The binding to Lipid II promotes two bactericidal activities, pore formation and inhibition of peptidoglycan biosynthesis (Breukink *et al.*, 1999).

The bacterial cell wall is a unique structure and consists mainly of a peptidoglycan polymer network located outside the cytosolic membrane, providing strength and shape to bacteria. The units of peptidoglycan layer consist of the

disaccharide *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) to which a pentapeptide is attached. This unit is assembled on the cytosolic side of the membrane on a very long bactoprenol hydrocarbon chain via a pyrophosphate linker. The enzymes MraY and MurG catalyze the final assembly steps, resulting in the formation of Lipid II.

Lipid II is a minor component of the bacterial cytoplasmic membrane. Estimates for gram-positive bacteria typically give values below 1 mol % of the membrane phospholipids (Kramer *et al.*, 2004). However, Lipid II plays an essential role in bacterial cell wall synthesis, carring the sugar-peptide subunits from the cytosolic side of the membrane – the site of Lipid II synthesis – to the peptidoglycan synthesis machinery outside the cell, where the penicillin-binding proteins catalyze the insertion of peptidoglycan uniting into a growing cell wall. The lipid anchor carrying the pyrophosphate is shuttled back to the cytosolic side of the membrane, where it can be reused (after dephosphorylation to the mono-phosphate form) for the next round of synthesis (Figure 1). Lipid II is also the target for antibiotics, such as ramoplanin and vancomycin, and other bacteriocins, such as mutacin, pediocin, subtilin, galidermin, epidermin and mersacidin.

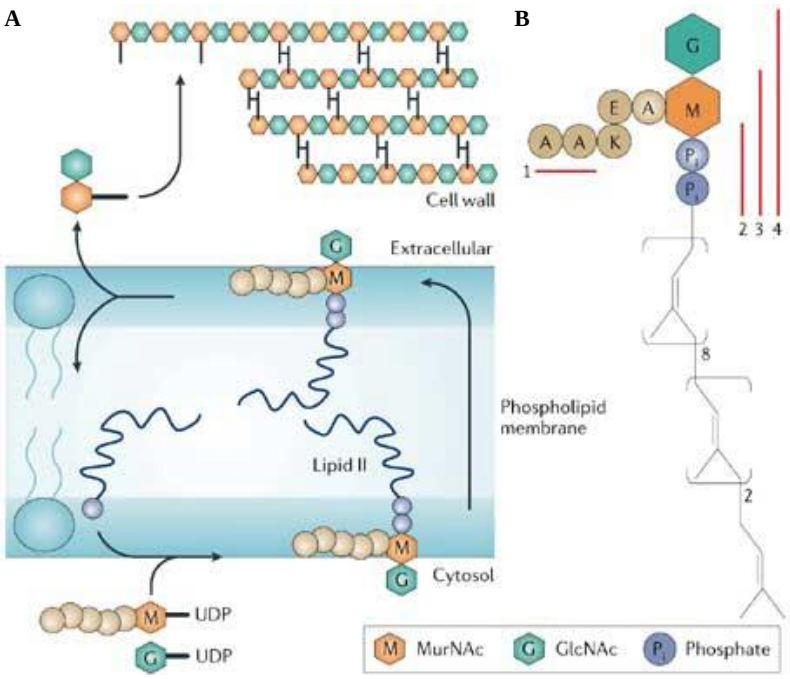


Figure 1: Cell wall assembly. (A) The cell wall biosynthesis starts on the cytosolic side of the bacterial plasma membrane. UDP-activated precursor sugars are assembled on a polyisoprenoid carrier and the coupling of which molecule produces Lipid II; Lipid II is transported across the membrane and peptidoglycan subunits are transferred to the growing peptidoglycan chain; the polyisoprenoid carrier is recycled back to the cytoplasmic side and the cycle is completed. (B) Lipid II structure, showing the polyisoprenoid anchor (eight *cis*-conformation isoprene units, two units in the *trans*-conformation and the terminal isoprene unit) and the pentapeptide. Red bars indicate the minimal binding sites in Lipid II of glycopeptide antibiotics (1), nisin (2), ramoplanin (3) and mersacidin (4). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid (Breukink and de Kruijff, 2006).

Lipid II moderates insertion of nisin into the bacterial membrane, which is followed by pore formation (van Heusden *et al.*, 2002). Structure-function studies identified that the interaction between nisin and Lipid II starts specifically with the high affinity binding of the N-terminal double-ring system of nisin to the hydrophilic headgroup (pyrophosphate) of the cell wall precursor, via direct hydrogen bonding with the polypeptide backbone. The N-terminus of nisin interacts with Lipid II and its C-terminus inserts into the membrane (Hsu *et al.*, 2002). The interaction between initially formed nisin-Lipid II complexes in the membrane results in the formation of complexes that consist of several nisin and Lipid II molecules, which assemble further into larger

complexes; the conversion of the large complexes into a pore requires the cooperative insertion of the nisin molecules into the lipid bilayer. The resulting nisin-Lipid II pore was supposed to contain eight nisin and four Lipid II molecules (Hasper *et al.*, 2004) (Figure 2).

The solution structure of the Lipid II-nisin complex was found to form a binding cage for the pyrophosphate linkage group of Lipid II; obviously, undecaprenylpyrophosphate, when released after transglycosylation, can also be bound (Hsu *et al.*, 2004).

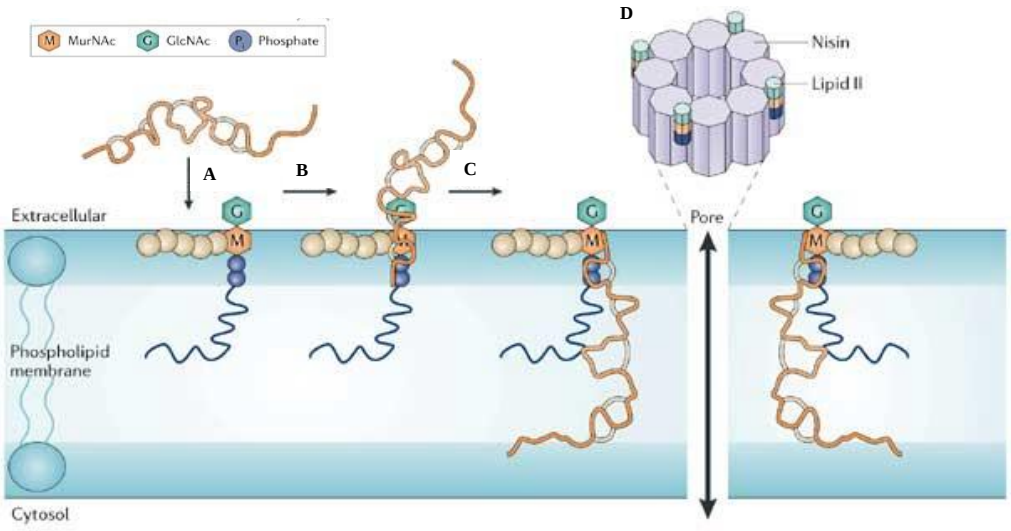


Figure 2: Nisin-Lipid II interaction. (A) Nisin reaches the bacterial membrane; (B) Nisin's N-terminal binds to the hydrophilic headgroup of Lipid II, with high affinity; (C) The pore formation starts, and nisin adopts a transmembrane orientation; (D) During or after assembly of four 1:1 (nisin:Lipid II) complexes, four additional nisin molecules are recruited and the final pore complex is formed (Breukink and de Kruijff, 2006).

Non-pore forming variants of nisin and other lantibiotics, such as mutacin 1140, bind with high affinity to Lipid II, but they kill bacteria without permeabilizing the membrane. This activity demonstrates that the lantibiotics have another Lipid II-mediated mechanism of cell killing. This mechanism was uncovered via fluorescence microscopy techniques, which revealed that lantibiotics that bind to Lipid II cluster the target molecules into patches when added to phospholipid bilayers, containing small amounts of Lipid II (Hasper *et al.*, 2006; de Kruijff *et al.*, 2008).

Since Lipid II is predominantly located into regions of the plasma membrane related to cell elongation and division, this mechanism of Lipid II abduction out of its

functional location leads to the inhibition of the bacterial cell wall synthesis, blocking the incorporation of murein precursor units into the cell wall (Hasper *et al.*, 2006). The Lipid II binding and sequestration can be considered the primary mode of action for type A lantibiotics (Hasper *et al.*, 2006;).

Furthermore, Lipid II-independent mechanisms of action have been described. Nisin can impair the integrity of microbial membranes at micromolar concentrations (Breukink *et al.*, 1999) or displace cationic autolytic enzymes from their anionic binding sites in the gram-positive cell wall, resulting in premature lysis of nascent cell-wall septa (Bierbaum and Sahl, 1985). Nisin can also promote the release of enzymes, such as *N*-acetylmuramoyl-*L*-alanin amidase and *N*-acetyilglucosaminidase (Héchard and Sahl, 2002). Nisin also inhibits the outgrowth of bacterial spores without specific molecular target: in the presence of nisin, the spores lose their heat resistance and become hydrated, but nisin does not inhibit germination initiation, acting immediately after the initiation of germinating process by preventing the establishment of oxidative metabolism or membrane potential in germinating spores (Gut *et al.*, 2008).

Type B lantibiotics can increase membrane permeability on target cells, cause protein translocation and ATP-dependent calcium uptake (as described to cinnamycin (Chen and Tai, 1987)), inhibit peptidoglycan synthesis at the transglycosylation level by forming a complex with the membrane-bound lipid II (mersacidin and actagardin (Brötz *et al.*, 1998)), and also inhibit the bacterial phospholipase A2 (duramycin-C (Héchard and Sahl, 2002)). Lacticin 3147, a two-component type B lantibiotic, requires the presence of both components to exert its maximal antimicrobial activity: one of the peptides, that resembles type B lantibiotic mersacidin, acts by inhibiting cell wall synthesis, while the other peptide, more similar to the type A lantibiotic, is responsible for pore formation via interaction with Lipid II molecule (Wiedemann *et al.*, 2001; Wiedemann *et al.*, 2006).

Recently, Yoneyama *et al.* (2009a,b) described a new mechanism of action for lacticin Q, a pore-forming type B lantibiotic that apparently does not require Lipid II or another docking molecule to exert its antimicrobial activity in the nanomolar range. According to the authors, lacticin Q quickly binds to the outer membrane leaflets (a process not restricted, but accelerated by the presence of negatively charged phospholipids (Yoneyama *et al.* 2010)), forming an amphiphilic α-helical structure; lacticin Q forms pores (average diameter greater than 4.6 nm) accompanied by lipid flip-flop and consequently protein leakage; some bacteriocin molecules migrate from the outer to the inner membrane leaflets when the pore closes; this antimicrobial

mechanism was called “huge toroidal pore” and the mode of action of lacticin Q shows greater similarity to the mode of action of self-defense antimicrobial peptides from multicellular eukaryotes (e.g. magainin 2), than to those of typical lantibiotics. Despite this mode of action, lacticin Q still exhibits selective toxicity, but the mechanisms used by this bacteriocin to recognize sensitive cells remain unclear.

Among the class II bacteriocins, the bacteriocins produced by *Pediococcus acidilactici*, such as pediocin PA-1/AcH, are the most studied. The subclass IIa bacteriocins dissipate the proton-motive force, preventing the occurrence of energy synthesis reactions (Mcauliffe *et al.*, 2001). Alternatively, these bacteriocins act through the formation of small pores in the membrane, with subsequent intracellular material efflux (Drider *et al.*, 2006; Oppegard *et al.*, 2007). Some bacteriocins belonging to subclass IIa are also active against viruses, and although the mode of action is not completely known, possible explanations could be the aggregation of virus particles, blockage of receptor sites on the host cell, or inhibition of key reactions in the multiplication cycle (Wachsman *et al.*, 2003).

Sub-class IIb bacteriocins require the combined activity of both partners to form hydrophilic pores that dissipate the membrane potential, by increasing the permeabilization of target cells or by specific cationic/anionic pore formation (Héchard and Sahl, 2002). Some two-peptide bacteriocins can also interfere with cellular ATP production (Willey and van der Donk, 2007). Sub-class IIc bacteriocins, with no structural similarity among the group members, have different modes of action, including membrane permeabilization, specific inhibition of septum formation and pheromone activity (Héchard *et al*., 2001).

It is still unclear if class II bacteriocins require a target molecule on the senstive cells. In this respect, it has been recognized that antibacterial peptides, which do not have a specific receptor, are equally active in their D and L forms, and some Class II bacteriocins synthesized as D-enantiomers have been shown to be inactive, indicating a stereospecific interaction with a target molecule (Nes and Holo, 2000). In addition, with nisin as an example, it was proven that some lantibiotics require a specific target to be active in the nanomolar range, and activity in nanomolar range has been demonstrated by Class II bacteriocins. These findings suggest Class II bacteriocins needs a receptor-like molecule on sensitive cells in order to exert maximum antimicrobial activity, and the components of the sugar phosphotransferases systems, such as the MptD, a membrane subunit of the mannose permease EII_t^{Man}, have been considered (Héchard *et al*., 2001).

Class III bacteriocins catalyze the hydrolysis of bacterial cell walls, promoving the autolysis of sensitive bacteria (Dobson *et al*, 2007) and the mode of action of Class IV bacteriocins remains unclear (Vermeiren *et al*., 2006).

1.4. Bacteriocin application and safety

The widespread use of antibiotics for both animal and human applications over the last 50 years has been related with an increase in antibiotic resistance among commensal and pathogenic micro-organisms. The emergence of multi-drug resistant bacteria can lead to treatment failure and increased mortality, and several bacterial strains have already developed resistance against most currently available antibiotics (Sang and Blecha, 2008). This process led the World Health Organization to announce antimicrobial drug resistance as a main public health concern and a global crisis (WHO, 1995).

Antibiotics have been extensively used in animal research, as therapeutic agents or growth promoters, and their efficacy and cost-effectiveness contribute to their popularity. Nevertheless, the intensive use of antibiotics in animal therapy and prophylaxis can lead to residue problems in animal products and in the environment (Molina *et al*., 2003). As more countries develop antibiotic-limiting policies, the need for alternative antimicrobials will probably increase the studies for novel products and improve the biochemical and genetic characterization of existing ones (Diez-Gonzalez, 2004).

In order to prevent outbreaks of infectious diseases and to reduce the selective pressure of antibiotics, many researchers have proposed the use of antimicrobial peptides as potential alternatives to antibiotics based on their broad-spectrum activity, mechanisms of action and little evidence of resistance, which indicates a promising future for industrial and agricultural applications of these peptides (Callaway *et al.*, 2004; Gordon *et al.*, 2005).

Although bacteriocins are traditionally associated with food applications, they also offer potential for other applications. Bacteriocins are stable to heat and low pH values, can be easily produced and show little activity against eukaryotic cells. Based on these features, bacteriocins have been suggested as the most promising class of antimicrobial peptides to replace or increase the efficacy of antibiotic in the field of animal and human medicine or for the design and production of new antimicrobials (Sahl and Bierbaum, 2008).

The combination of common therapeutic antibiotics with novel antimicrobial agents, known as combination therapy, is generally used to increase the activity of both antimicrobials, to reduce the increasing selection pressure by antibiotic and to broaden the antimicrobial spectrum (Cirioni *et al.*, 2006). Synergistic effects have been observed *in vitro* when some antimicrobial peptides are combined with clinically used antibiotics and tested against several clinically isolated bacterial strains (Giacometti *et al.*, 2005).

The presence of those synergistic effects make the antimicrobial peptides, specially bacteriocins or other peptides produced by bacteria, potentially valuable as an adjuvant for antimicrobial chemotherapy against antibiotic-resistant bacterial strains, reducing the antibiotic level required for the treatment of infectious diseases in human and veterinary medicine (Giacometti *et al.*, 2005; Todorov, 2010).

Particularly on animal trials, bacteriocin and bacteriocin-producing bacteria may be useful to improve animal nutrition and health through the manipulation of ruminal fermentation, the control of animal infections and the inhibition of enteric pathogens (Patra, 2011).

The specificity of bacteriocins can be particularly advantageous when single bacterial strain or specific groups of organisms are targeted (Russell and Mantovani, 2002). Bacteriocins that inhibit methanogenesis have the potential to improve feed efficiency in animal husbandry by reducing the amount of energy lost in the form of methane (Lee *et al.*, 2002). In addition, the reduction of amino acid degradation in the rumen can increase the amount of dietary protein that reaches the omasum and small intestine of ruminant animals (Rychlik and Russell, 2002).

In vitro experiments indicated that nisin has similar effects on ruminal fermentation when compared with monensin, the most commonly used ionophore fed to cows (Callaway *et al*., 1997). Nisin was also used in the treatment of bovine mastitis and it caused significant increases in cure rates of infections (Sears *et al.*, 1992; Wu *et al.*, 2007). In a similar way, the prevalence of mastitis-causing bacteria was reduced in animals treated with lacticin 3147 (Ross *et al.*, 1999; Twomey *et al.*, 2000; Klostermann *et al.*, 2008).

Independent on the effectiveness of bacteriocins demonstrated in some studies, the application of bacteriocins in industrial scale requires a comprehension of the role of the structural, biochemical and physical factors that influence the activity of bacteriocins, a detailed investigation of mechanisms of action, stability and the development of resistance (Hoskin and Ramamoorthy, 2008; Keymanesh *et al.*, 2009).

Additionally, in order to assess the safety use of bacteriocins it is important to evaluate the fate of the molecule after ingestion, and also the *in vivo* potential effects of the bacteriocins, which include cytotoxicity and immunogenicity (Penninks and Knippels, 2001; Gunn, 2008). Besides these requirements, the introduction of new proteins and/or biological varieties for industrial application demands the evaluation of the possible allergenic effects upon the administration of the desired compound (Knippels *et al.*, 1999a).

The allergic reaction is characterized by a change in the balance of cytokines produced by T lymphocytes of type T_H1 and T_H2 (T_H1/T_H2 balance), with increase of cytokines produced by T_H2 lymphocytes. Additionally, T_H2 lymphocytes, producers of IL-4, IL-5, IL-9 or IL-13, may play key role in the development and maintenance of the allergic response (Robinson and Kay, 1996). Besides the release of mediators as histamine, prostaglandins, leukotrienes and cytokines, depending on the severity of the reactions, the allergic response can cause local effects, such as the effects on the gastrointestinal tract (including a secretory response of the epithelium, increased permeability to macromolecules, changes in ion transport and intestinal motility), or systemic effects, on respiratory and cardiovascular systems (e.g. upper airway edema, severe asthma and circulatory collapse) (Majamaa and Isolauri, 1996; Crowe and Perdue, 1992; Monteleone and Sherman, 1997).

Almost no data is available about the allergenic or immunostimulation effects triggered upon the administration of bacteriocins. However, some studies have reported the effects related to the administration of LAB, including several bacteriocin-producing cells. Lactic acid bacteria belonging to different species can modulate the T_H1/T_H2 balance by reducing the release of T_H2 cytokines, especially IL-4 and IL-5 (in a dose dependent manner), and increasing the production of T_H1 cytokines, especially IFN-γ. Several LAB are also able to increase the secretion of IL-12, an important cytokine for activation of T_H1 lymphocytes and involved in controlling the development of allergic reactions. The mechanism by which certain LAB can affect the production of cytokines by T_H2 lymphocytes are not fully elucidated (Pochard *et al*., 2002).

As the local or systemic immune-mediated effects are difficult to be investigated in humans and, although there is no universal procedure for the evaluation of allergenicity *in vitro* or *in vivo, in vivo* antigenicity assays can be performed with animal models (guinea pigs, mice, rats, and occasionally dogs), by means of the parenteral or enteral via (Knippels *et al.*, 1999b).

According to Taylor and Leher (1996), an animal model should fill some important criteria: the sensitization and the challenge with the test protein should be performed preferably by oral via, since natural barriers such as gastrointestinal acid denaturation, enzymatic digestion and the epithelial/mucosal layer, may prevent, reduce or influence the allergenicity of proteins; as in real situations, the presence of adjuvant may contribute to the sensitization process, but the use of adjuvants should be avoided, in order to evaluate the inherent allergenicity of the protein; the animal model should produce significant amounts of specific T_H2 antibodies; animals must be able to tolerate most of the proteins and the clinical reactions triggered after challenge with the allergen should be similar to those observed in humans; at least two generations of animals should remain without contact with the allergen in the study, avoiding prior exposure to the allergen and the induction of tolerance; animals should be relatively easy to manipulate, enabling the reproducibility of the experiments.

1.5. Bovicin HC5

Bovicin HC5 is a lantibiotic produced by *Streptococcus bovis* HC5, an anaerobic gram-positive bacterium isolated from the bovine rumen (Mantovani *et al.*, 2002), and belonging to the group D of Lancefield (Whitehead e Cotta, 2000). *Streptococcus bovis* is an important ruminal LAB that is predominant when cattle are fed starch-based diets and is largely responsible for rumen acidosis (Hungate, 1966; Morovsky *et al.*, 1998).

The majority of the bacteriocin produced by *S. bovis* HC5 remains adsorbed to the cells during bacterial growth and the antimicrobial activity of bovicin HC5 is not detected in cell-free supernatant until the culture reaches the stationary phase. Bovicin HC5 is released from the producer cells by acidic solution of sodium chloride (100 mM, pH 2.0), without significant lysis of the cells. It is resistant to low pH, heat, proteinase K and chymotrypsin, which are useful characteristics to the industrial use of bovicin HC5 (Mantovani *et al.*, 2002).

Bovicin HC5 is a positively charged amphiphilic peptide of 2449 Da and contains posttranslational modified amino acids residues (Mantovani *et al.*, 2002), and the same pattern of lanthionine rings found in nisin and also in streptin, a lantibiotic produced by *Streptococcus pyogenes* (Wescombe and Tagg, 2003). Bovicin HC5 shares other similarities with nisin, such as the putative Lipid II-binding motif, but its mode of action is still unclear.

Previous results indicated that bovicin HC5 promotes the release of intracellular potassium from target cells, by forming pores on sensitive cell membranes (Mantovani *et al.*, 2002; Houlihan *et al.*, 2004). Besides this mechanism, bovicin HC5 is also able to promote the depletion in intracellular ATP, via F_1F_0ATPase, although a significant efflux of this molecule is not observed in bovicin-treated cells (Mantovani and Russell, 2008).

The activity of bovicin HC5 is higher in acidic pH (Houlihan *et al.*, 2004), and the decrease in intracellular potassium only happens if the extracellular pH is equal to or lower than 6.0 (Houlihan and Russell, 2006). However, the antimicrobial activity of bovicin HC5 persists even at more alkaline pH values, and the loss of viability is observed in bovicin-treated cells. This observation suggests that bovicin HC5 might have another mechanism of action besides the depletion of potassium and ATP.

Bovicin HC5 has a broad spectrum of activity, being able to inhibit the growth of some food-borne micro-organisms, such as *Listeria monocytogenes* (Mantovani and Russell, 2003), and also *Clostridium aminophilum* (Mantovani and Russell, 2002), *Clostridium sporogenes* (Mantovani *et al.*, 2002; Flythe and Russell, 2004), *Bacillus cereus*, *Bacillus thuringiensis* and *Clostridium* ssp. (de Carvalho *et al.*, 2007). Bovicin HC5 was also effective against strains of *Staphylococcus aureus*, coagulase-negative *Staphylococcus* sp., *Streptococcus agalactiae*, *Streptococcus bovis* and *Streptococcus uberis* isolated from cows with mastitis (Pinto, 2008).

Bovicin HC5 inhibits many gram-positive ruminal organisms, reducing approximately 50 % of the methane production when added to mixed ruminal cultures as semi-purified preparations (Lee *et al.*, 2002), and it has been proposed as a potential feed additive to manipulate ruminal fermentation, improving feed efficiency in cattle, as well as reducing ruminal acidosis (Mantovani and Russell, 2002; Lima *et al.*, 2009). Bacterial adaptation has not yet been demonstrated, even after repeated transfers of sensitive bacteria with sublethal doses of bovicin HC5 (Mantovani and Russell, 2003). According to Mantovani and Russell (2003), the bovicin HC5 can be more powerful than nisin, offering promising prospects.

Only high concentrations of bovicin HC5 (above 115 μg ml^{-1}), considerably higher than the concentrations needed for its antimicrobial activity, were able to exert toxic effects against Vero cells and hemolytic effect on sheep erythrocytes, which suggests that bovicin HC5 shows little activity against mammalian cells (Paiva *et al.*, *under review*).

Even though bovicin HC5 has been characterized as an effective and broad-spectrum bacteriocin, the potential *in vivo* application of this bacteriocin still requires the elucidation of its mechanism of action and immunostimulatory effects. Although bovine *S. bovis* strains are genetically and physiologically distinct from pathogenic isolates involved in human infections, the effects of the administration of *S. bovis* HC5 are yet to be elucidated.

1.6. References

Atrih, A.; Rekhif, N.; Moir, A.J.G.; Lebrihi, A.; Lefebvre, G. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. International Journal of Food Microbiology 68, 93-109, 2001.

Balakrishnan, M.; Simmonds, R.S.; Kilian, M.; Tagg, J.R. Different bacteriocin activities of *Streptococcus mutans* reflect distinct phylogenetic lineages. Journal of Medical Microbiology 51, 941-948, 2002.

Bernbom, N.; Licht, T.R.; Brogren, C.H.; Jelle, B.; Johansen, A.H.; Badiola, I.; Vogensen, F.K.; Norrung, B. Effects of *Lactococcus lactis* on composition of intestinal microbiota: role of nisin. Applied and Environmental Microbiology 72, 239-244, 2006.

Bierbaum, G.; Sahl, H.G. Induction of autolysis of staphylococci by the basic peptide antibiotic pep5 and nisin and their influence on the activity of autolytic enzymes. Archives in Microbiology 141, 249–254, 1985.

Boziaris, I.S.; Humpheso, L.; Adams, M.R. Effect of nisin on heat injury and inactivation of *Salmonella enteritidis* PT4. International Journal of Food Microbiology 43, 7–13, 1998.

Branen, J.K.; Davidson, P.M. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. International Journal of Food Microbiology 90, 63–74, 2004.

Breukink, E.; de Kruijff, B. Lipid II as a target for antibiotics. Nature Reviews Drug Discovery 5, 321-323, 2006.

Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O.P.; Sahl, H.G.; de Kruijff, B. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 286, 2361–2364, 1999.

Brötz, H.; Josten, M.; Wiedemann, I.; Schneider, U.; Götz, F.; Bierbaum, G. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Molecular Microbiology 30, 317–327, 1998.

Burkard, M.; Entian, K.D.; Stein, T. Development and application of a microtiter plate-based autoinduction bioassay for detection of the lantibiotic subtilin. Journal of Microbiological Methods 70, 179–185, 2007.

Callaway, T.R.; Melo, A.M.S.C.; Russell, J.B. The effect of nisin and monensin on ruminal fermentation in vitro. Current Microbiology 35, 90-96, 1997.

Callaway, T.R.; Anderson, R.C.; Edrington, T.S.; Genovese, K.J.; Harvey, R.B.; Poole, T.L.; Nisbet, D.J. Recent pre-harvest supplementation strategies to reduce carriage and shedding of zoonotic enteric bacterial pathogens in food animals. Animal Health Research Review 5, 35-47, 2004.

Cheikhyoussef, A.; Pogori, N.; Chen, H.; Tian, F.; Chen, W.; Tang, J.; Zhang, H. Antimicrobial activity and partial characteriztion of bacteriocin-like inhibitory substances (BLIS) produced by *Bifidobacterium infantis* BCRC 14602. Food Control, 20, 553-559, 2009.

Chen, L.L.; Tai, P.C. Effects of antibiotics and other inhibitors on ATP-dependent protein translocation into membrane vesicles. Journal of Bacteriology 169, 2372–2379, 1987.

Cirioni, O.; Slvestri, C.; Ghiselli, R.; Giacometti, A.; Orlando, F.; Mocchegiani, F.; Chiodi, L.; Vittoria, A.D.; Saba, V.; Scalise, G. Experimental study on the efficacy of

combination of α-helical antimicrobial peptides and vancomycin against *Staphylococcus aureus* with intermediate resistance to glycopeptides. Peptides 27, 2600-2606, 2006.

Claypool, L.; Hainemann, B.; Voris, L.; Stumbo, C.R. Residence time of nisin in the oral cavity following consumption of chocolate milk containing nisin. Journal of Dairy Science 49, 314-316, 1966.

Cleveland, J.; Montville, T.J.; Nes, I.F.; Chikindas, M.L. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71, 1-20, 2001.

Cotter, P.D.; Hill, C.; Ross, R.P. Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology 3, 777–788, 2005.

Crowe, S.E.; Perdue, M.H. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. Gastroenterology 103, 1075-1095, 1992.

de Carvalho, A.A.T.; Mantovani, H.C.; Vanetti, M.C.D. Bactericidal effect of bovicin HC5 and nisin against *Clostridium tyrobutyricum* isolated from spoiled mango pulp. Letters in Applied Microbiology 45, 68–74, 2007.

de Kruijff, B.; van Dam, V.; Breukink, E. Lipid II: a central component in bacterial cell wall synthesis and a target for antibiotics. Prostaglandins, Leukotrienes and Essential Fatty Acids 79, 117-121, 2008.

de Kwaadsteniet, M.; ten Doeschate, K.; Dicks, L.M. Characterization of the structural gene encoding nisin F, a new lantibiotic produced by a *Lactococcus lactis* subsp. *lactis* isolate from freshwater catfish (*Clarias gariepinus*). Applied and Environmental Microbiology 74, 547-549, 2008.

de Vuyst, L.; Leroy, F. Bacteriocins from lactic acid bacteria: production, purification, and food applications. Journal of Molecular Microbiology and Biotechnology 13, 194-199, 2007.

Delves-Broughton, J.; Blackburn, P.; Evans, R.J.; Hugenholtz, J. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek 69, 193-202, 1996.

Delves-Broughton, J. Nisin as a food preservative. Food Australia 57, 525-527, 2005.

Diez-Gonzalez, F. Applications of bacteriocins in livestock. Current Issues in Intestinal Microbiology 8, 15-24, 2004.

Dobson, A.E.; Sanozky-Dawes, R.B.; Klaenhammer, T.R. Identification of an operon and inducing peptide involved in the production of lactacin B by *Lactobacillus acidophilus*. Journal of Applied Microbiology 103, 1766–1778, 2007.

Draper, L.A.; Ross, R.P.; Hill, C.; Cotter, P.D. Lantibiotic immunity. Current Protein and Peptide Science 9, 39–49, 2008.

Drider, D.; Fimland, G.; Héchard, Y.; McMullen, L.M.; Prévost, H. The continuing story of class IIa bacteriocins. Microbiology and Molecular Biology Reviews 70, 564-582, 2006.

Drosinos, E.H.; Mataragas, M.; Metaxopoulos, J. Modeling of growth and bacteriocin production by Leuconostoc mesenteroides E131. Meat Science 74, 690–696, 2006.

Dufour, A.; Hindré, T.; Haras, D.; Le Pennec, J.P. The biology of lantibiotics from the lacticin 481group is coming of age. FEMS Microbiology Reviews 31, 134–167, 2007.

Dupuy, B.; Raffestin, S.; Matamouros, S.; Mani, N.; Popoff, M.R.; Sonenshein, A.L. Regulation of toxin and bacteriocin gene expression in *Clostridium* by interchangeable RNA polymerase sigma factors. Molecular Microbiology 60, 1044-1057, 2006.

Eijsink, V.G.; Axelsson, L.; Diep, D.B.; Harvarstein, L.S.; Holo, H.; Nes, I.F. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie van Leeuwenhoek 81, 639–654, 2002.

FDA (US Food and Drug Administration). Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Federal Register 53, 11247-11251, 1988.

FDA, 2001. US Food and Drug Administration, Department of Health and Human Services. Agency Response Letter GRAS Notice nº GRN000065. Available from http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn0065.pdf (accessed 08.09.10)

Flythe, M. D.; Russell, J. B. The effect of pH and a bacteriocin (bovicin HC5) on *Clostridium sporogenes* MD1, a bacterium that has the ability to degrade amino acids in ensiled plant materials. FEMS Microbiology Ecology 47, 215-222, 2004.

Gálvez, A.; López, R.L.; Abriouel, H.; Valdivia, E.; Ben Omar, N. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Critical Reviews in Biotechnology 28, 125-152, 2008.

Giacometti, A.; Cirioni, O.; Kamysz, W.; Silvestri, C.; Licci, A.; Riva, A.; Lukasizk, J.; Scalise, G. In vitro activity of amphibian peptides alone and in combination with antimicrobial agents against multidrug-resistant pathogens isolated from surgical wound infection. Peptides 26, 2111-2116, 2005.

Gillor, O. Bacteriocins' role in bacterial communication. In: Riley, M.A.; Chavan, M. (eds) Bacteriocins: ecology and evolution. Springer, Berlin, 135–146, 2007.

Gobbetti, M.; de Angelis, M.; Di Cagno, R.; Minervini, F.; Limitone, A. Cell–cell communication in food related bacteria. International Journal of Food Microbiology 120, 34–45, 2007.

Gordon, Y.J.; Romanowski, E.G.; McDermott, A.M. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curret Eye Research 30, 505–515, 2005.

Goto, Y.; Li, B.; Claesen, J.; Shi, Y.; Bibb, M.J.; van der Donk, W.A. Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. PloS Biology 8, e1000339. doi:10.1371/journal.pbio.1000339, 2010.

Gray, E.J.; Di Falco, M.; Souleimanov, A.; Smith, D.L. Proteomic analysis of the bacteriocin thuricin 17 produced by *Bacillus thuringiensis* NEB17. FEMS Microbiology Letters 255, 27-32, 2006a.

Gray, E.J.; Lee, K.D.; Souleimanov, A.M; Di Falco, M.R; Zhou, X.; Ly, A.; *et al.* A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: isolation and classification. Journal of Applied Microbiology 100, 545-554, 2006b.

Gross, D.C.; Vidaver, A.K. Bacteriocins of phytopathogenic *Corynebacterium* species. Canadian Journal of Microbiology 25, 367-374, 1979.

Gunn, J.S. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends in Microbiology 16, 284–290, 2008.

Gut, I.M.; Prouty, A.M.; Ballard, J.D.; van der Donk, W.A.; Blanke, S.R. Inhibition of *Bacillus anthracis* spore outgrowth by nisin. Antimicrobial Agents and Chemotherapy 52, 4281-4288, 2008.

Hancock, R.E.; Rozek, A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiology Letters 206, 143–149, 2002.

Hancock, R.E.; Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24, 1551-1557, 2006.

Hasper, H.E.; de Kruijff, B.; Breukink, E. Assembly and stability of nisin-Lipid II pores. Biochemistry 43, 11567-11575, 2004.

Hasper, H.E.; Kramer, N.E.; Smith, J.L.; Hillman, J.D.; Zachariah, C.; Kuipers, O.P.; de Kruijff, B.; Breukink, E. An alternative bactericidal mechanism of action for lantibiotic peptides that target Lipid II. Science 313, 1636-1637, 2006.

Héchard, Y.; Sahl, H.G. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84, 545-557, 2002

Héchard, Y.; Pelletier, C.; Cenatiempo, Y.; Frère, J. Analysis of σ-54 dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EIIMan) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology 147, 1575–1580, 2001.

Helmann, J.D.; Moran, C.P. RNA polymerase and σ factor in *B. subtilis* and its closest relatives. In From Genes to Cells. Sonenshein, A.L.; Hoch, J.A.; Losick, R. (eds). Washington, DC: ASM Press, 289-312. 2002.

Hoskin, D.W.; Ramamoorthy, A. Studies on anticancer activity of antimicrobial peptides. Biochimica et Biophysica Acta 1778, 357-375, 2008.

Houlihan, A.J.; Russell, J.B. Factors affecting the activity of bovicina HC5, a bacteriocin from *Streptococcus bovis* HC5: release, stability and binding to target bacteria. Journal of Applied Microbiology 100, 168-174, 2006.

Houlihan, A.J.; Mantovani, H. C.; Russell, J. B. Effect of pH on the activity of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. FEMS Microbiology Letters 231, 27-32, 2004.

Hsu, S.T.D.; Breukink, E.; de Kruijff, B.; Kaptein, R.; Bonvin, A.M.; van Nuland, N.A. Mapping the targeted membrane pore formation mechanism by solution NMR: the nisin Z and Lipid II interaction in SDS micelles. Biochemistry 41, 7670-7676, 2002.

Hsu, S.T.D.; Breukink, E.; Tischenko, E.; Lutters, M.A.G.; de Kruijff, B.; Kaptein, R.; Bonvin, A.M.J.J.; van Nuland, N.A.J. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature Structural & Molecular Biology 11, 963-967, 2004.

Hungate, R.E. The rumen and its microbes. New York, Academic Press, 1966.

Ivanova, I.; Miteva, V.; Stefanova, T.S.; Pantev, A.; Budakov, I.; Danova, S.; *et al.* Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. International Journal of Food Microbiology 42, 147-158, 1998.

Jarvis, B.; Mahoney, R.R. Inactivation of nisin by alpha chymotrypsin. Journal of Dairy Science 52, 1448-1449, 1969.

JECFA. Specifications for the identity and purity of foods additives and their toxicological evaluation: some antibiotics. Twelfth Report of the Joint FAO/WHO

Expert Committee on Food Additives, Geneva, 1-8 July 1968, 33-35. FAO Nutrition Meeting Series 45, 1969.

Joerger, R.D. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. Poultry Science 82, 640–647, 2003.

Jung, G. Lantibiotics — ribosomally synthesized biologically active polypeptides containing sulfide bridges and α,β-didehydroamino acids. Angewandte Chemie International Edition English 30, 1051-1068, 1991.

Kamoun, F.; Mejdoub, H.; Aouissaoui, H.; Reinbolt, J.; Hammami, A.; Jaoua, S. Purification, amino acid sequence and characterization of bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. Journal of Applied Microbiology 98, 881-888, 2005.

Keymanesh, K.; Soltani, S.; Sardari, S. Application of antimicrobial peptides in agriculture and food industry. World Journal of Microbiology and Biotechnology 25, 933–944, 2009.

Klaenhammer, T.R. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Review 12, 39-85, 1993.

Klostermann K, Crispie F, Flynn J, Ross RP, Hill C, Meaney W. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. Journal of Dairy Research 75, 365-373, 2008.

Knippels, L.M.J.; Houben, G.F.; Spanhaak, S.; Penninks, A.H. An Oral Sensitization Model in Brown Norway Rats to Screen for Potential Allergenicity of Food Proteins. Methods 19, 78-82, 1999a.

Knippels, L.M.J.; Penninks, A.H.; Smit, J.J.; Houben, G.F. Immune-Mediated Effects upon Oral Challenge of Ovalbumin-Sensitized Brown Norway Rats: Further Characterization of a Rat Food Allergy Model. Toxicology and Applied Pharmacology, 156, 161–169, 1999b.

Kramer, N.E. et al. Resistance of Gram-positive bacteria to nisin is not determined by lipid II levels. FEMS Microbiology Letters 239, 157-161, 2004.

Lee, S.S.; Hsu, J.T.; Mantovani, H.C.; Russell, J.B. The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production *in vitro*. FEMS Microbiology Letters 217, 51-55, 2002.

Lia, B.; Sherb, D.; Kelly, L.; Shi, Y.; Huang, K.; Knerr, P.J.; Joewono, I.; Rusch, D.; Chisholm, S.W.; van der Donk, W.A. Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. PNAS 107, 10430–10435, 2010.

Lima, J.R.; Ribon, A.O.; Russell, J.B.; Mantovani, H.C. Bovicin HC5 inhibits wasteful amino acid degradation by mixed ruminal bacteria *in vitro*. FEMS Microbiology Letters 292, 78-84, 2009.

Majamaa, H.; Isolauri, E. Evaluation of the gut mucosal barrier: Evidence for increased antigen transfer in children with atopic eczema. Journal of Allergy and Clinical Immunology 97, 985–990, 1996.

Mantovani, H.C.; Hu, H.; Worobo, R.W.; Russell, J.B. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology 148, 3347-3352, 2002.

Mantovani, H.C.; Russell, J.B. The ability of a bacteriocin of *Streptococcus bovis* HC5 (bovicin HC5) to inhibit *Clostridium aminophilum*, an obligate amino acid fermenting bacterium from the rumen. Anaerobe 8, 247-252, 2002.

Mantovani, H.C.; Russell, J.B. Inhibition of *Listeria monocytogenes* by bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5. International Journal of Food Microbiology 89, 77-83, 2003.

Mantovani, H.C.; Russell, J.B. Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5, catalyzes the efflux of intracellular potassium but not ATP. Antimicrobial Agents and Chemotherapy 52, 2247–2249, 2008.

McAuliffe, O.; Ross, R.P.; Hill, C. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiology Reviews 25, 285–308, 2001.

Messi, P.; Bondi, M.; Sabia, C.; Battini, R.; Manicardi, G. Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. International Journal of Food Microbiology 64, 193-198, 2001.

Molina, A.; Molina, M.P.; Althaus, R.L.; Gallego, L. Residue persistence in sheep milk following antibiotic therapy. Veterinary Journal 165, 84–89, 2003.

Monteleone, C.A.; Sherman, A.R. Nutrition and asthma. Archives of Internal Medicine 157, 23–24, 1997.

Morovsky, M.; Pristas, P.; Czikkova, S.; Javorsky, P. A bacteriocin-mediated antagonism by *Enterococcus faecium* BC25 against ruminal *Streptococcus bovis.* Microbiology Research 153, 277-281, 1998.

Nes, I.F.; Holo, H. Class II antimicrobial peptides from lactic acid bacteria, Biopolymers 55, 50-61, 2000.

Nolan, E.M.; Walsh, C.T. How nature morphs peptide scaffolds into antibiotics. Chem Bio Chem 10, 34-53, 2009.

Ondaa, T.; Yanagidab, F.; Tsujia, M.; Shinoharab, T.; Yokotsuka, K. Production and purification of a bacteriocin peptide produced by *Lactococcus* spp. strain GM005, isolated from Miso-paste. International Journal of Food Microbiology 87, 153-159, 2003.

Oppegard, C.; Rogne, P.; Emanuelsen, L.; Kristiansen, P.E.; Fimland, G.; Nissen-Meyer, J. The two-peptide class II bacteriocins: structure, production, and mode of action. Journal of Molecular Microbiology and Biotechnology 13, 210-219, 2007.

Pag, U.; Sahl, H.G. Multiple activities in lantibiotics - models for the design of novel antibiotics? Current Pharmaceutical Design 8, 815–833, 2002.

Paiva, A.D.; de Paula, S.O.; Baracat-Pereira, M.C.; Breukink, E.; Mantovani, H.C. Assessment of the *in vitro* cytotoxicity of the lantibiotics bovicin HC5 and nisin on eukaryotic cells and model membranes. Microbiology, 2011. *Under review.*

Patra, A.K. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environmental Monitoring and Assessment 2011. *In press*.

Pavlova, O.A.; Severinov, K.V. Posttranslationally modified microcins. Russian Journal of Genetics 42, 1380-1389, 2006.

Penninks, A.H.; Knippels, L.M.J. Determination of protein allergenicity: studies in rats. Toxicology Letters 120, 171-180, 2001.

Pinto, M.S. Atividade de própolis verde e bovicina HC5 sobre bactérias isoladas de mastite bovina. Universidade Federal de Viçosa. Dissertação de Mestrado. Viçosa: UFV, 2008. 94 p.

Pochard, P.; Gosset, P.; Grangette, C.; Andre, C.; Tonnel, A.B.; Pestel, J.; Mercenier, A. Lactic acid bacteria inhibit T_H2 cytokine production by mononuclear cells from allergic patients. Journal of Allergy and Clinical Immunology 110, 617-623, 2002.

Rayman, K.; Malik, N.; Hurst, N. Failure of nisin to inhibit outgrowth of *Clostridium botulinum* in a model cured meat system. Applied and Environmental Microbiology 46, 1450-1452, 1983.

Reddy, K.V.; Aranha, C.; Gupta, S.M.; Yedery, R.D. Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: in vitro and in vivo studies. Reproduction 128, 117-126, 2004.

Riley, M.A.; Wertz, J.E. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 84, 357-364, 2002.

Robinson, D.S.; Kay, A.B. Role of TH1 and TH2 cells in human allergic disorders. Chemical Immunology 63, 187-203, 1996.

Rood, J.J.; Cole, S.T. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiology Reviews 55, 621-648, 1991.

Ross, R.P.; Galvin, M.; McAuliffe, O.; Morgan, S.M.; Ryan, M.P.; Twomey, D.P.; Meaney, W.J.; Hill, C. Developing applications for lactococcal bacteriocins. Antonie Van Leeuwenhoek 76, 337-346, 1999.

Rossi, L.M.; Rangasamy, P.; Zhang, J.; Qiu, X.Q.; Wu, G.Y. Research advances in the development of peptide antibiotics. Journal of Pharmaceutical Sciences 97, 3, 1060–1070, 2008.

Russell, J.B.; Mantovani, H.C. The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics. Journal of Molecular Microbiology and Biotechnology 4, 347-355, 2002.

Rychlik, J.L.; Russell, J.B. The adaptation and resistance of *Clostridium aminophilum* F to the butyrivibriocin-like substance of *Butyrivibrio fibrisolvens* JL5 and monensin. FEMS Microbiology Letters 209, 93-98, 2002.

Sahl, H.G.; Bierbaum, G. Multiple activities in natural antimicrobials. Microbe 3, 467–473, 2008.

Salzman, R.A.; Koiwa, H.; Ibeas, J.I.; Pardo, J.M.; Hasegawa, P.M.; Bressan, R.A. Inorganic cations mediate plant PR5 protein antifungal activity through fungal Mnn1- and Mnn4-regulated cell surface glycans. Molecular Plant-Microbe Interaction 17, 780-788, 2004.

Sang, Y.; Blecha, F. Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Animal Health Research Reviews 9, 227–235, 2008.

SCF, 1992. Opinions of the Scientific Committee for Food 26th Series. Commission of the European Communities. Available from http://europa.eu.int/comm/food/fs/sc/scf/reports/scf_reports_26.pdf (accessed 08.09.2010).

Sears, P.M.; Smith, B.S.; Stewart, W.K.; Gonzalez, R.N. Evaluation of a nisin-based germicidal formulation on teat skin of live cows. Journal of Dairy Science 75, 3185-3190, 1992.

Sit, C.S.; Vederas, J.C. Approaches to the discovery of new antibacterial agents based on bacteriocins. Biochemistry and Cell Biology 86, 116–123, 2008.

Tagg, J.R.; Ragland, N.L. Applications of BLIS typing to studies of the survival on surfaces of salivary streptococci and staphylococci. Journal of Applied Bacteriology 71, 339-342, 1991.

Taylor, S.L.; Leher, S.B. Principles and characteristics of food allergens. Critical Reviews in Food Science and Nutrition 36, 91–118, 1996.

Todorov, S.D.; Dicks, L.M.T. Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. Biotechnology Journal 1, 405-409, 2006.

Todorov, S.D.; Wachsman, M.; Tomé, E.; Dousset, X.; Destro, M.T.; Dicks, L.M.T.; Franco, B.D.G.M.; Vaz-Velho, M.; Drider, D. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faeciem*. Food Microbiology 27, 869-879, 2010.

Todorov, S.D. Bacteriocins from *Lactobacillus plantarum* – production, genetic organization and mode of action. A review. Brazilian Journal of Microbiology 40, 209-221, 2009.

Todorov, S.D. Diversity of bacteriocinogenic lactic acid bacteria isolated from boza, a cereal-based fermented beverage from Bulgaria. Food Control 21, 1011-1021, 2010

Twomey, D.P.; Wheelock, A.I.; Flynn, J.; Meaney, W.J.; Hill, C.; Ross, R.P. Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. Journal of Dairy Science 83, 1981-1988, 2000.

van Heusden, H.E.; de Kruijff, B.; Breukink, E. Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin. Biochemistry 41, 12171-12178, 2002.

van Kraaij, C.; Breukink, E.; Rollema, H.S.; Bongers, R.S.; Kosters, H.A.; de Kruijff, B.; Kuipers, O.P. Engineering a disulfide bond and free thiols in the lantibiotic nisin Z. European Journal of Biochemistry 267, 901-909, 2000.

Vermeiren, L.; Devlieghere, F.; Vandekinderen, I.; Debevere, J. The interaction of the non-bacteriocinogenic *Lactobacillus sakei* 10A and lactocin S producing *Lactobacillus sakei* 148 towards *Listeria monocytogenes* on a model cooked ham. Food Microbiology 23, 511–518, 2006.

Wachsman, M.B.; Castilla, V.; Holgado, A.P.D.; De Torres, R.A.; Sesma, F.; Coto, C.E.. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication *in vitro*. Antiviral Research 58, 17-24, 2003.

Wescombe, P.A.; Tagg, J.R. Purification and characterization of streptin, a type A1 lantibiotic produced by *Streptococcus pyogenes*. Applied and Environmental Microbiology 69, 2737-2747, 2003.

Whitehead, T.R.; Cotta, M.A. Development of molecular methods for identification of *Streptococcus bovis* from human and ruminal origins. FEMS Microbiology Letters, 182, 237-240, 2000.

[WHO] World Health Organization. The use of essential drugs. Sixth Report of the WHO expert committee, WHO Tech. Rep. Ser. No. 850. Rome:WHO, 1995.

Wiedemann, I.; Breukink, E.; van Kraaij, C.; Kuipers, O.P.; Bierbaum, G.; de Kruijff, B.; Sahl, H.G. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. Journal of Biological Chemistry 276, 1772–1779, 2001.

Wiedemann, I.; Böttiger, T.; Bonelli, R.R.; Wiese, A.; Hagge, S.O.; Gutsmann, T.; Seydel, U.; Deegan, L.; Hill, C.; Ross, P.; Sahl, H.G. The mode of action of the

lantibiotic lacticin 3147 – a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Molecular Microbiology 61, 285-296, 2006.

Willey, J.M.; van der Donk, W.A. Lantibiotics: peptides of diverse structure and function. Annual Review of Microbiology 61, 477-501, 2007.

Wu, J.; Hu, S.; Cao, L. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. Antimicrobial Agents and Chemotherapy 51, 3131-3135, 2007.

Xie, L.; Miller, L.M.; Chatterjee, C.; Averin, O.; Kelleher, N.L.; van der Donk, W.A Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity. Science 303, 679–681, 2004.

Yoneyama, F.; Imura, Y.; Ichimasa, S.; Fujita, K.; Zendo, T.; Nakayama, J.; Matsuzaki, K.; Sonomoto, K. Lacticin Q, a lactococcal bacteriocin, causes high-level membrane permeability in the absence of specific receptors. Applied and Environmental Microbiology 75, 538-541, 2009a.

Yoneyama, F.; Imura, Y.; Ohno, K.; Zendo, T.; Nakayama, J.; Matsuzaki, K.; Sonomoto, K. Peptide-lipid huge toroidal pore, a new antimicrobial mechanism mediated by a lactococcal bacteriocin, lacticin Q. Antimicrobial Agents and Chemotherapy 53, 3211-3217, 2009b.

Yoneyama, F.; Shioya, K.; Zendo, T.; Nakayama, J.; Sonomoto, K. Effect of a negatively charged lipid on membrane lacticin Q interaction and resulting pore formation. Bioscience, Biotechnology, and Biochemistry 74, 218-221, 2010.

Yount, N.Y.; Bayer, A.S.; Xiong, Y.Q.; Yeaman, M.R. Advances in antimicrobial peptide immunobiology. Biopolymers 84, 435-458, 2006.

Zelezetsky, I.; Pacor, S.; Pag, U.; Papo, N.; Shai, Y.; Sahl, H.G.; Tossi, A. Controlled alteration of the shape and conformational stability of alpha-helical cell-lytic peptides: effect on mode of action and cell specificity. Biochemical Journal 390, 177-188, 2005.

CHAPTER 2

The role of Lipid II and membrane thickness in the mechanism of action of the lantibiotic bovicin HC5.

2.1. Abstract

Lantibiotics are antimicrobial peptides produced by gram-positive bacteria, being nisin the most well known member. Nisin inhibits peptidoglycan synthesis and forms pores at sensitive membranes upon interaction with Lipid II, the essential bacterial cell wall precursor. Bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5, has the putative N-terminal Lipid II binding motif, and we investigated the mode of action of bovicin HC5 using both living bacteria and model membranes, with special emphasis on the role of Lipid II. Bovicin HC5 showed activity against *Staphylococcus conhii* and *Staphylococcus warneri*, but bovicin HC5 hardly interfered with the membrane potential of *S. conhii*. In model membranes, bovicin HC5 was not able to cause carboxyfluorescein release or proton influx from DOPC vesicles containing Lipid II. Bovicin HC5 blocked Lipid II-dependent pore-formation activity of nisin, and a high affinity interaction with Lipid II was observed ($Ka=3.1x10^{6}\ M^{-1}$), with a 1:1 stoichiometry. In DOPC vesicles containing Lipid II, bovicin HC5 was able to assemble with Lipid II into a pre-pore-like structure. We furthermore observed pore formation activity of bovicin HC5 in thin membranes, which was stimulated by the presence of Lipid II. Moreover, bovicin HC5 induced the segregation of Lipid II into domains in giant model membrane vesicles. In conclusion, bovicin HC5 has a primary mode of action similar to nisin, but some differences regarding the pore forming capacity were demonstrated.

2.2. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by species of *Bacteria* and certain strains of the *Archaea* domain (Drosinos *et al.*, 2006). They differ in size, amino acid sequence, mechanisms of action and range of antimicrobial specificities (Cleveland *et al.*, 2001). Different peptides exhibit bactericidal or bacteriostatic activity, even at low concentrations, acting by inducing pore formation on sensitive cell membranes or inhibiting the peptidoglycan, nucleic acid or protein synthesis (Cotter *et al.*, 2005).

Nisin, the most well known bacteriocin, is a heat-stable antibacterial peptide produced by the lactic acid bacterium *Lactococcus lactis* subsp. *lactis*. Nisin belongs to the type A group of lantibiotics and it is a small (3.5 kDa), cationic, hydrophobic peptide with five characteristic (beta-methyl) lanthionine rings. So far, nisin is the only bacteriocin which has been approved for use in over 50 countries as food preservative (code E234), including USA, European Union, Australia and New Zealand (Delves-Broughton, 2005).

The antimicrobial activity of nisin is based on both low-affinity membrane and high-affinity target interactions (Pag and Sahl, 2002). Nisin can disrupt microbial membranes by interacting with membrane phospholipids (Breukink *et al.*, 1999) and it is also able to displace or release enzymes resulting in lysis of cell-wall (Bierbaum and Sahl, 1985; Héchard and Sahl, 2002). Nisin binds with high affinity to the Lipid II molecule, a hydrophobic carrier of peptidoglycan monomers, using this compound as a docking molecule for its pore-forming activity and leading to the inhibition of bacterial cell wall synthesis (Brötz *et al.*, 1998; Breukink *et al*., 1999; Wiedemann *et al.*, 2001; Breukink *et al.*, 2003). Nisin also inhibits the outgrowth of bacterial spores, by uncoupling the establishment of oxidative metabolism or membrane potential and the shedding of external spore structures (Gut *et al.*, 2008). Recently, Gut and co-workers (2011) showed that nisin utilizes Lipid II as the target during inhibition of spore outgrowth and the membrane disruption induced by nisin is important to inhibit the development of spores into vegetative cells.

Bovicin HC5 is a lantibiotic (2449 Da) produced by *Streptococcus bovis* HC5, a lactic acid bacterium isolated from the bovine rumen. Bovicin HC5 contains posttranslational modified amino acids residues, is stable at high temperatures and low pH, and has a broad spectrum of activity, including some food-borne microorganisms,

such as *Listeria monocytogenes* (Mantovani *et al.*, 2001; Mantovani *et al.*, 2002; Mantovani and Russell, 2003).

Bovicin HC5 shares activity similarities with nisin, and the former peptide also carry the two lanthionine rings at its N-terminus, which form the Lipid II-binding motif in nisin molecule (Figure 1). Based on these features, we hypothesized that bovicin HC5, like nisin, could target the essential bacterial cell wall precursor, Lipid II. In this paper, we characterized the Lipid II-based activity of bovicin HC5 using live bacterial cells and artificial model membranes approaches, and compared the activities of bovicin HC5 and nisin.

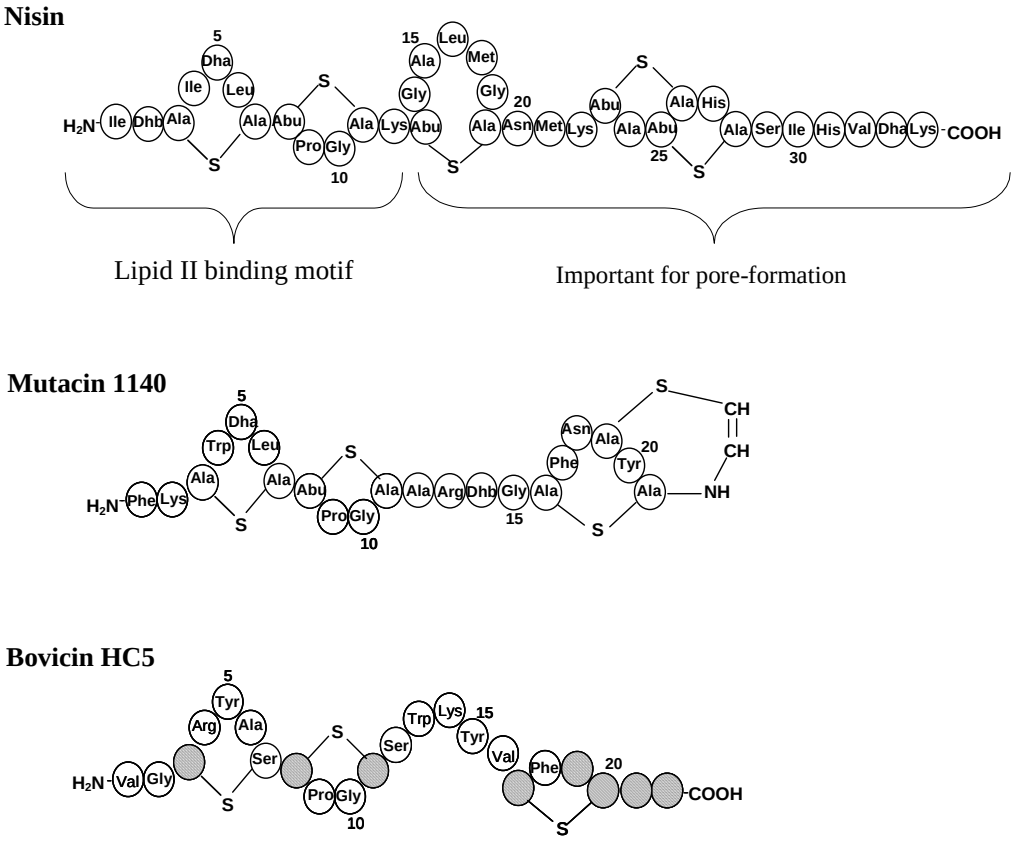


Figure 1: Comparison of primary structures of nisin, mutacin 1140 and bovicin HC5. In nisin's and mutacin`s structures, the posttranslationally modified amino acids residues, dehydroalanine (Dha) and deydrobutyrine (Dhb), as well as the lanthionine rings (Abu-S-Ala and Ala-S-Ala) are shown. In bovicin HC5's structure, the residues indicated by striped circles do not correspond to any 20 amino acids commonly found in proteins, and represent putative posttranslational modified amino acids residues; the suggested positions of lanthionine linkages are indicated by the traces.

2.3. Experimental procedures

2.3.1. Chemicals and materials

Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocoline ($C_{18:1}$, DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocoline ($C_{14:1}$, DMoPC), and 1,2-dilauroyl-*sn*-glycero-3-phosphocoline ($C_{12:0}$, DLPC) were purchased from Avanti Polar Lipids, Inc. Phospholipids were dissolved in chloroform:methanol (1:1) to a stock concentration of 10 mM and stored at -20 ºC. After destruction of phospholipids, the sample concentrations were determined by inorganic phosphate analysis, according to the procedure described by Rouser *et al.* (1970).

Carboxyfluorescein (CF) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS, MW = 524.4) were purchased from Invitrogen. Lipid II with and without the pyrene label was synthesized and purified as described by Breukink *et al.* (2003). All other chemicals used were of analytical or reagent grade.

2.3.2. Bacterial strains and culture conditions

The bovicin HC5-producing strain *Streptococcus bovis* HC5 was cultivated under anaerobic conditions in basal medium containing (per liter): 0.292 g K_2HPO_4; 0.292 g KH_2PO_4; 0.48 g $(NH_4)_2SO_4$; 0.48 g NaCl; 0.1 g $MgSO_4.7H_2O$; 0.064 g $CaCl_2.2H_2O$; 0.5 g cystein hydrochloride; 4 g Na_2CO_3; 0.1 g trypticase; 0.5 g yeast extract at 39 ºC, overnight. Glucose was added as carbon source (4 g/L).

Staphylococcus cohnii and *Staphylococcus warneri* were grown in Müeller-Hinton medium (Sigma) at 30 ºC, with aeration, for 5 hours. Stock cultures were stored at – 70 °C, in solutions containing their own growth medium and 50 % glycerol. Working cultures were maintained on agar plates and subcultured weekly.

2.3.3. Bacteriocins

Nisin A was isolated from commercial products (Chrisin (2.5% w/w nisin), 1000 IU/mg, Christian Hansen, Denmark). Extracts of bovicin HC5 were prepared as described by Mantovani *et al.* (2002). Purification of bovicin HC5 was performed by reverse-phase high performance liquid chromatography (RP-HPLC) using a semipreparative column (Shimadzu C18; 5 μm; 150 by 4.6 mm [inner diameter]). The column was equilibrated with buffer A (0.1 % trifluoroacetic acid (TFA) in ultrapure water) and the peptide was eluted using a linear gradient from 35 to 50 % buffer B (80

% acetonitrile, 0.1 % TFA in ultrapure water), 22°C, and at a flow rate of 1 ml min^{-1}. The absorbance was monitored at 214 and 280 nm and the eluted fraction corresponding to pure bovicin HC5 was lyophilized. Analytical HPLC and electrospray mass spectrometry was used to confirm the correct mass and the purity of bovicin HC5.

Bacteriocin stock solutions (1 mM, in 0.05 % acetic acid) were stored at -20 ºC until use. Protein concentration was determined using a bicinchoninic acid protein assay reagent (Pierce Chemical Corp., Bonn, Germany), with bovine serum albumin as the standard.

2.3.4. MIC determinations

The minimal growth-inhibitory concentrations of bovicin HC5 and nisin were determined against *Staphylococcus cohnii*, a nisin-sensitive strain, and *Staphylococcus warneri*, a nisin-resistant strain. Overnight cultures of the strains were diluted 100-fold in Mueller-Hinton broth ($OD_{600\ nm}$=0.05). Different concentrations of purified bovicin HC5 and nisin in acetic acid (0.05 %) were added and the cultures were incubated at 30 ºC, with aeration; the samples were collected after 8 h of growth and the optical density was determined (600 nm). The lowest antimicrobial agent concentration resulting in less than 1 % outgrowth was determined as the MIC value. Results given are means of three independent determinations.

2.3.5. Membrane depolarization assay using intact cells

The cytoplasmic membrane depolarization activity of bovicin HC5 and nisin was determined using the membrane potential-sensitive fluorophore $DiSC_2(5)$ (Hope *et al.*, 1985) and *Staphylococcus cohnii*. Bacterial cells in logarithmic phase (OD_{600nm}= 0.6-0.7) were centrifuged (1742 x *g*, 15 min, 4 ºC), washed in 5 mM HEPES buffer (pH 7.8) containing glucose (0.4 %) and Mg^{+2} (2.5 mM), and ressuspended in the same buffer to an OD_{600nm} of 0.05 in a 1-cm quartz cuvette. The dye $DiSC_2(5)$ was added to a final concentration of 0.4 μM, followed by an incubation at room temperature for 2 min.

Subsequently, different concentrations of the bacteriocins were added and the fluorescence was followed over time with a SLM Aminco Spectrofluorometer (SPF-500C). An excitation wavelength of 622 nm and an emission wavelength of 650 nm were used.

2.3.6. Preparation of large unilamellar vesicles (LUVs)

LUVs containing DOPC or DLPC/DMoPC (1:1) with and without Lipid II were prepared for isothermal titration calorimetry, carboxyfluorescein leakage and proton influx experiments by the extrusion technique (Hope *et al.*, 1985). The dried lipid films were hydrated by the addition of 10 mM Tris and 150 mM NaCl (pH 7.5), 10 mM Tris and 50 mM CF (pH 7.5), or HPTS buffer (2 mM HPTS in 0.2 M NaH_2PO_4/Na_2HPO_4, pH 7.0), and followed by vigorous stirring.

The LUVs were prepared by repeated extrusion through polycarbonate filters with a 0.2 μm pore size (Isopore membrane filters; Millipore, Ireland). Following the extrusion, vesicles were passed through a Sephadex G-50 spincolumn (equilibrated with 10 mM Tris and 150 mM NaCl (pH 7.5) 10 mM MES, 0.2 M Na_2SO_4 buffer (pH 5.5) to remove the non-enclosed probe (CF or HPTS, respectively).

Vesicle concentrations were determined by phosphate determination, according to the method described by Rouser *et al.* (1970). In the case of HPTS vesicles, the lipids from the vesicles were firstly extracted according to Bligh-Dyer procedure (1959) in order to exclude the phosphate present in the buffer, and the inorganic phosphate determination was performed.

The final vesicle concentration used was 25 μM (final lipid-Pi) and the final concentration of Lipid II varied according to the experiment performed (0.1 mol % for leakage experiments; 0.5 mol % for pyrene labeled Lipid II experiment; 2 mol % for ITC).

2.3.7. Carboxyfluorescein leakage assay

Different concentrations of the bacteriocins were added to 10 mM Tris and 150 mM NaCl buffer (pH 7.5) containing CF loaded vesicles (final concentration of 25 mM (lipid-Pi)). The CF leakage from the vesicles was monitored by measuring the fluorescence intensity at 516 nm (excitation wavelength at 492 nm), using a SLM Aminco Spectrofluorometer (SPF-500C). The samples were continuously stirred in a 10 x 4 mm quartz cuvette, kept at 20 ºC using a continuous circulation water bath. To disrupt the vesicles, Triton X-100 (final concentration of 0.2 %) was added 2 min after the addition of the desired peptide, and the resulting fluorescence was taken as 100 % leakage value.

The percentage of CF leakage was calculated according to the equation:

$$\% \text{ CF efflux} = (F_t - F_0)/ (F_\infty - F_0) \times 100$$

where F_t was the fluorescence at time t; F_0 was the fluorescence at time t_0 (baseline fluorescence for CF-loaded vesicles before the addition of peptides); F_∞ was the maximum fluorescence (100 %) after addition of Triton X-100.

2.3.8. Proton permeability assay

The effects of the bacteriocins on the proton permeability was monitored by adding different concentrations of the peptides to 1 ml of 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) containing HPTS loaded vesicles (final concentration of 25 mM (lipid-Pi)). The fluorescence intensity was detected at 508 nm (excitation wavelength at 450 nm) on a SLM Aminco Spectrofluorometer (SPF-500C). The samples were continuously stirred in a 10 x 4 mm quartz cuvette, kept at 20 ºC using a continuous circulation water bath. Triton X-100 was added after 2 min to disintegrate the vesicles and the resulting fluorescence was taken as 100 % of proton influx.

2.3.9. Isothermal Titration Calorimetry (ITC) measurements

Titration experiments were performed on an Auto-iTC_{200} isothermal titration calorimeter (MicroCal Inc.). DOPC LUVs with or without 2 mol % Lipid II were prepared as described, in 10 mM Tris and 150 mM NaCl (pH 7.5). The vesicles were injected into the sample cell (volume=0.2 ml) containing 20 μM bovicin HC5 in the same buffer as used for the vesicles. All solutions were degassed before starting the experiment.

The ITC-runs consisted of 16 injections, 5 μL each, of a vesicle stock solution at 25 ºC (20 mM final phospholipid concentration), and the experiment proceeded over 33 min. The results were analyzed using the Origin® software (version 7.0) provided by MicroCal Inc.

2.3.10. Assessing assembly using pyrene labeled Lipid II

LUVs were prepared in 10 mM Tris and 150 mM NaCl (pH 7.5) and the fluorescence spectrum was recorded between 360 and 550 nm (excitation wavelength at 350 nm) in the absence and presence of the bacteriocins after an incubation time of 5 min, on a SLM Aminco Spectrofluorometer (SPF-500C) and the samples were continuously stirred in a 10 x 4 mm quartz cuvette, kept at 20 ºC using a water bath with continuous circulation.

To prevent the influence of quenching effects of the peptides on the monomer fluorescence of pyrene Lipid II on the excimer/monomer ratios, these were calculated

using the monomer fluorescence on the sample in the absence of peptide, as described before (Hasper *et al.*, 2004).

2.3.11. Preparation of Giant Unilamellar Vesicles (GUVs)

GUVs were prepared using a homemade electroformation setup with ITO cover slips (Ramadurai *et al.*, 2010). On one slip, 5 μL from a chloroform-methanol (1:1, v/v) solution containing 2.5 mM DOPC, 0.2 mol % NBD-labeled Lipid II was deposited and dried in a vacuum desiccator (1 h), in order to remove the remaining solvents. With this slip, the electroformation chamber was assembled and filled with a solution of 100 mM sucrose.

Via two clamps, the GUV cell was connected to the power supply, and an alternating current was applied (3 V, 10 Hz). GUVs formation was carried out for 3 h and the chambers were analyzed directly using confocal fluorescence microscopy.

Peptides were added by pipetting the desired volume from a 1 mM solution directly into the GUV chamber.

2.3.12. Confocal fluorescence microscopy

The NBD label was detected using an Argon Laser (488 nm, Spectra-Physics), and it appeared green upon excitation. Difference Interference Contrast (DIC) (Nomarski optics) was used for detection of GUVs, as a control for their shape.

Nikon EZ-C1 Software Version 2.20 Gold was used for analysis of the images.

2.4. Results

2.4.1. Antimicrobial activity

The minimum inhibitory concentration (MIC) value of nisin determined against the nisin-sensitive strain, *S. cohnii*, was 0.05 μM, while the MIC against *S. warneri*, the nisin-resistant strain, was determined to be 10 μM. The MIC values of bovicin HC5 were 0.2 μM and 2 μM, for *S. cohnii* and *S. warneri*, respectively. It should be noted here that the activity of bovicin HC5 persisted in time for both strains tested, and even after 24 hours of incubation bacterial growth was not observed at the MIC values. However, in the same period of incubation, nisin was not able to prevent the growth of *S. cohnii* and *S. warneri* and, at the MIC values, an OD_{600nm} of 3.3 and 4.4 was reached by each culture, respectively. This suggests that the mode of action of bovicin HC5 differs from that of nisin despite their similar Lipid II binding motif.

2.4.2. Membrane depolarization

To verify whether bovicin HC5 caused disruption of the membrane potential of living bacterial cells, membrane depolarization assays were performed with exponentially growing *S. cohnii* cultures, and the fluorescence of $DiSC_2(5)$ was used as an indicator for the presence of a membrane potential.

In spite of the low MICs determined for both bacteriocins, at the MIC values, neither bovicin HC5 nor nisin caused significant membrane depolarization in *S. cohnii* cells (Figure 2, traces A and B). Upon addition of 4 μM bovicin HC5 (20 times the MIC) only a minor effect in the membrane potential of *S. cohnii* could be observed (Figure 2, trace C). Nisin was more efficient in dissipating the membrane potential of *S. cohnii*, since at 4x the MIC (0.2 μM final concentration) this peptide caused major release of the dye from the *S. cohnii* membrane, showing that the membrane potential was dissipated upon addition of nisin (Figure 2, trace D). These results suggest that the interaction between bovicin HC5 and the target cells was different when compared to nisin.

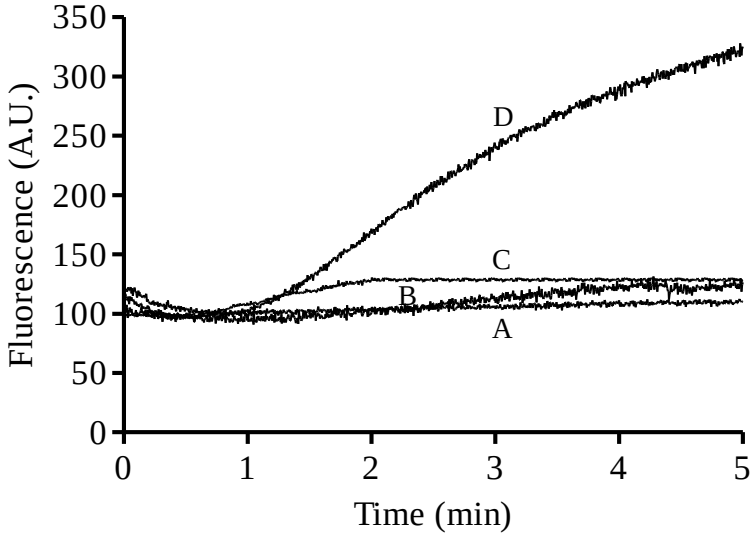


Figure 2: Effect of bovicin HC5 and nisin on the membrane potential of glucose-energized cells of *Staphylococcus cohnii*, determined using $DiSC_2$ (5), a membrane potential sensitive fluorophore. (A) 0.2 μM bovicin HC5; (B) 0.05 μM nisin; (C) 4 μM bovicin HC5; (D) 0.2 μM nisin. The bacterial cells were diluted to an OD_{600nm} of 0.05 and $DiSC_2$ (5) was added to a final concentration of 0.4 μM. The bacteriocin addition was performed when a baseline was reached. Dye release was monitored at excitation and emission wavelengths of 622 and 650 nm, respectively.

2.4.3. Specific interaction with Lipid II

To determine if bovicin HC5, as nisin, could exhibit Lipid II-binding ability, model membranes containing Lipid II and different lipid compositions were used in several different assays. The CF-leakage assay was initially performed to investigate the pore-forming capacity of bovicin HC5 in neutral conditions (pH 7.5), using DOPC vesicles.

In DOPC/DOPG vesicles, with a negative surface charge, and in liposomes made of pure DOPC, bovicin HC5 or nisin did not cause CF efflux, even at a concentration of 1 μM (data not shown). In the presence of DOPC vesicles containing 0.1 mol % Lipid II, the addition of 0.1 μM or 1 μM bovicin HC5 (Figure 3, traces A and B) did not result in significant efflux activity (about 10 % when 1 μM bovicin HC5 was used). This strongly contrasted with the effect of 0.1 μM nisin, which caused an immediate leakage at CF-loaded DOPC/Lipid II vesicles, due to the Lipid II-dependent pore formation capacity of nisin; in this case, a leakage of 80 % was observed (Figure 3, trace C).

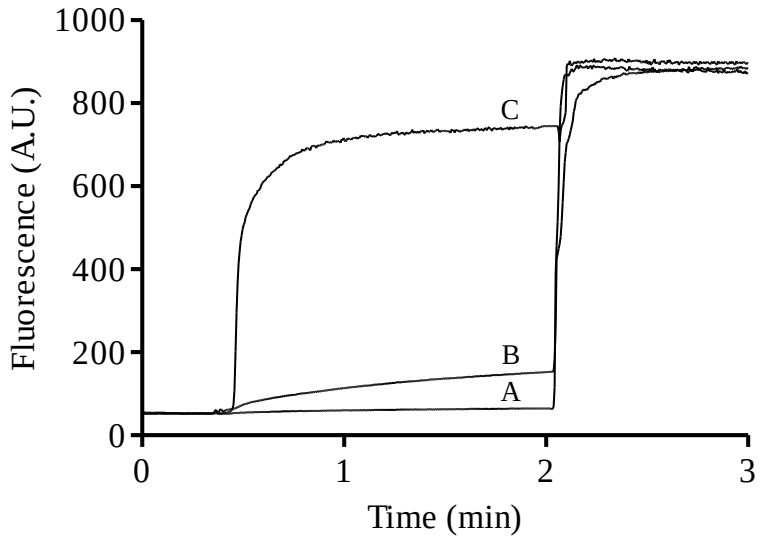


Figure 3: Activity of 0.1 μM (A) and 1 μM (B) bovicin HC5 towards DOPC Lipid II vesicles. Fluorescence of samples containing CF-loaded DOPC vesicles with 0.1 mol % Lipid II was recorded for 3 min. Nisin or bovicin HC5 were added after 20 s and Triton X-100 solution was added to yield the 100 % leakage value. Activity of 0.1 μM nisin (C) is shown as a positive control.

Some antibiotics are known to form small pores on sensitive membranes and a similar situation could be the case for bovicin HC5. To check the possibility that bovicin HC5-induced pores could be smaller than the pores formed by nisin and too narrow to allow CF leakage (1 nm of molecular radius), we performed a proton leakage assay, using HPTS. This is a fluorescent compound that can be used as a pH dependent probe and the influx of H^+ can be measured by the amount of quenching obtained (van Kan *et al.*, 2002).

The ability of bovicin HC5 and nisin to dissipate the applied proton gradient was measured as a change in HPTS emission. No decrease of fluorescence intensity was observed when the bacteriocins were added to DOPC containing vesicles (25 μM Lipid-Pi) in the absence of Lipid II (data not shown). The addition of 0.1 μM bovicin HC5 at HPTS-loaded DOPC/Lipid II vesicles did not result in proton influx (Figure 4, trace A), but the influx of H^+ was observed when 1 μM bovicin HC5 was used (Figure 4, trace B). The addition of nisin (0.1 μM final concentration) resulted in an immediate dissipation of the proton gradient over the DOPC Lipid II containing vesicles (Figure 4, trace C), and this effect was approximately four-fold higher when compared to the effect obtained with 1 μM bovicin HC5.

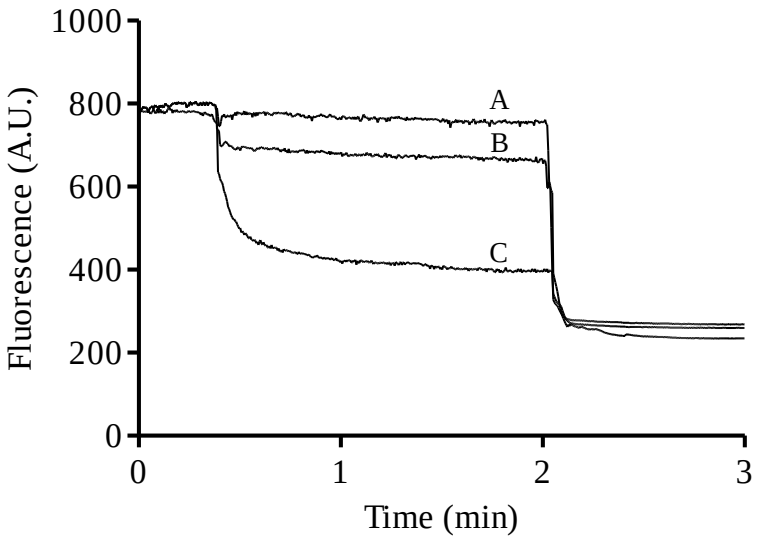


Figure 4: Effect of bovicin HC5 and nisin on the proton permeability of DOPC Lipid II containing vesicles. Time courses of HPTS fluorescence are shown after addition of the bacteriocins to model membranes: (A) 0.1 μM bovicin HC5; (B) 1 μM bovicin HC5; (C) 0.1 μM nisin. Measurements were performed in 10 mM MES, 0.2 M Na_2SO_4 buffer (pH 5.5) and DOPC Lipid II vesicles (25 μM final lipid-Pi).

The results obtained with CF and HPTS leakage assays showed that bovicin HC5, in contrast to nisin, did not induce significant leakage in DOPC/Lipid II vesicles, even at micromolar range, which indicates that bovicin HC5 does not perturb the membrane barrier in DOPC vesicles in a Lipid II-dependent way.

To check if bovicin HC5 was able to interact with Lipid II even in the absence of leakage, we performed competition assays, by sequential addition of nisin after bovicin HC5. A similar approach had previously been used with vancomycin in the study that determined the capacity of nisin (Breukink *et al.*, 1999) and mutacin (Hasper *et al.*, 2004) to bind to Lipid II.

Similar to the results presented in Figure 3, a CF-leakage of 80 % is observed when 0.1 μM nisin is added to DOPC vesicles containing Lipid II (Figure 5, trace A). In the presence of 0.02 μM bovicin HC5, CF leakage was significantly reduced by 50 % (Figure 5, trace C), and increasing concentrations of bovicin HC5 resulted in higher reductions until complete inhibition of nisin induced-CF leakage was obtained at 0.1 μM bovicin HC5 (Figure 5, trace G).

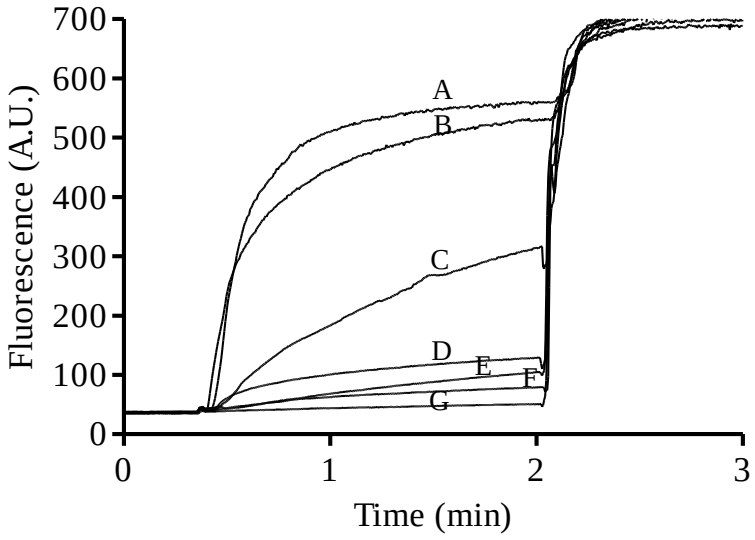


Figure 5: Interference of Lipid II-dependent pore formation activity of nisin by bovicin HC5. Different concentrations of bovicin HC5 (0.01-0.1 μM) were added 20 seconds prior nisin addition (0.1 μM final concentration) to DOPC vesicles (25 μM lipid-Pi) containing 0.1 mol % Lipid II. CF leakage was monitored for 2 minutes, and after that, Triton X-100 was added to determine the maximum CF leakage. (A) 0.1 μM nisin; (B) 0.01 μM bovicin HC5; (C) 0.02 μM bovicin HC5; (D) 0.03 μM bovicin HC5; (E) 0.04 μM bovicin HC5; (F) 0.05 μM bovicin HC5; (G) 0.1 μM bovicin HC5.

These results clearly show that bovicin HC5 was able to bind to Lipid II. This binding inhibited the pore-forming activity of nisin, most likely because Lipid II binding sites were already occupied by bovicin HC5, which indicates that both bacteriocins compete for the same receptor and bovicin HC5 binds with high affinity to the Lipid II, which becomes unavailable to nisin binding.

In order to gain more insight into the interaction between bovicin HC5 and Lipid II, ITC measurements were performed with DOPC vesicles with and without Lipid II, which were titrated into a solution of 20 μM bovicin HC5. In the absence of Lipid II, bovicin HC5 displayed little interaction with the DOPC vesicles (data not shown). However, in the presence of 2 mol % Lipid II containing LUVs, significant interaction between bovicin HC5 and Lipid II was evidenced by the heat effects, and a clear saturation of the interaction could be observed above molar ratio of 1.5 (Figure 6). The apparent binding constant (Ka) between bovicin HC5 and Lipid II was estimated to be 3.1×10^6 M^{-1} and the stoichiometry of this binding was determined to be 1:1, when all the amount of Lipid II present at the vesicles was considered.

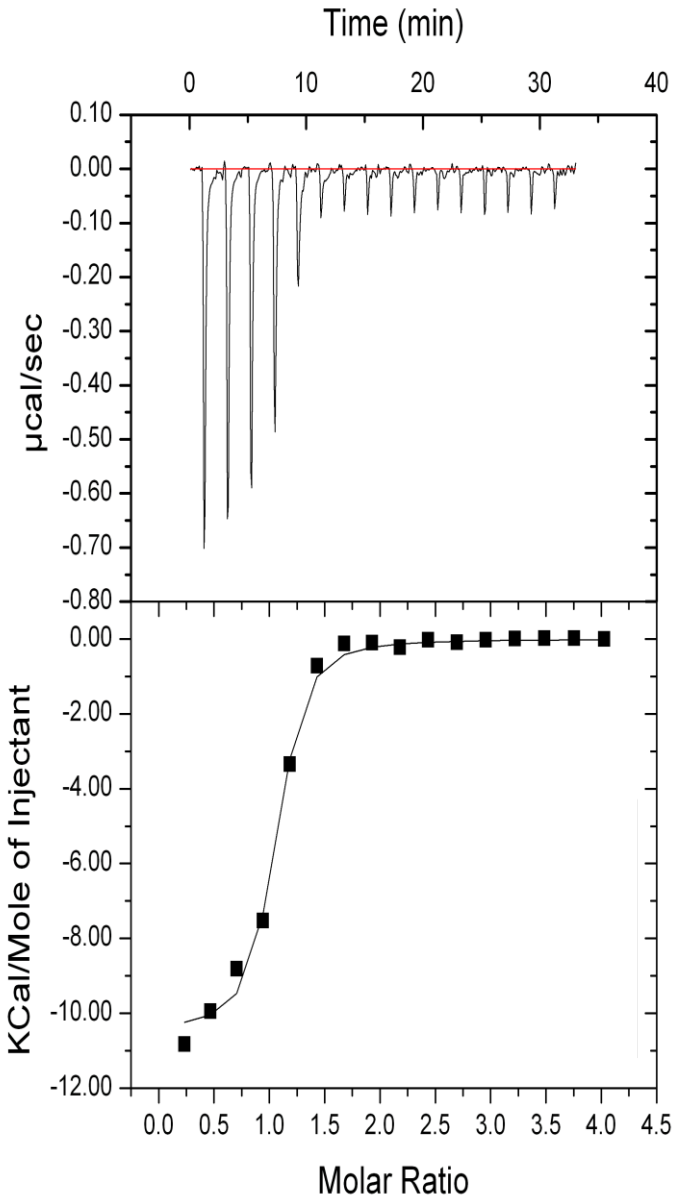


Figure 6: Calorimetric titrations of bovicin HC5 with DOPC vesicles containing 2 mol % Lipid II. Vesicles were dissolved in 10 mM Tris and 150 mM NaCl (pH 7.5). The graph on the top shows the heat peaks after injections of 5 µL vesicles (20 mM final phospholipid concentration) into the sample cell containing 20 µM bovicin HC5 in the same buffer. The bottom graph displays the integrated heat per injection, normalized to the injected amount of moles of Lipid II and is displayed against the molar ratio of Lipid II versus bovicin HC5.

In order to investigate if bovicin HC5 could assemble into a pre-pore-like structure (together with Lipid II) as was shown for a nisin hinge-mutant that lacked the ability to form pores (Hasper *et al.*, 2004), we used pyrene labeled Lipid II. This same

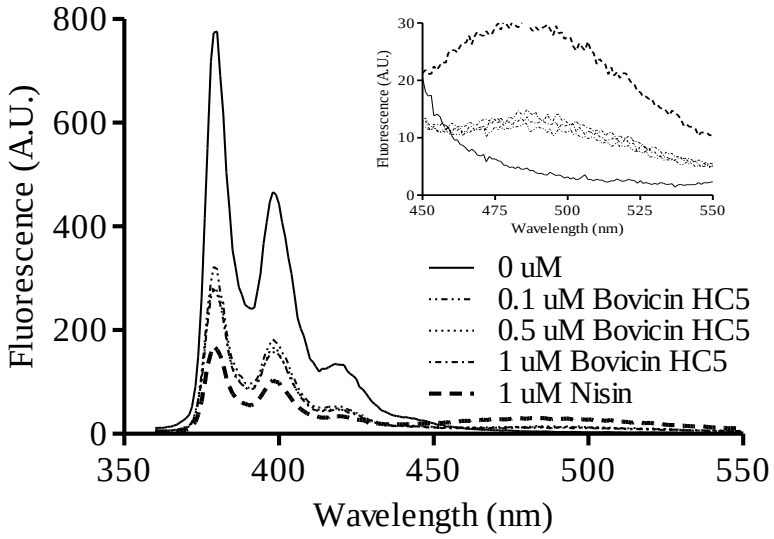
approach was previously used to characterize the nisin-Lipid II pore complex (Breukink *et al.*, 2003; Hasper *et al.*, 2004).

Pyrene labeled Lipid II can be used as a sensor of pore assembly, because pyrene shows a distance dependent signal: pyrene monomers exhibit characteristic fluorescence emission maxima at three wavelengths (378, 398 and 417 nm). In addition, pyrene can display a fluorescence peak around 495 nm, which occurs when two pyrene rings become stacked at a distance of 0.35 nm of each other and form an excited state dimer, which is called excimer (Somerharju, 2002).

Addition of bovicin HC5 to pyrene labeled Lipid II containing DOPC vesicles caused a large decrease of monomers signals intensity, likely due to the interaction of bovicin HC5 with Lipid II. However, only a small increase, not proportional to the reduction at monomer signals, was determined between 480 and 510 nm, where the excimer fluorescence was expected to be observed (Figure 7A).

The reduction of monomer fluorescence caused by addition of bovicin HC5 had a similar extent as the reduction caused by nisin, but the induced excimer formation was more evident with nisin. A more quantitative picture is presented in Figure 8B where the ratio between monomer and excimer signals (E/M) is depicted as a function of the peptide concentration. The maximal ratio that was obtained was 0.018 and 0.050 for bovicin HC5 and nisin, respectively. This plateau value was reached at concentrations of 0.1 μM bovicin HC5 and 0.2 μM nisin (Figure 7B).

A



B

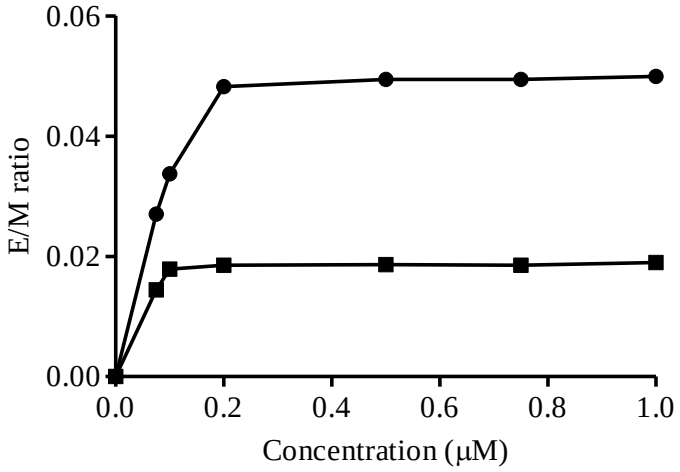


Figure 7: (**A**) Fluorescence spectra of pyrene labeled Lipid II (0.5 mol %) in DOPC vesicles (25 μM lipid-Pi). Pyrene labeled Lipid II fluorescence was recorded in the absence of bovicin HC5 and after incubation for 5 min with different concentrations of bovicin HC5 (0.1; 0.5; 1 μM). The fluorescence spectrum in the absence of bacteriocins (0 μM) and after addition of 1 μM nisin are shown as negative and positive controls, respectively. The inset shows an enlarged view of the excimer emission part of the spectra. Spectral recordings were performed between 360 and 550 nm, and at an excitation wavelength of 350 nm. (**B**) Ratio between monomer and excimer signals (E/M ratio) of pyrene labeled Lipid II obtained for DOPC LUVs, after addition of nisin (circles) and bovicin HC5 (squares). The E/M ratio is shown as a function of bacteriocin concentration.

These latter results indicated that bovicin HC5 does assemble into some kind of complex with Lipid II, but there might be some differences between bovicin HC5 and nisin with respect to the pore-forming ability. Aiming to investigate if this assemble involved a pre-pore-complex or if the membranes were too thick to allow actual pore-formation, we decided to check the possible Lipid II-mediated pore formation of bovicin HC5 using another membrane system, composed of phospholipids with shorter acyl chain length, DLPC/DMoPC ($C_{12:0}$/$C_{14:1}$; 1:1 mol/mol). Such thin membranes were stable and no spontaneously CF-leakage was detected.

A different picture emerged upon testing the effects of the lantibiotics on these shorter acyl chain length membrane system compared to the results above, obtained with DOPC vesicles. In the presence of Lipid II, considerable CF-leakage was detected when bovicin HC5 was added to the DLPC/DMoPC vesicles (0.1 μM and 1 μM, traces A and C, figure 8). However, even in the absence of Lipid II, leakage of CF was observed when 1 μM bovicin HC5 was added (trace B, figure 8). The addition of 0.1 μM nisin caused more than 80 % or 95 % of CF-leakage from DLPC/DMoPC vesicles, without and with 0.1 mol % Lipid II, respectively (traces D and E, figure 8).

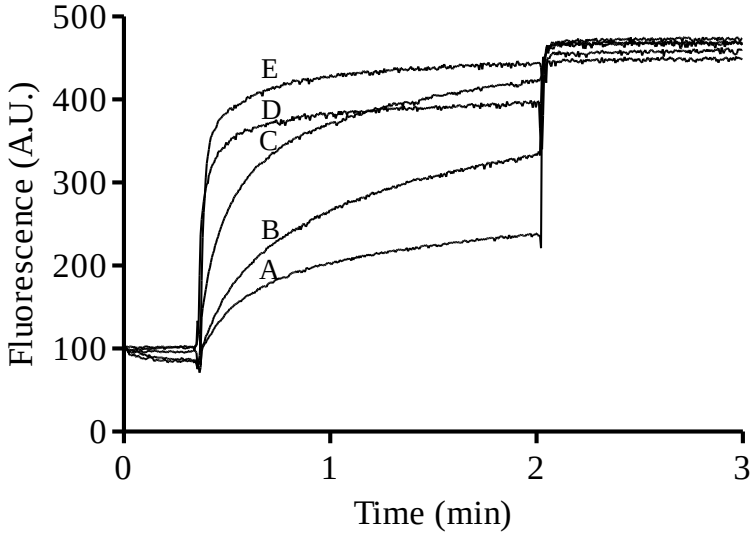


Figure 8: Activity of bovicin HC5 ((A) 0.1 μM; (C) 1 μM) and nisin ((E) 0.1 μM) towards DLPC/DMoPC Lipid II vesicles. Activity of 1 μM bovicin HC5 (B) and 0.1 μM nisin (D) towards DLPC/DMoPC vesicles without Lipid II are also shown. Fluorescence of samples containing CF-loaded DLPC/DMoPC vesicles with 0.1 mol % Lipid II was recorded for 3 min. Nisin or bovicin HC5 were added after 20 s and 100 % leakage level was determined by addition of Triton X-100.

The leakage caused by bovicin HC5 towards DLPC/DMoPC vesicles was stimulated by the presence of Lipid II, although the rate and the efficiency of CF release was lower than the leakage caused by nisin in the presence or absence of Lipid II. Since Lipid II-mediated pore formation by bovicin HC5 is dependent on the membrane thickness, we decided to check whether the segregation of Lipid II into domains could be part of bovicin HC5 mode of action, as already demonstrated for mutacin 1140 and mutants of nisin (Hasper *et al.*, 2006; Wiedemann *et al.*, 2001).

We visualized the bovicin HC5's capacity of assembly with Lipid II into a complex in a macro scale using GUVs composed of DOPC and 0.2 mol % NBD-labeled Lipid II. The GUVs were prepared in homemade chambers and analyzed directly on a confocal microscope. Bovicin HC5 or nisin (few µl from a 1 mM stock solution) were added to one edge of the sample chamber, and they diffused into the field of focus.

Before addition of peptides to the chamber, the GUVs had a uniform fluorescent membrane, as a consequence of the homogeneous distribution of NBD-labeled Lipid II over the membrane (Figures 9A and 10A).

As already demonstrated by Hasper *et al.* (2006), after 2 min of exposure to 20 µM nisin, the green fluorescence used started to segregate into patches (Figure 9B), and after 7 min, the typical Lipid II patches were obtained (Figure 9C). Moreover, after 10 min of exposure to nisin, the vesicles in close proximity started to adhere to each other, the adhesion sites became more fluorescent and the rest of the fluorescence remained spread into patches over the membrane (Figure 9D). Those results confirmed the sequestration of Lipid II into large domains induced by nisin, as a result of the high-affinity interaction between this lantibiotic and its receptor in membranes.

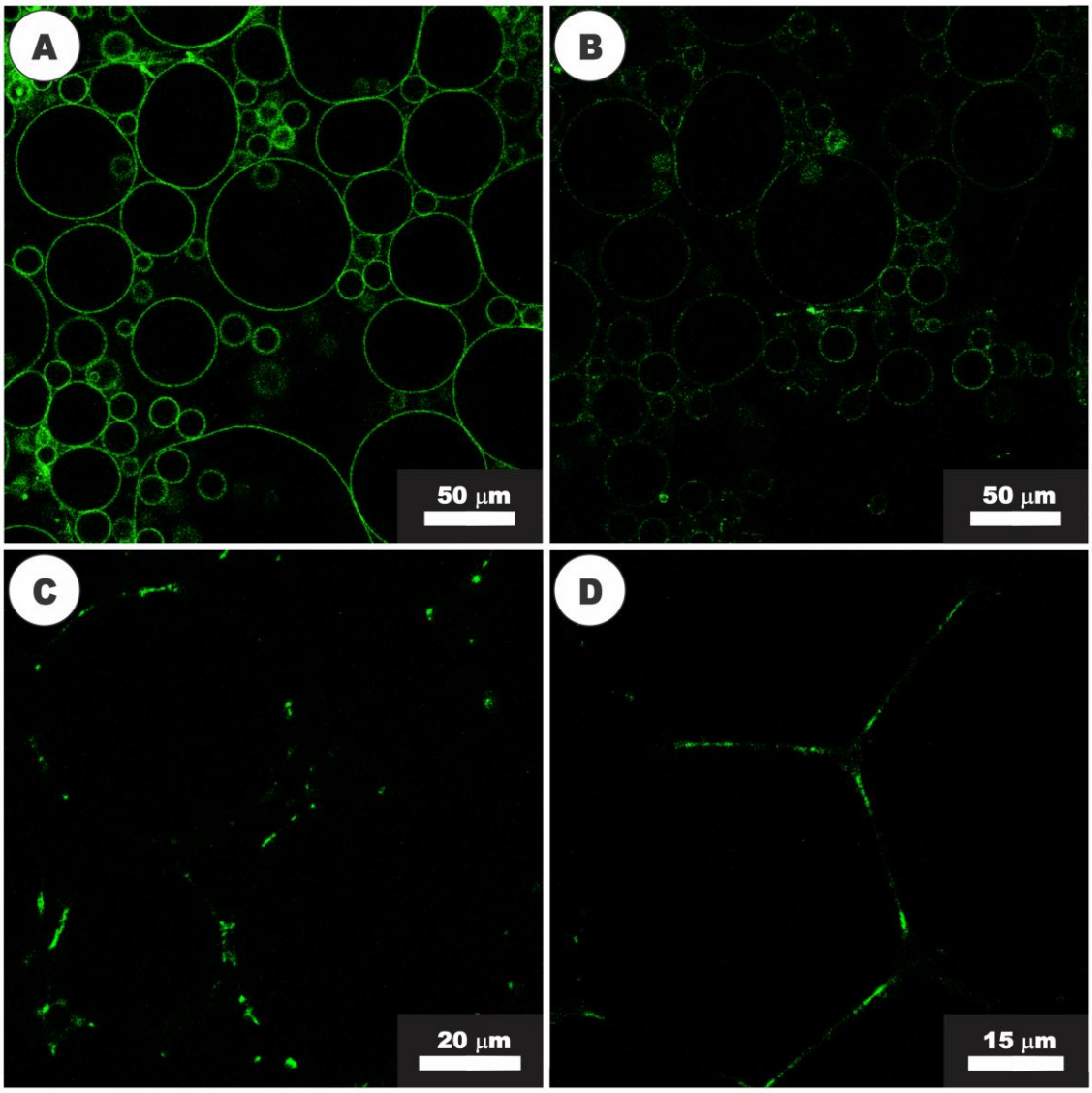


Figure 9: Activity of nisin (20 µM) visualized with confocal fluorescence microscopy. (A) GUVs composed of DOPC and NBD-labeled Lipid II before the addition of nisin; (B) GUVs after 2 min of exposure to nisin, showing the beginning of Lipid II segregation process; (C) After 7 min of nisin activity, the clustering of Lipid II into large domains was observed. (D) Adhesion of GUVs in close proximity, after 10 min of exposure to nisin.

The addition of bovicin HC5 to the GUVs also resulted in a heterogeneous spreading of the fluorescence, but a different picture was obtained. After 5 min of exposure to 20 µM bovicin HC5, the vesicles changed their common shape (Figure 10B). However, the Lipid II sequestration into separate domains was observed only when the vesicles were exposed for long periods of time to the same concentration of

bovicin HC5 (30 min; Figure 10C) or if higher amounts of the peptide (40 µM) were used (Figure 10D).

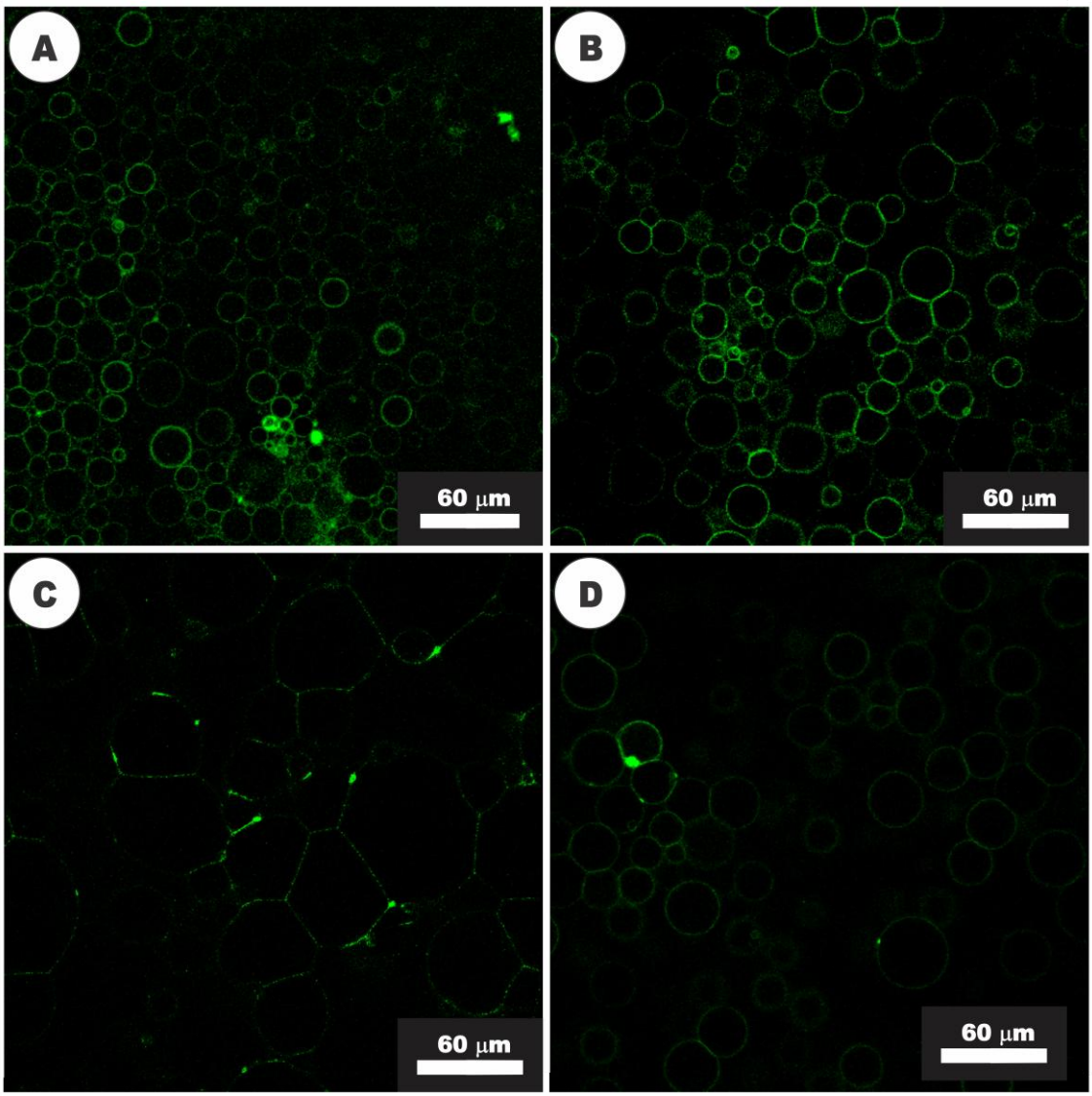


Figure 10: Activity of bovicin HC5 visualized with confocal fluorescence microscopy. (A) GUVs composed of DOPC and NBD-labeled Lipid II before the addition of bovicin HC5; (B) Changing of the common shape of GUVs, after 5 min of exposure to bovicin HC5 (20 µM); (C) After 30 min of bovicin HC5 activity or (D) at higher concentrations of this peptide (40 µM), the segregation of Lipid II into domains was observed.

2.5. Discussion

Several antimicrobial peptides are known to use Lipid II as a target to exert their antimicrobial activity in nanomolar range, such as vancomycin (Sheldrick *et al.*, 1978) and members of the lantibiotic family of bacteriocins, as nisin (Hasper *et al.*, 2006), plantaricin C (Wiedemann *et al.*, 2006), gallidermin (Bonelli *et al.*, 2006) and mutacin 1140 (Smith *et al.*, 2006). Comparison of primary structures and activities of lantibiotics reveals the complexity of lantibiotic's mode of action as well as the different interactions displayed by molecules that share the same target, such as Lipid II (Wiedemann *et al.*, 2006).

In the present study, we show that bovicin HC5, another bacteriocin belonging to the type A lantibiotic group, has a primary mode of action similar to nisin, which involves specific interaction with the cell wall precursor Lipid II. However, some differences regarding the pore forming capacity of both bacteriocins were demonstrated.

Bovicin HC5 was able to inhibit nisin-sensitive and nisin-resistant bacteria, but the patterns of activity were quite different from nisin-treated cells. Bovicin HC5 show a more persistent antibacterial activity, inhibiting the growth of the indicator strains even after 24 hours of incubation. Bovicin HC5 was also 5-fold more effective than nisin in inhibiting the growth of the nisin-resistant bacteria, *S. warneri*. However, despite the sensitivity, the membrane potential in intact *S. conhii* cells was only slightly affected by high concentrations of bovicin HC5 (20x the MIC). This effect differed from nisin, which caused major membrane depolarization on *S. conhii* cells.

Bovicin HC5 did not produce significant CF leakage or proton efflux from liposomes composed of DOPC and Lipid II. These results contrast the effect of nisin, which caused an efficient and rapid CF release and HPTS efflux from the vesicles, even at small concentrations (0.1 μM). The pore formation capacity of bovicin HC5 in DOPC containing Lipid II vesicles was markedly reduced if compared with nisin.

Nevertheless, independent of the presence of detectable leakage, bovicin HC5 was able to bind to Lipid II, and this binding was confirmed when competition assays and ITC experiments were performed. The addition of increasing concentrations of bovicin HC5 reduced the activity of nisin and blocked the nisin pore-forming capacity when equal concentrations of bovicin HC5 and nisin were used. The ITC results show that bovicin HC5 bound with an apparent high affinity to Lipid II ($K_a=3.4 \times 10^6\ M^{-1}$), with a stoichiometry of 1:1.

All these results indicate that bovicin HC5 efficiently binds to Lipid II and the stability of this interaction is strong enough to prevent an interaction between nisin and its target. A possible explanation for this high affinity to Lipid II is that bovicin HC5 has an arginine in position 4 (instead of isoleucine in nisin), which provides an additional positive charge to the putative Lipid II-motif, and might enhance binding to the pyrophosphate moiety of Lipid II, considered the primary binding site for nisin (Hsu *et al.*, 2004).

Using pyrene labeled Lipid II we show that bovicin HC5 is able to bring some Lipid II molecules into close proximity, assembling into a pre-pore-like structure. Mutacin 1140 and mutants of nisin that lack the hinge region are also not able to form pores on DOPC vesicles containing Lipid II, but they cause some pyrene excimer fluorescence by assembling with Lipid II into a pre-pore-like structure (Hasper *et al.*, 2006; Wiedemann *et al.*, 2001).

The structure of bovicin HC5/Lipid II complex has not yet been resolved, but it seems to be different from the nisin/Lipid II complex. In the presence of bovicin HC5, the orientation of the pyrenes within the pore is probable different, and they stack to a much lesser extent than in the pore formed by nisin (the distance between two Lipid II molecules within the pore complex formed by nisin was estimated to be 18 Å (Breukink *et al.*, 2003)), leading to a lower excimer signal.

The thickness of the lipid bilayer in liposomes composed of monounsaturated phosphorylcholines depends on the number of acyl chain carbons and can be estimated by the equation: $dL = (15.9 \pm 1.4) + (1.5 \pm 0.2)n$ Å, where dL is the bilayer thickness, and n is the number of carbon atoms of the acyl chain (Kucerka *et al.*, 2004). Then we can assume that the thickness of the membranes composed of DOPC was approximately 43 Å. Nisin has an overall length of 50 Å, while the predicted bovicin HC5's length is 33 Å. This observation suggests that bovicin HC5 is too short to permeabilize thick lipid bilayers, composed of phospholipids with C_{18} or longer acyl chains.

This hypothesis was confirmed when the bovicin HC5 activity was determined towards vesicles composed of DLPC/DMoPC or shorter acyl chains (data not shown). In such conditions, bovicin HC5 was able to form pores, causing the efflux of CF from the liposomes, which was more efficient when Lipid II was present in the bilayer. According to the equation described by Kucerka *et al*. (2004), the thickness of the membranes composed of DLPC/DMoPC can be estimated to be 38 Å, thus shorter enough to be spanned by bovicin HC5.

This membrane thickness dependency has also been demonstrated to gallidermin's pore formation capacity. Gallidermin, a peptide that has 22 amino acids residues and an inflexible C-terminus (forming a closed structure due to a lanthionine ring), also interacts with Lipid II and its pore-formation capacity was only observed in model membranes composed of short-chain phospholipids (Cotter *et al.*, 2005). More recently, Christ *et al.* (2008) demonstrated that besides membrane thickness, the composition of the sensitive membrane, such as the presence of branched membrane lipids, can modulate the membrane properties and facilitate the access to Lipid II by gallidermin.

Non-targeted amphiphilic peptides, such as magainin and dermaseptin, do not form a defined pore structure on sensitive cell membranes but instead they can be present in multiple aggregational states (Shai, 1999). This can also be the case when high concentrations of nisin and bovicin HC5 are used towards thin membranes, since we demonstrated the occurrence of CF leakage caused by both bacteriocins in DLPC/DMoPC liposomes in the absence of Lipid II, which suggest that they are able to disturb the membrane integrity in special cases, independent of Lipid II.

Early studies indicated that bovicin HC5 may impair membrane functions. It was previously visualized through atomic force microscopy that the treatment of sensitive cells with bovicin HC5 induces severe cell-shape deformations (H.C. Mantovani, unpublished results), and these characteristics are typical for compounds that interfere with cell wall biosynthesis. In this study, we demonstrated that bovicin HC5 also induces the segregation of Lipid II into domains in giant model membranes, a mechanism that strengthened the idea that bovicin HC5 interferes with the cell wall synthesis.

When used together, bovicin HC5 and nisin show additive effects and they inhibit sensitive bacteria in a more efficient way than when they are used separately (H.C. Mantovani, unpublished results). Moreover, bacteria that can readily become resistant to nisin did not become significantly resistant to bovicin, even after they were repeatedly transferred with sublethal doses of the peptide (Mantovani and Russell, 2003), suggesting that these two lantibiotics, although sharing the same target, still have different modes of action or different mechanisms to reach their targets.

In this study, we determine the mode of action of bovicin HC5 based on the interaction with its specific target, the Lipid II. The pore-forming activity of bovicin HC5 is clearly dependent on the membrane thickness and stimulated by the presence of Lipid II, although a generalized cell membrane disruption activity, independent of Lipid

II, can also be observed in thin membranes when higher concentrations of bovicin HC5 are used. Independent on the membrane thickness, bovicin HC5 maintains its antibacterial activity, by binding to Lipid II and recruiting some Lipid II molecules as a pre-pore-like structure. Besides those activities, bovicin HC5 acts by sequestering Lipid II into domains, inhibiting the bacterial cell wall biosynthesis. These varied mechanisms can be combined toward sensitive cells and might explain the differences observed in sensitivity to the lantibiotics depending on the bacterial strain tested.

2.6. References

Bierbaum, G.; Sahl, H.G. Induction of autolysis of staphylococci by the basic peptide antibiotic pep5 and nisin and their influence on the activity of autolytic enzymes. Archives in Microbiology 141, 249–254, 1985.

Bligh, E.G.; Dyer, W.J. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917, 1959.

Bonelli, R.R.; Schneider, T.; Sahl, H.G.; Wiedemann, I. Insights into in vivo activities of lantibiotics from gallidermin and epidermin mode-of-action studies. Antimicrobial Agents and Chemotherapy 50, 1449-1457, 2006.

Breukink, E.; van Heusden, H.E.; Vollmerhaus, P.J.; Swiezewska, E.; Brunner, L.; Walker, S.; Heck, A.J.R.; de Kruijff, B. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. The Journal of Biological Chemistry 278, 19898-19903, 2003.

Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O.P.; Sahl, H.; de Kruijff, B. Use of the cell wall precursor Lipid II by a pore-forming peptide antibiotic. Science 286, 2361-2364, 1999.

Brötz, H.; Josten, M.; Wiedemann, I.; Schneider, U.; Götz, F.; Bierbaum, G. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Molecular Microbiology 30, 317–327, 1998.

Christ, K.; Al-Kaddah, S.; Wiedemann, I.; Rattay, B.; Sahl, H.S.; Bendas, G. Membrane lipids determine the antibiotic activity of the lantibiotic gallidermin. Journal of Membrane Biology 226, 9-16, 2008.

Cleveland, J.; Montville, T.J.; Nes, I.F.; Chikindas, M.L. Bacteriocins: safe, natural antimicrobials for food preservation. The International Journal of Food Microbiology 71, 1–20, 2001.

Cotter, P.D.; Hill, C.; Ross, R.P. Bacterial lantibiotics: strategies to improve therapeutic potential. Current Protein & Peptide Science 6, 61–75, 2005.

Delves-Broughton, J. Nisin as a food preservative. Food Australia 57, 525-527, 2005.

Drosinos, E.H.; Mataragas, M.; Metaxopoulos, J. Modeling of growth and bacteriocin production by *Leuconostoc mesenteroides* E131. Meat Science 74, 690–696, 2006.

Gut, I.M.; Prouty, A.M.; Ballard, J.D.; van der Donk, W.A.; Blanke, S.R. Inhibition of *Bacillus anthracis* spore outgrowth by nisin. Antimicrobial Agents and Chemotherapy 52, 4281-4288, 2008.

Gut, I.M.; Blanke, S.R.; van der Donk, W.A. Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. ACS Chemical Biology 6, 744-752, 2011.

Hasper, H.E.; de Kruijff, B.; Breukink, E. Assembly and stability of nisin-Lipid II pores. Biochemistry 43, 11567-11575, 2004.

Hasper, H.E.; Kramer, N.E.; Smith, J.L.; Hillman, J.D.; Zachariah, C.; Kuipers, O.P.; de Kruijff, B.; Breukink, E. An alternative bactericidal mechanism of action for lantibiotic peptides that target Lipid II. Science 313, 1636-1637, 2006.

Héchard, Y.; Sahl, H.G. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84, 545-557, 2002.

Hope, M.J.; Bally, M.B.; Webb, G.; Cullis, P.R. Production of large unilamellar vesicles by a rapid extrusion procedure – characterization of size distribution, trapped volume and ability to maintain a membrane-potential. Biochimica et Biophysica Acta, 812, 55-65, 1985.

Hsu, S.T.; Breukink, E.; Tischenko, E.; Lutters, M.A.; de Kruijff, B.; Kaptein, R.; Bonvin, A.M.; van Nuland, N.A. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature Structural & Molecular Biology 11, 963-967, 2004.

Kucerka, N.; Uhriková, D.; Teixeira, J.; Balgavý, P. Bilayer thickness in unilamellar phosphatidylcholine vesicles: small-angle nêutron scattering using contrast variation. Physica B: Physics of Condensed Matter 350, 639-642, 2004.

Mantovani, H.C.; Kam, D.K.; Ha, J.K.; Russell, J.B. The antibacterial activity and sensitivity of *Streptococcus bovis* strains isolated from the rumen of cattle. FEMS Microbiology Ecology 37, 223-229, 2001.

Mantovani, H.C.; Hu, H.; Worobo, R.W.; Russell, J.B. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology 148, 3347–3352, 2002.

Mantovani, H.C.; Russell, J.B. Inhibition of *Listeria monocytogenes* by bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5. The International Journal of Food Microbiology 89, 77–83, 2003.

Pag, U.; Sahl, H.G. Multiple activities in lantibiotics - models for the design of novel antibiotics? Current Pharmaceutical Design 8, 815–833, 2002.

Ramadurai, S.; Holt, A.; Schafer, L.V.; Krasnikov, V.V.; Rijkers, D.T.S.; Marrink, S.J.; Killian, J.A.; Poolman, B. Influence of hydrophobic mismatch and amino acid composition on the lateral diffusion of transmembrane peptides. Biophysical Journal 99, 1447-1454, 2010.

Rouser, G.; Fkeischer, S.; Yamamoto, A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. Lipids 5, 494-496, 1970.

Shai, Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochimica et Biophysica Acta 1462, 55-70, 1999.

Sheldrick, G.M.; Jones, P.G.; Kennard, O.; Williams, D.H.; Smith, G.A. Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. Nature 271, 223–225, 1978.

Smith, L.; Hasper, H.; Breukink, E.; Novak, J.; Cerkasov, J.; Hillman, J.D.; Wilson-Stanford, S.; Orugunty, R.S. Elucidation of the antimicrobial mechanism of mutacin 1140. Biochemistry 47, 3308-3314, 2008.

Somerharju, P. Pyrene-labeled lipids as tools in membrane biophysics and cell biology. Chemistry and Physics of Lipids 116, 57-74, 2002.

van Kan, E.J.; Demel, R.A.; Breukink, E.; van der Bent, B.A.; de Kruijff, B. Clavanin permeabilizes target membranes via two distinctly different pH-dependent mechanisms. Biochemistry 41, 7529-7539, 2002.

Wiedemann, I.; Breukink, E.; van Kraaij, C.; Kuipers, O.P.; Bierbaum, G.; de Kruijff, B.; Sahl, H.G. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. Journal of Biological Chemistry 276, 1772–1779, 2001.

Wiedemann, I.; Bottiger, T.; Bonelli, R.R.; Schneider, T.; Sahl, H.G.; Martínez, B. Lipid II-based antimicrobial activity of the lantibiotic plantaricin C. Applied and Environmental Microbiology 72, 2809-2814, 2006.

CHAPTER 3

The effects of Lipid II binding on bovicin HC5.

3.1. Abstract

Bovicin HC5 is an antimicrobial peptide belonging to class I lantibiotics and produced by *Streptococcus bovis* HC5. It consists of 22 amino acids residues and has a tryptophan residue located at position 13. This peptide binds to Lipid II, the essential bacterial cell wall precursor. In this study, the interaction of bovicin HC5 with Lipid II was examined using tryptophan fluorescence and circular dichroism spectroscopy with model membrane systems that do or do not allow pore-formation by bovicin HC5. A blue-shift of 12 nm could be observed for the fluorescence emission maximum of the tryptophan residue for all membrane systems tested, in the presence of Lipid II. This change in fluorescence emission was paralleled by a decrease in accessibility towards acrylamide and phospholipids carrying a spin-label at the 12 position of the acyl chain, the most efficient quencher. The presence of Lipid II changed the orientation of bovicin HC5 in membranes from parallel to perpendicular with respect to the membrane surface. Moreover, the binding of Lipid II by bovicin HC5 causes a significant change in secondary structure although the nature of this change remains to be solved. The interaction of bovicin HC5 with Lipid II was highly stable even at pH 2.0.

3.2. Introduction

Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5, is a positively charged amphiphilic peptide consisting of 22 amino acids residues, including some post-translational modified amino acids residues. Bovicin HC5 is resistant to low pH, heat, proteinase K and chymotrypsin; it has a broad spectrum of activity and bacterial resistance has not yet been demonstrated among sensitive strains (Mantovani *et al.*, 2002).

Bovicin HC5 shares some similarities with nisin, the most well known bacteriocin, regarding the mechanism of action and Lipid-II binding domains. However, unlike nisin, the pore-forming activity of bovicin HC5 is membrane thickness-dependent, being detected only in membranes composed of DLPC/DMoPC ($C_{12:0}$/$C_{14:1}$) or shorter acyl chains. Since bovicin HC5 is about 12 amino acids residues shorter than nisin, its ability to permeabilize thick lipid bilayers (composed of phospholipids with C_{18} or longer acyl chains) appears to be limited. Independent on the membrane thickness, bovicin HC5 is still able to bind to Lipid II, recruiting some Lipid II molecules as a pre-pore-like structure and sequestering Lipid II into domains, with consequent inhibition of the bacterial cell wall biosynthesis (Paiva *et al.*, 2011).

Nisin shows a distinct Lipid II-binding mechanism and the pyrophosphate moiety of Lipid II is considered the primary binding site for nisin. Upon binding to Lipid II, the N-terminal part of nisin folds back onto the first two lanthionine rings, forming a cage-like structure. The backbone architecture of such pyrophosphate cage leads to the formation of five intermolecular hydrogen bonds between the amide groups of nisin and the highly electronegative pyrophosphate group of Lipid II. This Lipid II-recognition mechanism may be generalized to other lantibiotics that have the conserved N-terminal lanthionine rings (Hsu *et al.*, 2004).

The topology of bovicin HC5 upon interaction with its target Lipid II is still unknown, but as demonstrated for nisin (Breukink *et al.*, 1998), it might represent an important aspect of bovicin HC5`s mode of action. Bovicin HC5 contains an intrinsic tryptophan residue at position 13, just after the pyrophosphate cage determined to nisin and, in this study, the tryptophan residue of bovicin HC5 was used in fluorescence spectroscopy experiments to characterize the bovicin HC5-membrane association after interacting with Lipid II. Additionally, information about the secondary structure of bovicin HC5, in solution and upon binding to Lipid II, was gathered by circular dichroism spectroscopy.

3.3. Experimental procedures

3.3.1. Chemicals and materials

Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocoline ($C_{18:1}$, DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocoline ($C_{14:1}$, DMoPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocoline ($C_{12:0}$, DLPC), and the spin-labeled (SL) lipids 1,2-dioleoyl-*sn*-glycero-3-TEMPO-phosphocoline (TEMPO-PC), 1-palmitoyl-2-stearoyl(5-DOXYL)-*sn*-glycero-3-phosphocoline (5DOX-PC) and 1-palmitoyl-2-stearoyl(12-DOXYL)-*sn*-glycero-3-phosphocoline (12DOX-PC) were purchased from Avanti Polar Lipids, Inc.

Lipid II and water-soluble Lipid II (3-LII, a Lipid II variant that consists of a shortened prenyl chain, with three isoprene repeats, instead of 11 as on the natural occurring full-length lipid II) were synthesized and purified as described by Breukink *et al.* (2003).

Phospholipids were dissolved in chloroform:methanol (1:1) to a stock concentration of 10 mM and stored at -20 ºC. After destruction of phospholipids by adding perchloric acid, the sample concentrations were determined by inorganic phosphate analysis, according to the procedure described by Rouser *et al.* (1970).

All other chemicals used were of analytical or reagent grade.

3.3.2. Bovicin HC5

Streptococcus bovis HC5 was cultivated under anaerobic conditions in basal medium containing (per liter): 0.292 g K_2HPO_4; 0.292 g KH_2PO_4; 0.48 g $(NH_4)_2SO_4$; 0.48 g NaCl; 0.1 g $MgSO_4.7H_2O$; 0.064 g $CaCl_2.2H_2O$; 0.5 g cysteine hydrochloride; 4 g Na_2CO_3; 1 g trypticase; 0.5 g yeast extract at 39 ºC, overnight. Glucose was added as carbon source (4 g/L). Stock cultures were stored at – 70 °C, in solutions containing their own growth medium and 50 % glycerol.

Extracts of bovicin HC5 were prepared as described by Mantovani *et al*. (2002). Purification of bovicin HC5 was performed by RP-HPLC using a semipreparative column (Shimadzu C18; 5 μm; 150 by 6 mm [inner diameter]). The column was equilibrated with buffer A (0,1 % trifluoroacetic acid (TFA) in ultrapure water) and the peptide was eluted using a linear gradient of 35 to 50 % buffer B (80 % acetonitrile, 0,1 % TFA in ultrapure water), at a temperature of 22°C and flow rate of 1 ml min^{-1}. The absorbance was monitored at 214 and 280 nm and the eluted fraction corresponding to

pure bovicin HC5 was lyophilized. The purity of bovicin HC5 was confirmed using analytical HPLC and electrospray mass spectrometry.

Bovicin HC5 stock solution (1 mM, in 0.05 % acetic acid) was stored at -20 ºC until use. Protein concentration was determined using a bicinchoninic acid protein assay reagent (Pierce Chemical Corp., Bonn, Germany), with bovine serum albumin as a standard.

3.3.3. Large unilamellar vesicles and soluble Lipid II

Large unilamellar vesicles (LUVs) containing DOPC, DOPC with DOPG (3 or 12 %) or DLPC/DMoPC (1:1 mol/mol), with and without Lipid II, were prepared by the extrusion technique, as described by Hope *et al.* (1985). Buffer containing 10 mM Tris and 150 mM NaCl (pH 7.5) or buffer containing 10 mM potassium phosphate and 40 mM potassium sulphate (pH 6.0) were used to hydrate the dried lipid films, depending on the experiment performed (tryptophan and CD spectroscopy, respectively), and followed by vigorous stirring. The LUVs were prepared by repeated extrusion through polycarbonate filters with a 0.2 μm pore size (Isopore membrane filters; Millipore, Ireland).

Lipid and vesicle concentrations were based on inorganic phosphate determination (Rouser *et al.*, 1970). The final vesicle concentration used was 25 μM (final lipid-Pi) and the final concentration of Lipid II varied according to the experiment performed (1 or 4 mol % of the total lipid amount for tryptophan or CD spectroscopy, respectively). Control experiments were performed using vesicles without Lipid II, but containing the same phospholipid composition.

3.3.4. Emission spectra and intensity measurements

The fluorescence emission spectra of the intrinsic bovicin tryptophan residue were performed on a SLM Aminco Spectrofluorometer (SPF-500C), with spectral recordings between 300 and 450 nm (bandwidth 5 nm) as well as single-wavelength recordings at 340 nm, with excitation at 280 nm (bandwidth 5 nm), in the absence or presence of vesicles of the indicated composition at a concentration of 100 μM lipid-Pi. The measurements were performed after an incubation time of 5 min and the spectral changes were followed for 6 min.

The samples were continuously stirred in a 10 x 4 mm quartz cuvette and kept at 20 ºC, using a continuous circulation water bath. Bovicin HC5 was used at a

concentration of 1 μM and fluorescence spectra and single-wavelength recordings were corrected by blank subtraction. The bovicin HC5:Lipid II ratio was 1:1.

Titration experiments using individual samples were performed to investigate the concentration dependency of the spectral changes observed in the fluorescence spectra of bovicin HC5. Fluorescence intensities were measured as a function of the amount of added vesicles, at 340 nm, and averaged over recordings times of 20 s. Intensities and complete spectra were measured before and after addition of membranes (containing or not Lipid II). The Lipid II:bovicin HC5 ratios used for the individual samples varied from 0:1 to 4:1, and the experiments were performed at least in quadruplicate.

3.3.5. Acrylamide quenching

Insertion of bovicin HC5 into Lipid II-containing membranes was investigated with the water-soluble quencher acrylamide (Eftink and Ghiron, 1976). Acrylamide quenching experiments were carried out at an excitation wavelength of 280, recorded at 340 nm and averaged over measuring times of 20 s. Small aliquots from an aqueous 3 M acrylamide stock solution were added stepwise, and an equilibration time of 60 s was taken to allow homogeneous distributions of the acrylamide. Acrylamide titration of bovicin samples, in the absence or in the presence of Lipid II containing membranes, was started 5 min after the addition of samples.

Titrations were performed in triplicate and the data were analyzed according to the Stern-Volmer equation (Eftink and Ghiron, 1976).

$$F_0 / F = 1 + K_{SV} \times [Q]$$

Where F_0 is the fluorescence in the absence and F is the fluorescence in the presence of increasing quencher concentrations [Q]. The Stern-Volmer quenching constant K_{SV} is the product of the bimolecular quenching rate k_q and the fluorescence lifetime τ_0:

$$K_{SV} = k_q \times \tau_0$$

Both the Stern-Volmer constant and the bimolecular quenching rate can be used as measure for the accessibility of the tryptophan residue to acrylamide. The K_{SV} was calculated as the slope of the Stern-Volmer plot, which is linear for acrylamide concentrations up to 60 mM.

3.3.6. Spin-labeled lipid quenching

The position of the tryptophan residue of bovicin HC5 in the Lipid II containing membranes was assessed by considering the quenching effect of three different SL-

phospholipids on the fluorescence of bovicin tryptophan. Single-wavelength recordings at 340 nm and spectral recordings were performed in the absence and presence of DOPC or DLPC/DMoPC vesicles containing 1 mol % Lipid II and an additional amount of 25 % of one of the spin-labeled lipids (TEMPO-PC, 5DOX-PC or 12DOX-PC).

The change of fluorescence intensity upon addition of 100 μM lipid-Pi of the spin-labeled vesicles to a solution of 1 μM bovicin HC5 was compared to the fluorescence intensity change upon addition of the same amount of vesicles containing unlabeled PC (F_0). The data were analyzed as quenching efficiency (Q_{ef}) of each SL-lipid, via the equation

$$Q_{ef} = (1 - F_h / F_0) \times 100$$

In which F_h is the fluorescence in the presence of quencher at depth h and F_0 is the fluorescence in the absence of quencher.

3.3.7. Circular dichroism measurements

The circular dichroism spectra of bovicin HC5 were recorded on a Jasco-810 CD spectrometer in a quartz cuvette of 1-mm light path. The temperature was kept at 20 ºC by a Jasco Peltier CDF 426S and the samples were scanned in the wavelength range of 195-280 nm. Data were digitally collected every 0.2 nm at a scan speed of 20 nm/min, with 1-s response time and 2 mm bandwidth.

The spectra were recorded at a bovicin HC5 concentration of 20 μM in 10 mM potassium phosphate/40 mM potassium sulphate buffer (different pH values) or 0-30 μM Lipid II final concentration or 0-700 μM vesicles without Lipid II (concentrations based on Lipid II containing vesicles). Each spectrum was an average of five recordings.

The effect of Lipid II on the secondary structure of bovicin HC5 was determined by comparison of the bovicin HC5 individual spectra obtained in buffer and the spectra obtained in the presence of Lipid II. In all cases, blank spectra were subtracted from the bovicin HC5 spectra. The results were given in deg cm^{-2} $dmol^{-1}$.

3.4. Results

3.4.1. Tryptophan fluorescence - Emission spectra

Tryptophan fluorescence spectroscopy can be considered a valuable tool to study the membrane association of peptides (Breukink *et al.*, 1998). Bovicin HC5 has one intrinsic tryptophan residue, which enabled the use of fluorescence spectroscopy to study the interaction of bovicin HC5 with membranes.

The fluorescence emission spectra of this tryptophan residue were recorded in the absence or presence of model membranes with different phospholipid compositions that would either allow pore-formation (DLPC/DMoPC vesicles, 1:1 mol/mol) or where pore-formation has not been demonstrated (DOPC vesicles) (Paiva *et al.*, 2011).

When bovicin HC5 was resuspended in buffer, the emission spectrum of the tryptophan showed a fluorescence emission maximum at 353 nm (Figure 1, dotted line). Upon the addition of vesicles containing Lipid II a blue-shift of the emission maximum of 12 nm (maximal emission at 341 nm) could be observed, which was paralleled by an increase in fluorescence intensity (2.45- and 2.12-fold, in the presence of DOPC and DLPC/DMoPC vesicles, respectively) (Figure 1, tracings A and B).

The two model membranes also showed a consistent difference in fluorescence intensity, being the emission spectrum for the DLPC/DMoPC less intense than the observed for DOPC vesicles. This result may be due to pore-assembly of bovicin HC5 in thinner membranes, where in the pore-configuration the tryptophan residue is quenched by self-quenching of the tryptophan residue or by another residue from the neighboring bovicin HC5.

No effect was observed when the emission spectra were recorded in the presence of vesicles without Lipid II or in the presence of negatively charged liposomes, composed by DOPC and 3 mol % DOPG (data not shown).

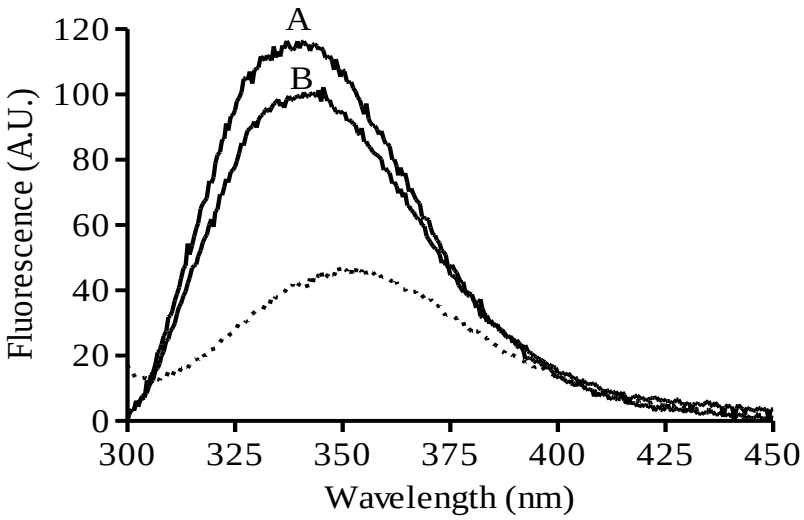


Figure 1: Fluorescence emission spectra of the tryptophan residue of bovicin HC5 in the absence and presence of model membranes. Spectra were recorded for 1 μM bovicin HC5 in the absence (dotted line) and in the presence of model membranes, composed of DOPC (tracing A), or DLPC/DMoPC (tracing B) containing 1 mol % Lipid II. An excitation wavelength of 280 nm was applied, and emission was recorded between 300 and 450 nm. Spectra were corrected by blank subtraction. The vesicle final concentration used was 100 μM vesicles (lipid-Pi). A.U., arbitrary units.

To investigate how the bovicin tryptophan fluorescence quantitatively depends on the presence of Lipid II molecule, a solution of 1 μM bovicin HC5 was titrated into Lipid II-containing vesicles. Fluorescence intensities were measured at 340 nm before and after addition of varying amounts of Lipid II containing membranes (0-200 μM vesicles, corresponding to 0-2 μM Lipid II).

With increasing amount of Lipid II-containing vesicles, an increase in the intensity of the tryptophan fluorescence was observed, until the Lipid II to bovicin HC5 ratio reached 0.5. After this point, the addition of Lipid II-containing vesicles did not influence the tryptophan fluorescence (Figure 2). Also in this experiment, a consistent difference in fluorescence intensity between the two vesicle systems was observed. No differences were observed when the tritation was performed with vesicles that did not contain Lipid II (data not shown).

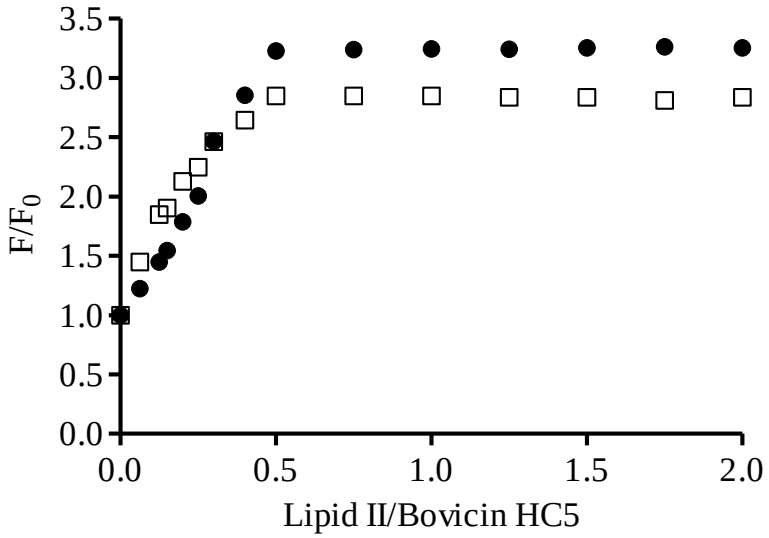


Figure 2: Lipid II:Bovicin HC5 ratio-dependent change of the bovicin HC5's tryptophan fluorescence. Different amounts of DOPC (filled circles) or DLPC/DMoPC (open squares) vesicles containing 1 mol % Lipid II were added to 1 μM bovicin HC5. Single-wavelength recordings were performed at 340 nm using an excitation wavelength of 280 nm. Fluorescence intensities before (F_0) and after addition of Lipid II-containing membranes (F) were used to calculate F/F_0 values, which were plotted against the Lipid II:Bovicin HC5 ratio.

3.4.2. Acrylamide quenching

The blue-shifts of the tryptophan residue indicated that it was inserted into the lipid phase of DOPC and DLPC/DMoPC Lipid II containing vesicles. To assess this in a more direct manner, the accessibility to acrylamide was determined in the presence and absence of DOPC and DLPC/DMoPC membranes. Acrylamide is a neutral water soluble quencher of the tryptophan fluorescence, and no charge interactions occur with the headgroups of negatively charged lipids.

Acrylamide was titrated from a stock solution into samples containing bovicin HC5 in the presence or absence of vesicles containing or not Lipid II. In the absence of vesicles, the addition of acrylamide caused an efficient quenching of the bovicin HC5's tryptophan fluorescence, as demonstrated by an increase of the F_0/F ratio (Figure 3, filled triangles), indicating that the tryptophan residue is readily accessible when bovicin HC5 is in solution. In the presence of Lipid II containing membranes, protection of quenching by acrylamide was observed and this reduction was the same, independent on the membrane composition (DOPC (Figure 3, filled circles) or DLPC/DMoPC

(Figure 3, open squares) phospholipids). Control experiments using vesicles lacking Lipid II, composed of DOPC and 3 % DOPG, revealed the same quenching effect as observed in buffer (data not shown).

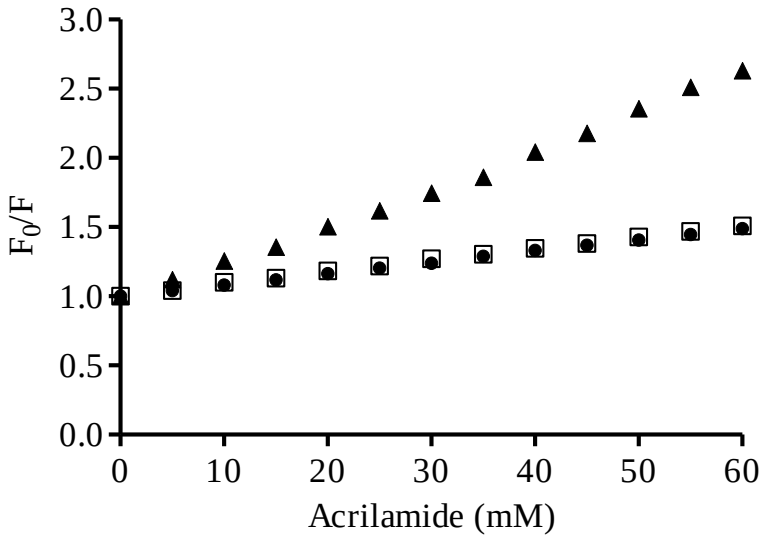


Figure 3: Stern-Volmer plot, showing the acrylamide quenching of bovicin HC5's tryptophan residue measured for samples containing 1 μM bovicin HC5 in the absence (filled triangles) and presence of DOPC (filled circles) or DLPC/DMoPC (open squares) vesicles containing 1 mol % Lipid II (100 μM lipid-Pi). Single-wavelength recordings were performed at 340 nm using an excitation wavelength of 280 nm. F_0: Fluorescence measured in the absence of the quencher; F: Fluorescence measured in the presence of the quencher.

The F_0/F plots were used to calculate the Stern-Volmer quenching constant (K_{sv}), as described in Experimental procedures. This Stern-Volmer constant is a measure for the accessibility of the tryptophan residue for the quencher. When bovicin HC5 was in solution (absence of vesicles), the Stern-Volmer constant was 27.9 M^{-1} and in the presence of Lipid II containing membranes the K_{sv} value was significantly lower (8.2 M^{-1} and 8.4 M^{-1} for DOPC and DLPC/DMoPC vesicles, respectively). This result demonstrated that the tryptophan residue was significantly less accessible for acrylamide on both model membranes tested, provided that Lipid II was present, and indicated that bovicin HC5 inserted into the membrane in a Lipid II-dependent way.

3.4.3. Spin-labeled lipid quenching

Depth-dependent quenching of fluorescence by spin-labeled lipids was used in order to obtain more information about the depth of insertion of the tryptophan residue of bovicin HC5 in Lipid II containing membranes. In this method, quenching depends on the distance between the tryptophan residue and the spin-label and occurs over a very short distance.

Spin-labeled lipids consist of a covalently linked nitroxide group with an unpaired electron (spin-label) positioned either at the headgroup (TEMPO-PC) or attached to the hydrocarbon chain at C-atom number 5 (5DOX-PC) or 12 (12 DOX-PC). The fluorescence of the tryptophan residue of bovicin HC5 was measured in the presence of Lipid II containing membranes that were enriched with each spin-labeled lipids.

In the presence of vesicles containing spin-labeled lipids, the fluorescence intensity of tryptophan residue became reduced as compared to the fluorescence in the presence of vesicles of the same composition, but without spin-labeled lipids. In both systems tested, the tryptophan fluorescence intensity was more effectively quenched by 12DOX-PC, the deepest quencher tested in this study. However, in DOPC system (Figure 4, black bars), the quenching efficiency was lower if compared to the DLPC/DMoPC system (Figure 4, white bars), although the relative differences have been larger within the DOPC system, when the different spin-labeled lipids were compared.

The presence of spin-labeled lipids in DOPC/DOPG vesicles did not affect the tryptophan fluorescence (data not shown).

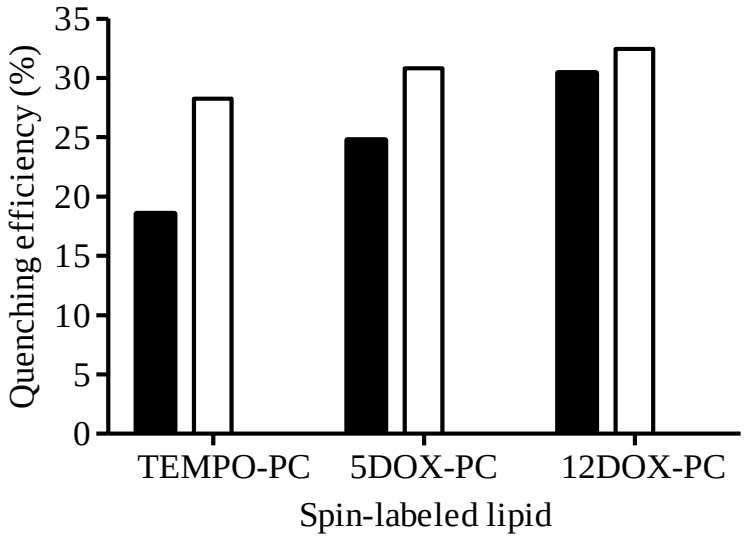


Figure 4: Quenching efficiency of the fluorescence emitted by the tryptophan residue of bovicin HC5 by spin-labeled lipids (TEMPO-PC, 5DOX-PC and 12DOX-PC) incorporated at 25 mol % in DOPC (black bars) or DLPC/DMoPC (white bars) vesicles containing 1 mol % Lipid II. Single-wavelength recordings were performed at 340 nm using an excitation wavelength of 280 nm. The quenching efficiencies were calculated from tryptophan fluorescence in the presence of membranes with and without spin-labeled lipids (n=5).

3.4.4. Circular dichroism spectroscopy

Circular dichroism (CD) is a good method of gathering information about the secondary structure and folding of proteins, as the proteins' spectra are dependent on their conformation. This is possible because all amino acids residues (except glycine) are chiral and right-handedly and left-handedly polarized light differently. This difference is presented as the ellipticity, which is a measure for circular dichroism (Pelton and McLean, 2000; Greenfield, 2006). CD is also a good tool to monitor conformational changes due to temperature, mutations, heat, denaturants or binding interactions (Greenfield, 2006).

The CD spectra of bovicin HC5 were recorded in different environments: in solution, in DOPC or DLPC/DMoPC vesicles (with and without Lipid II), and in the presence of water-soluble Lipid II (at different pH values). When the spectra of bovicin HC5 was assessed in solution (buffer), no structural change was verified, independently of the pH tested (from 2 to 10), and the minima was obtained at 198 nm (Figure 5).

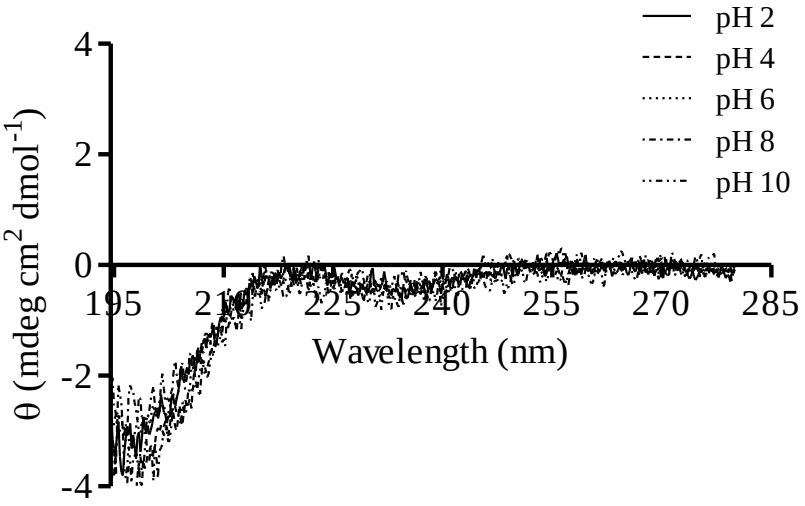
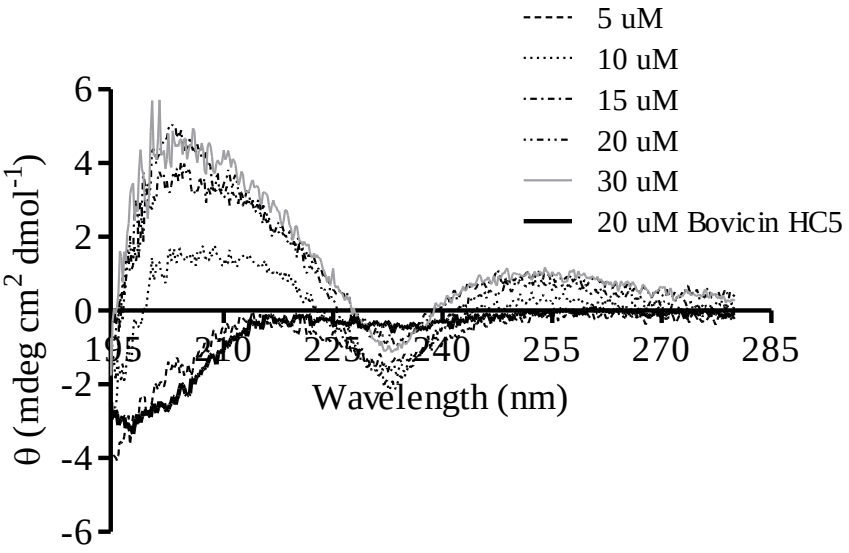


Figure 5: CD spectra of 20 μM bovicin HC5 in solution (10 mM potassium phosphate/40 mM potassium sulphate) and in different pH values (from 2 to 10). The samples were scanned from 195 to 280 nm, at 20 ºC. Each spectrum represents an average of five recordings after subtracting the blank spectrum from each bovicin HC5 spectrum.

The spectrum of bovicin HC5 was significantly changed upon the addition of Lipid II-containing vesicles or water-soluble Lipid II. In the presence of model membranes containing Lipid II (DOPC, Figure 6A, or DLPC/DMoPC, Figure 6B), the minimum appeared centered at about 234 nm. This minimum is flanked by two maxima, one at approximately 250 nm and another at approximately 204 nm (Figure 6A). The changes in CD-signal were specifically caused by the interaction of bovicin HC5 with Lipid II present in both model membranes, because addition of vesicles lacking Lipid II did not induce any spectral changes (data not shown).

Parallel control experiments were performed with negatively charged membranes, composed of DOPC with 3 % DOPG (since the negative charge of Lipid II is 3) and no changes in the spectrum of bovicin HC5 were observed.

A



B

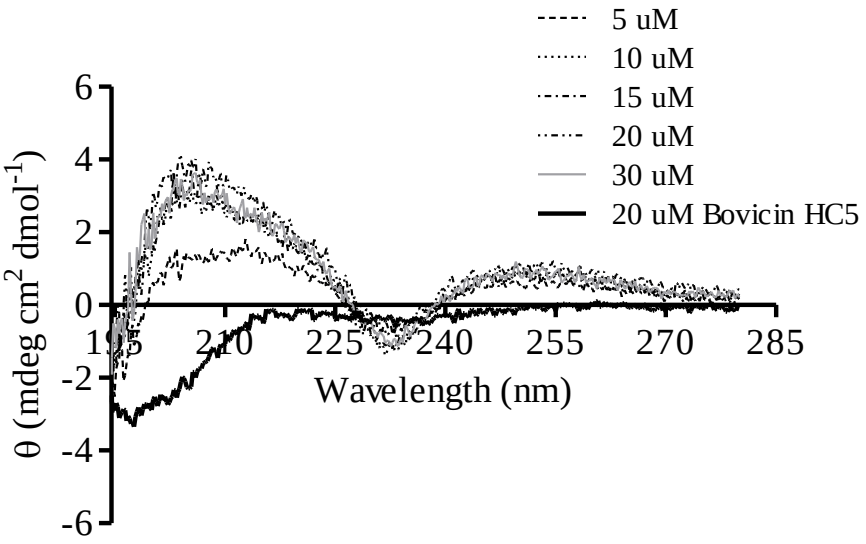


Figure 6: CD spectra of bovicin HC5 in the presence of model membranes composed of DOPC (A) or DLPC/DMoPC (B) containing Lipid II. Each spectrum was recorded for bovicin HC5 (20 μM) in buffer at pH 6, and in the presence of 5-30 μM Lipid II. The samples were scanned from 195 to 280 nm, at 20 ºC. Each spectrum was an average of five recordings and blank spectrum (in the absence of bovicin HC5) was subtracted from each spectrum obtained.

The CD spectrum of bovicin HC5 was also recorded in the presence of water-soluble Lipid II (3-LII), at pH 6.0. In this case, the inflection demonstrated in the presence of vesicles containing Lipid II, between 225 and 240 nm (minimum centered at approximately 234 nm), was also observed. However, in the presence of 3-LII, the spectrum of bovicin HC5 was flanked by two maxima, at 220 and 250 nm, which contrasted the effects on peptide`s spectrum observed in the presence of vesicles containing Lipid II, in which a maximum at about 204 nm was also observed (Figure 7).

Using increasing concentrations of 3-LII we determined the stoichiometry of the bovicin HC5/Lipid II interaction as 1:1, since the intensity and the pattern of the bovicin HC5`s spectrum did not alter when recorded with concentrations of 3-LII above 20 µM (Figure 7).

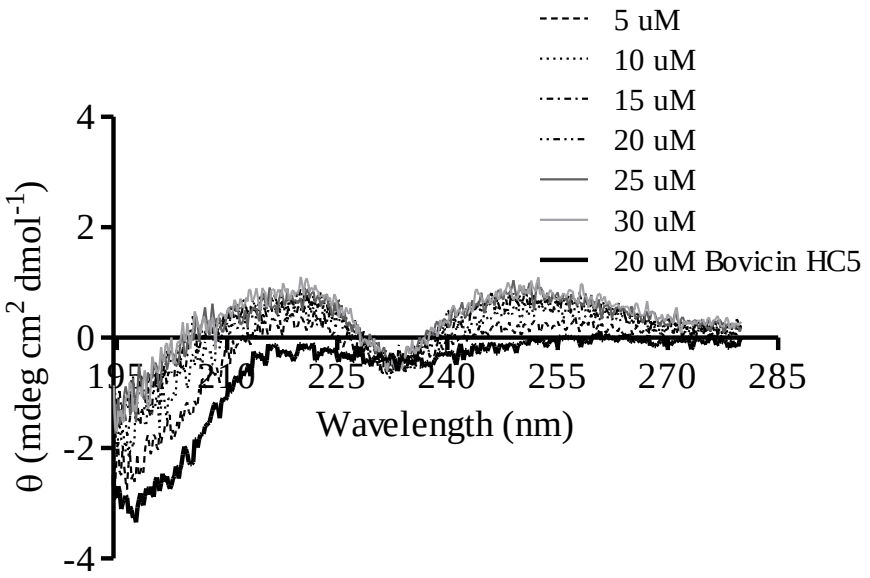


Figure 7: CD spectra of bovicin HC5 in the presence of different concentrations of water-soluble Lipid II (3-LII). Each spectrum was recorded for bovicin HC5 (20 µM) in buffer at pH 6 and in the presence of 5-30 µM 3-LII. The samples were scanned at 20 ºC from 195 to 280 nm. Each spectrum was an average of five recordings and blank spectrum (in the absence of bovicin HC5) was subtracted from each spectrum obtained.

Based on these results and to compare the influence of pH on the stability of the complexes formed by bovicin HC5 and Lipid II, the spectrum of bovicin HC5 was recorded in equimolar concentrations of 3-LII, but in different pH values (pH 2.0-10.0). In all additional pH values tested, the same inflection between 225 and 240 nm was

observed. Nevertheless, in pH 2.0, the spectrum of bovicin HC5 was not flanked by two maxima, as observed from pH 4 to 10, but a maxima wavelength was observed at 203 nm (Figure 8).

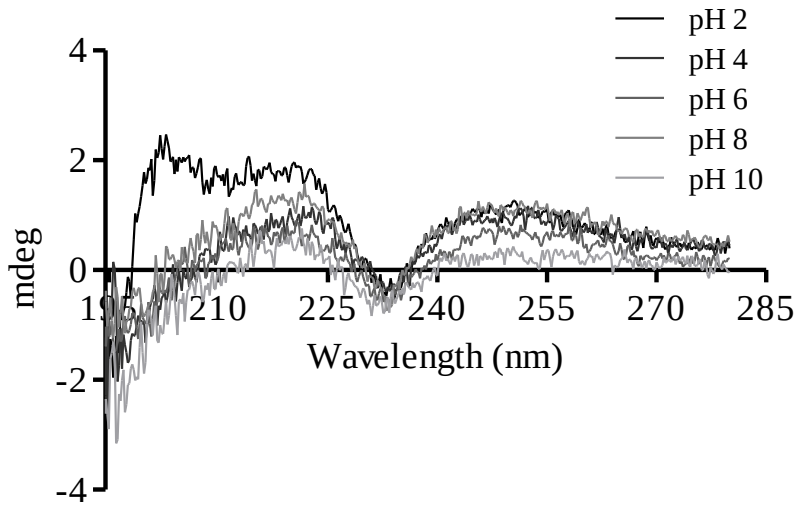


Figure 8: CD spectra of bovicin HC5 in the presence of water-soluble Lipid II (3-LII), in different pH values. Each spectrum was recorded in the presence of 20 μM bovicin HC5 and 20 μM 3-LII. The samples were scanned from 195 to 280 nm, at 20 ºC. Each spectrum was an average of five recordings and blank spectrum (in the absence of bovicin HC5) was subtracted from each spectrum obtained.

3.5. Discussion

Bovicin HC5 has the ability to translocate potassium (Mantovani *et al.*, 2002) and to interact specifically with Lipid II, inhibiting the bacterial cell wall synthesis (Paiva *et al.*, 2011). Bovicin HC5 is able to form pores after interacting with Lipid II, but in a membrane-thickness dependent way. Independent on the membrane thickness, bovicin HC5 maintains its antibacterial activity, by binding to Lipid II and recruiting some Lipid II molecules in a pore-like structure (Paiva *et al.*, 2011).

In the present study, we focused on the modification of the orientation and conformational changes of bovicin HC5 upon binding to its docking molecule, Lipid II. Tryptophan fluorescence as well as circular dichroism spectroscopy were used in order to determine the effect of the interaction between Lipid II and bovicin HC5, on depth of insertion and conformation under conditions that either allow (thin lipid membranes,

composed of DLPC/DMoPC phospholipids) or not allow (thick lipid membranes, composed of DOPC phospholipids) pore-formation in model membranes.

When bovicin HC5 was diluted into buffer (absence of vesicles), the fluorescence maxima of the tryptophan spectra occurred at 353 nm, which indicates that the tryptophan residue was localized in a hydrophilic environment (Lakowicz, 1999). Independent on the membrane phospholipid composition, upon addition of vesicles containing Lipid II, the fluorescence maximum of the tryptophan residue of bovicin HC5 displayed a blue-shift (maximally 12 nm) and a concomitant intensity increase, indicating that the tryptophan residue became located in a more hydrophobic environment.

In conjunction with this blue-shift, the tryptophan residue became much less accessible to acrylamide (K_{SV} values of 8.2 M^{-1} and 8.4 M^{-1} for DOPC and DLPC/DMoPC vesicles, respectively). These results showed that the bovicin HC5`s tryptophan residue became inserted into the membrane when bovicin HC5 interacted with Lipid II. Spin-labeled phospholipids indicated that the tryptophan residue was located near the 12^{th} position of the acyl chains of the membrane phospholipids.

Tryptophan spectroscopy had successfully been used before to determine the orientation of bacteriocins in model membranes, such as nisin (Breukink *et al.*, 1998; van Heusden *et al.*, 2002) and pediocin PA-1 (Chen *et al.*, 1997). Quenching of fluorescence by spin-labeled lipids has been used as a valuable tool to determine the topology and the depth of penetration of tryptophan- or tyrosine-containing peptides in model membranes (Ren *et al.*, 1997; Liu and Deber, 1997).

The changes in bovicin HC5's tryptophan emission spectra, in addition with the results from quenching experiments, are useful to propose a model for the orientation adopted by bovicin HC5 upon interaction with Lipid II in the cytoplasmic membrane. As previously reported for nisin (van Heusden *et al.*, 2002), the interaction with Lipid II changes the orientation of bovicin HC5 from a parallel to a perpendicular orientation, regarding to the membrane surface.

Circular dichroism spectroscopy was used in this study to examine the influence of binding to Lipid II on the structure of bovicin HC5. Furthermore, the stoichiometry and the pH dependency on the bovicin HC5/Lipid II interaction were examined.

In aqueous solution, the individual spectrum of bovicin HC5 was similar to the archetypal random coil spectrum (very low ellipticity above 210 nm and negative bands near 195 nm (Venyaminov *et al.*, 1993)), and it did not differ in a wide range of pH tested. This spectrum could be the result of the small size of bovicin HC5, which could

prevent the peptide with 22 amino acids residues to adopt a defined secondary structure in solution.

The presence of post-translationally modified amino acids residues and lanthionine-rings within the primary structure of bovicin HC5 prevented the quantitative structural analysis from the spectra obtained by circular dichroism measurements (van den Hooven *et al.*, 1993). Therefore, it was not possible to calculate the average secondary structure from the CD data, but comparisons of individual spectra and spectra upon interaction with Lipid II provided valuable information about the structural changes of bovicin HC5 upon interaction with Lipid II. Earlier studies with nisin (Hasper *et al.*, 2004) also used CD measurements for qualitative purposes, to get insight into the changes in nisin molecule after interacting to Lipid II.

As mentioned before, bovicin HC5 is able to form pores in DLPC/DMoPC vesicles containing Lipid II, but not on DOPC vesicles containing Lipid II, since bovicin HC5 is too small to permeabilize thick membranes. However, independent of this characteristic, the CD spectra from the bovicin HC5/Lipid II interaction were basically the same on both model membranes, confirming that bovicin HC5 is capable of binding to Lipid II even in the absence of pore formation.

The inflexion observed on the spectrum of bovicin HC5 in the presence of Lipid II containing membranes was caused strictly by the interaction between bovicin HC5 and Lipid II, since the same spectral change was demonstrated in the presence of water-soluble Lipid II. Moreover, the maximum centered at 204 nm is probably due to the effect of the interaction between the lateral chains of bovicin HC5`s amino acid residues and the phospholipids that compose the model membranes tested.

The spectrum of bovicin HC5 obtained in the presence of increasing concentrations of water-soluble Lipid II have shown a stoichiometry of 1:1 for the bovicin HC5/Lipid II interaction. The binding of bovicin HC5 to Lipid II occurred in a broad range of pH (from 2 to 10), and similar changes on bovicin HC5's spectrum were observed from pH 4 to 10; however, distinct spectrum was obtained at pH 2, suggesting that the interaction between bovicin HC5 and Lipid II is different at low pH when compared to less acidic conditions. These latter results indicate that bovicin HC5 can adopt a different conformation on highly acidic pH.

Nisin/Lipid II interaction is not observed in pH values below 4 and the changes observed at nisin spectrum upon binding to Lipid II are more evident in pH 6 (E. Breukink, unpublished results). In contrast, bovicin HC5 does not lose its affinity for Lipid II, even in highly acidic conditions, which is an important feature considering

practical applications for bovicin HC5, especially the preservation of acidic food products (e.g. tropical fruit juices and yogurt).

These results suggest that bovicin HC5, in spite of its ability to form pores, may have a binding affinity to Lipid II higher than nisin, especially at acidic conditions, which may help to explain the greater *in vivo* activity of bovicin HC5 against several bacterial strains in such conditions (Houlihan *et al.*, 2004).

Taken together, the results obtained in this study clearly show that upon the interaction with Lipid II bovicin HC5 changes its conformation, inserts into the membrane and adopts an overall perpendicular orientation with respect to the membrane surface, with the C-terminus inserted deeper into the membrane in comparison with the N-terminus. The bovicin HC5/Lipid II interaction occurs even in the absence of pore formation and in highly acidic conditions.

3.6. References

Breukink, E.; van Kraaij, C.; van Dalen, A.; Demel, R.A.; Siezen, R.J.; de Kruijff, B.; Kuipers, O.P. The orientation of nisin in membranes. Biochemistry 37, 8153-8162, 1998.

Breukink, E.; van Heusden, H.E.; Vollmerhaus, P.J.; Swiezewska, E.; Brunner, L.; Walker, S.; Heck, A.J.R.; de Kruijff, B. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. The Journal of Biological Chemistry 278 (22), 19898–19903, 2003.

Chen, Y.; Ludescher, R.D.; Montville, T.J. Electrostatic interactions, but ot the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipid vesicles. Applied and Environmental Microbiology 63 (12), 4770–4777, 1997.

Eftink, M.R.; Ghiron, C.A. Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. Biochemistry 15, 672-680, 1976.

Greenfield, N.J. Using circular dichroism spectra to estimate protein secondary structure. Nature Protocols 1 (6), 2876-2890, 2006.

Hasper, H.E.; de Kruijff, B.; Breukink, E. Assembly and stability of nisin-lipid II pores. Biochemistry 43 (36), 11567-11575, 2004.

Hope, M.; Bally, M.B.; Webb, G.; Cullis, P.R. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochimica et Biophysica Acta 812, 55-65, 1985.

Houlihan, A.J.; Mantovani, H.C.; Russell, J.B. Effect of pH on the activity of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. FEMS Microbiology Letters 231, 27-32, 2004.

Hsu, S.T.D.; Breukink, E.; Tischenko, E.; Lutters, M.A.G.; de Kruijff, B.; Kaptein, R.; Bonvin, A.M.J.J.; van Nuland, N.A.J. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature Structural & Molecular Biology 11, 963-967, 2004.

Lakowicz, J.R. Principles of fluorescence spectroscopy. Kluwer Academic/Plenum Publishers, New York. 1999.

Liu, L.P.; Deber, C.M. Anionic phospholipids modulate peptide insertion into membranes. Biochemistry 36, 5476-5482, 1997.

Mantovani, H.; Hu, H.; Worobo, R.W.; Russell, J.B. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology 148, 3347–3352, 2002.

Pelton, J.T.; McLean, L.R. Spectroscopic methods for analysis of protein secondary structure. Analytical Biochemistry 277, 167–176, 2000.

Paiva, A.D.; Breukink, E.; Mantovani, H.C. The role of Lipid II and membrane thickness in the mechanism of action of the lantibiotic bovicin HC5. Antimicrobial Agents and Chemotherapy, 2011. *In press.*

Ren, J.; Lew, S.; Wang, Z.; London, E. Transmembrane orientation of hydrophobic-helices is regulated by the relationship of helix length to bilayer thickness and by cholesterol concentration. Biochemistry 36, 10213-10220, 1997.

Rouser, G.; Fkeischer, S.; Yamamoto, A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. Lipids 5, 494-496, 1970.

van den Hooven, H.W.; Fogolari, F.; Rollema, H.S.; Konings, R.N.H.; Hilbers, C.W.; van de Ven, F.J. NMR and circular dichroism studies of the lantibiotic nisin in non-aqueous environments. FEBS Letters 319, 189-194, 1993.

van Heusden, H.E.; de Kruijff, B.; Breukink, E. Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin. Biochemistry 41, 12171-12178, 2002.

Venyaminov, S.; Baikalov, I.A.; Shen, Z.M.; Wu, C.S.; Yang, J.T. Circular dichroic analysis of denatured proteins: inclusion of denatured proteins in the reference set. Analytical Biochemistry 214, 17-24, 1993.

CHAPTER 4

Morphologic and immunostimulatory effects of the lantibiotic bovicin HC5 upon oral administration to an animal model.

4.1. Abstract

Cytotoxicity and immunostimulatory activity are important issues to address the safety of bacteriocins and other antimicrobial peptides. Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5, shows little inhibitory activity towards eukaryotic cells *in vitro* and weak immunogenicity *in vivo*. However, the effects of bovicin HC5 after ingestion have not been elucidated. In this study, we evaluated the effects of orally administrated bovicin HC5 to BALB/c mice, and results were compared with a model of intestinal inflammation (positive control). The oral administration of bovicin HC5 (0.004 mg/g animal weight/day) for 58 days to BALB/c mice resulted in low weight gain and some impairment of small intestine, characterized by moderate edema, villous enlargement and alteration of the apical portion of the villi, although no physiological changes in small intestine have been detected. No histological alterations were observed in the heart, liver and large intestine of animals treated with bovicin HC5. There was a cellularity reduction at the spleen, but no difference in the cytokine relative expression was detected in this organ. An increase of TNF-α, INF-γ and IL-12 relative expression in small intestine occurred upon administration of bovicin HC5. The results obtained indicate that orally administrated bovicin HC5 caused morphological alterations that were less pronounced than the alterations observed in the positive control group. Additionally, bovicin HC5 was also able to stimulate the host immune system, at local level.

4.2. Introduction

Bacteriocins are antimicrobial peptides produced by many species of bacteria and some members of the Archaea domain. Several bacteriocins produced by lactic acid bacteria (LAB) have been screened for application in the food industry, in an attempt to reduce the dependency on chemical preservatives (Belguesmia *et al*, 2011). Nisin, a well-know bacteriocin produced by *Lactococcus lactis*, has GRAS status (generally recognized as safe) and is currently the only bacteriocin with regulatory approval for use as a food preservative (Delves-Broughton, 2005). Other bacteriocins, such as pediocin PA-1/AcH and lacticin 3147, are also commercially available, but are marketed as fermentates of LAB having GRAS status, and are not approved as food additives (Gálvez *et al.*, 2008).

Despite the effectiveness in the food industry, many other bacteriocins have potential for biotechnological and therapeutical applications, including the treatment of topical infections, control of bovine mastitis and eradication of multi-resistant pathogens. To be used in these applications, a bacteriocin should show some desirable properties, such as simplicity for production and extraction, stability to low pH and heat, and little inhibitory activity towards eukaryotic cells (Toke, 2005; Parisien *et al.*, 2008). Bovicin HC5, a lantibiotic produced by *Streptococcus bovis* HC5, fits all these requirements (Mantovani *et al.*, 2002; Paiva *et al.*, 2011), and promising results have been obtained both *in vitro* and *in vivo* experiments.

Similar to other bacteriocins belonging to the lantibiotic group, bovicin HC5 has post-translationally modified amino acids residues and thioether linkages, which provide it with remarkable stability. Bovicin HC5 acts by binding to its specific receptor Lipid II, inhibiting the bacterial cell wall synthesis and forming pores in the membranes of the sensitive cells (Paiva *et al.*, *under review*). Bovicin HC5 showed little cytotoxicity against mammalian cells *in vitro*, even at micromolar concentrations, a dose much higher than the concentration needed to inhibit sensitive bacterial cells (Paiva *et al.*, 2011). Moreover, bovicin HC5 is only weakly immunogenic to New Zealand rabbits and BALB/c mice immunised via sub-cutaneous route (unpublished data).

The industrial use of bacteriocins remains limited due to the lack of data concerning their safety, such as the efficacy of antimicrobial activity, the destiny of the bacteriocin after ingestion, the cytotoxicity and the immune-stimulatory effects of the

peptide (Maher and McClean, 2006; Vaucher *et al.*, 2010). All these parameters are prerequisite for further application and they have been little addressed.

In order to gain insight on the safety of bacteriocins, we focused this study on bovicin HC5 and analyzed the effects of orally administrated bovicin HC5 to BALB/c mice. The morphologic alterations and the immunostimulatory effects of the peptide were evaluated.

4.3. Experimental procedures

4.3.1. *Streptococcus bovis* HC5 and bovicin HC5

Streptococcus bovis HC5 was cultivated under anaerobic conditions, at 39 ºC, in basal medium containing (per liter): 0.292 g K_2HPO_4; 0.292 g KH_2PO_4; 0.48 g $(NH_4)_2SO_4$; 0.48 g NaCl; 0.1 g $MgSO_4.7H_2O$; 0.064 g $CaCl_2.2H_2O$; 0.5 g cystein hydrochloride; 4 g Na_2CO_3; 0.1 g trypticase; 0.5 g yeast extract. Glucose was added as sole carbon source (4 g l^{-1}).

Bovicin HC5 extracts were prepared as described by Mantovani *et al*. (2002). Purification of bovicin HC5 was performed by RP-HPLC using a semi preparative column (Shimadzu C18; 5 μm; 150 by 6 mm [inner diameter]), equilibrated with buffer A (0.1 % trifluoroacetic acid (TFA) in ultrapure water). The peptide was eluted using a linear gradient of 35 to 50 % buffer B (80 % acetonitrile, 0.1 % TFA in ultrapure water), 22 °C, and at a flow rate of 1 ml min^{-1}. The eluted fraction corresponding to pure bovicin HC5 was lyophilized and the correct mass and purity of bovicin HC5 was confirmed by analytical HPLC.

Bovicin HC5 stock solutions (1 mg ml^{-1} in PBS, 10 mM, pH 7.2) were stored at -20 ºC until use. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce Chemical Corp., Bonn, Germany), with bovine serum albumin as the standard.

4.3.2. Animals

Five-week-old female BALB/c mice weighing 18±1 g were provided by the animal breeding colony of the Federal University of Viçosa. The animals were randomly divided into three experimental groups, containing 6 animals each: Group 1, mice given PBS (negative control, NC group); Group 2, mice given purified bovicin HC5 (Bov group); Group 3, mice given ovalbumin (Sigma®, 99 % of purity) (positive control, PC group).

The mice were housed in an animal room maintained at 24±2 °C, with a light/dark cycle of 12 h and a relative humidity of 55±15% during the experiment and for 10 days prior to initiate the study. The mice were fed a standard laboratory rodent chow (Purina®) and water *ad libitum* over the experiment. All procedures were conducted in accordance with the Guidelines for Animal Experiments adopted by the Ethical Committee in Animal Research of the Federal University of Viçosa.

The animals were immunised as described by Malo and Morin (1986) with modifications. Initially, the mice were subcutaneously immunised with bovicin HC5 (0,004 mg/g animal weight/day) (Akiyama *et al.*, 2001)) or ovalbumin (100 µl of a 1 mg ml^{-1} stock solution in sterile ultrapure water) in alum (50 µl of a 20 mg ml^{-1} alum hydroxide solution in sterile saline) (first immunization, day 0); after three weeks, the mice were subcutaneously boosted with the proteins, but without the use of adjuvant (second immunization, day 21). The group 1-mice were immunised with sterile PBS (10 mM, pH 7.2), using the same conditions described above.

PBS, bovicin HC5 or ovalbumin (100 µl) were administered without the use of adjuvant, by daily gavages, using *18-gauge* needles made of stainless steel. The oral administration started one week after the second sensitization injection (day 28) and continued for the full experiment period (day 58). The mice were weighted weekly and monitored daily, regarding to behavior, general appearance and adverse reactions at the immunization sites.

Blood samples were collected at the beginning and at the end of the experiment (days 0 and 58), from the orbital plexus under light ether anesthesia. The samples were kept at room temperature for 2 hours, and the sera were centrifuged at 12,000 x *g* for 5 min (Eppendorf®, Centrifuge 5415C, Hamburg, Germany), at room temperature (Ausubel *et al*., 1999). The samples were stored at -20 °C until use.

4.3.3. Gut permeability

The possible changes in gut permeability were determined by the uptake of β-lactoglobulin following challenge, as described by Knippels *et al*. (1999), with some modifications. After the experimental period (day 58), animals sensitized with PBS, bovicin HC5 or ovalbumin were orally challenged with 200 µl of the respective samples. After thirty minutes, the animals received an additional intragastric dose of β-lactoglobulin (β-LG, 90 % of purity, 0.2 ml of a 100 mg ml^{-1} solution in tap water; obtained from Sigma Chemicals Co., St. Louis, MO), and blood samples were collected

from the orbital plexus under light ether anesthesia, after 0.5, 1, 2 and 5 h of the β-LG administration.

The blood samples were processed as described and stored at -20 °C until use.

Sera were used for the quantification of β-lactoglobulin by FPLC, using a cationic change column (Mono Q; 5 μm; 150 by 6 mm [inner diameter]). The column was equilibrated with buffer A (20 mM Tris in ultrapure water) and the β-LG was eluted using a linear gradient of 25 to 50 % buffer B (20 mM Tris, 1 M NaCl, in ultrapure water), 22 °C, and at a flow rate of 1 ml min^{-1}. The absorbance was monitored at 220 and 280 nm.

In order to determine the concentration of β-lactoglobulin in animal sera, a calibration curve was prepared, using solutions with known concentrations (0; 6.25; 12.5; 25.0; 50.0 mg ml^{-1}), mixed to pre-immune serum of the animals from each group. The standard curve was constructed by plotting the peak areas related to β-lactoglobulin against the concentration of standard solutions used.

Serum samples before β-lactoglobulin administration were used as negative control. Analyses were performed in duplicate and the chromatographic profiles obtained were compared to the calibration curve.

4.3.4. Histological and morphometric analysis

After 58 days of experiment, the animals were sacrificed by cervical dislocation. The organs, heart, liver, spleen and gut, of the all mice were aseptically removed, washed in PBS and fixed in Carson formalin solution (Carson *et al.*, 1973), for 24 hours, at room temperature. The fixed organs were sectioned, dehydrated in ethanol (70º, 80º, 95º and absolute ethanol, during 30 min each), and embedded in resin (Historesin®, Leica). The fragments were incubated at 37 ºC, for 24 hours.

Transverse and longitudinal histological sections were obtained by microtome. Semi-serial cuts, with a thickness of 3 μm and interval between cuts of 30 μm, were obtained. The slides were stained with toluidine blue/sodium borate (1 %), hematoxylin and eosin (HE), Alcian Blue (pH 2.5) combined with periodic acid-Schiff (PAS) (Bancroft and Stevens (1996), with modifications), depending on the histological analysis that would be performed.

Sections stained with HE were used for morphologic analysis. To each animal, twenty fields of longitudinal gut sections stained with toluidine blue/sodium borate (1 %) or HE with increases of 10x were randomly selected in order to determine the villi

height (from the basal region, just above the crypt, to the top of the villi), villi width (taking the average of three points, located in the upper, middle and basal region of the villi), and mucosal thickness.

Sections stained with PAS were used for visualization of goblet cells and the determination of mucopolysaccharides (acidic, in blue; neutral, in dark red; acidic/neutral, in dark purple). Ten fields of 353 x 265 µm with increases of 20x were randomly selected, and the goblet cells PAS^+ and AB^+ were counted for the analysis of the presence of different mucins.

Sections stained with toluidine blue/sodium borate (1 %) were used to detect mast cells. An area equivalent to 20 jejunum villi (mucosa and submucosa), in each animal, was evaluated. Data were reported as number of cells per field.

Images of histological sections were captured with the light microscope Olympus AX 60, coupled to a micro camera. The morphometric analyzes were performed with the image analysis program Image Pro Plus 4.0 for Windows (Media Cybernetcs). The results were shown as the mean value $\pm$ standard deviation.

4.3.5. Analysis of relative gene expression by real-time PCR

Jejunum segments and the whole spleen (100 mg of tissue) were removed aseptically from the animals, washed in sterile PBS, and individually manipulated.

The spleen was processed with 1 ml of saline (0.85 %) for cell extraction. The cells were transferred to micro centrifuge tubes and kept on ice for up to 1 h; splenocytes were sedimented by centrifugation (7500 x *g*, 5 min.) and suspended in 1 ml erythrocytes lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 2 mM EDTA), for 10 min on ice. The splenocytes were centrifuged again and the supernatant was discarded. The cells were frozen in liquid nitrogen and stored at - 80 ºC until use.

A jejunum segment of 6 cm was removed and washed three times with saline (0.85 %), for removal of waste. The segments were transferred to micro centrifuge tubes and kept on ice for up to 1 h. The organs were frozen in liquid nitrogen and then stored at - 80 ºC until use.

The mRNA was extracted from jejunum segments and spleen using the Tri Reagent (Sigma®), following the protocol recommended by the manufacturer. Tissue samples were homogenized with 1 ml Tri Reagent, and the samples were incubated at room temperature for 5 min. Then, 0.2 ml chloroform was added and the samples were vigorously shaken for 15 s; the samples were kept at room temperature for 15 min and centrifuged (12000 x *g*, 15 min, 4 ºC). The RNA containing aqueous phase was

collected and transferred to a new and sterile tube; 50 µl of isopropanol were then added and the samples were incubated at room temperature for 5 min; the samples were centrifuged (12000 x *g*, 15 min, 4 ºC) and the supernatant was transferred to another new and sterile tube. After that, 450 µl of isopropanol were added and the samples were kept at room temperature for 10 min.

Following, the samples were centrifuged (12000 x *g*, 10 min, 4 ºC) and the supernatant was discarded. The precipitate was washed by adding 1 ml ethanol (75 %) and suspended in vortex; the sample was centrifuged (7500 x *g*, 5 min, 4 ºC) and 1 ml ethanol was added to the *pellet*; the samples were stored *overnight* at - 80 ºC. The RNA samples was dried at room temperature, suspended in 40 µl of DEPC water, and maintained in water bath at 37 ºC, for 15 min, to facilitate the dissolution of RNA samples. An aliquot of 5 µl was used to obtain the concentration of RNA per µl in the samples, using a Genesis 10S UV-VIS Spectrophotometer (Thermo Scientific, Ridgefield Court, Asheville, USA).

Complementary DNA (cDNA) was synthesized through a reverse transcription reaction (M-MuLV reverse transcriptase, Promega). Real-time PCR relative quantification of mRNA were performed on the Gene Amp® 5700 Sequence Detection System Version 1.3 (Applied Biosystems) using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantification of amplicons. The standard PCR conditions were 95 °C for 10 min, 40 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, followed by the standard denaturation curve. The sequences of murine primers were designed using the Primer Express software (Applied Biosystems) and the nucleotide sequences present in the Gen Bank data base (the primers sequences are depicted in Table 1). PCR conditions for each target were optimized with regard to primer concentration, absence of primer dimer formation, and efficiency of amplification of target genes and housekeeping gene control. In each reaction, 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 450 nM specific primers, and 2.5 ng of cDNA were used.

Threshold for positivity of real-time PCR was determined based on negative controls. The results were demonstrated as mRNA expression of the test groups, relative to negative control group. Instructions from Applied Biosystems User's Bulletin #2 (P/N 4303859) were used to calculate the relative level of gene expression, by reference to the β-actin in each sample, using the cycle threshold (Ct) method. Briefly, Ct value was calculated by determining the point at which the exponential increase in signal (fluorescence) exceeds a somewhat arbitrary signal level (usually 10 times the standard

deviation of the baseline). The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to an internal control (β-actin), and then compared with the target–internal control in the control animals (negative control group) to calculate fold increase expression, using the expression 2^DCt, according to the User's Bulletin. Negative controls without RNA and without reverse transcriptase were also performed. Results were shown as mean values ± standard deviation (n=3).

Table 1: Sequences of sense (S) and antisense (AS) *primers* used for real time-PCR analysis.

Primers	**Sequences**
β-actin S	5' AGC TGC GTT TTA CAC CCT TT 3'
β-actin AS	5' AAG CCA TGC CAA TGT TGT CT 3'
IL-10 S	5' TGG ACA ACA TAC TGC TAA CC 3'
IL-10 AS	5' GGA TCA TTT CCG ATA AGG CT 3'
IL-4 S	5' CTG ACG GCA CAG AGC TAT TGA 3'
IL-4 AS	5' TAT GCG AAG CAC CTT GGA AGC 3'
IL-5 S	5' GAG GTT ACA GAC ATG CAC CAT T 3'
IL-5 AS	5' TCA GTT GGT AAC ATG CAC AAA G 3'
IL-13 S	5' ACC AAC ATC TCC AAT TGC AA 3'
IL-13 AS	5' ATG CAA TAT CCT CTG GGT CC 3'
TNF-α S	5' TGT GCT CAG AGC TTT CAA CAA 3'
TNF-α AS	5' CTT GAT GGT GGT GCA TGA GA 3'
IL-12 p40 S	5' AGC ACC AGC TTC TTC ATC AGG 3'
IL-12 p40 AS	5' GCG CTG GAT TCG AAC AAA G 3'
IFN-γ S	5' GCA TCT TGG CTT TGC AGC T 3'
IFN-γ AS	5' CCT TTT TCG CCT TGC TGT TG 3'
TGF-β S	5' GCT GAA CCA AGG AGA CGG AAT 3'
TGF-β AS	5' GCT GAT CCC GTT GAT TTC CA 3'
IL-17 S	5' GCT CCA GAA GGC CCT CAG A 3'
IL-17 AS	5' CTT TCC CTC CGC ATT GAC A 3'

4.3.6. Statistical analysis

The results were initially evaluated by one-way analysis of variance. For histological and morphometric data, when differences among groups were identified, the Dunn`s multiple comparison test or Student`s t test was conducted to examine significant differences among the treatments; for cytokine gene expression, the groups were compared by t test. A probability value of less than 0.05 was considered statistically significant.

All the comparisons were performed using the GraphPad Prism 5 software.

4.4. Results

We investigated the effects of the oral administration of bovicin HC5 to BALB/c mice. Daily administration of the purified bacteriocin (1 mg ml^{-1}, 100 µl final volume) started at day 28 and continued uninterruptedly for 30 days, until the sacrifice day (day 58). All animals survived throughout the study and no differences in the general appearance or adverse reactions were observed.

4.4.1. Weight gain

The weight gain of BALB/c mice belonging to the three experimental group, negative control (NC), bovicin HC5 (Bov) and positive control (PC), was weekly monitored after the first immunization. Monitoring weight gain allowed to verify if the sensitization followed by challenge with bovicin HC5 or ovalbumin could affect the weight of the animals, which is frequently associated with the clinical manifestation of allergy or gastrointestinal disorder.

There was no significant difference among the average initial weight of the mice at the beginning of the experiment (18.51, 18.4 and 18.34 g to NC, Bov and PC groups, respectively). Among the mice of the negative control group, the average weight ranged from 18.51 $\pm$ 0.35 g (day 0) to 20.8 $\pm$ 0.31 g (day 58) during the experiment, which means a weight gain percent of 11.01 %. Animals treated with bovicin HC5 or ovalbumin gained weight during the three initial weeks of the experiment, but weight was either maintained or lost after starting the oral administration of bovicin HC5 or ovalbumin, a condition that persisted throughout the experimental period.

At the end of the experiment, the percent of weight gain was 0.91 and – 1.8 % for animals in the Bov and PC groups, respectively, which was statistically lower

compared to the negative control group ($p < 0.05$). There was no significant difference regarding the percent of weight gain between Bov and PC groups (Figure 1).

The mice were also monitored for clinical signs of diarrhea, intestinal bleeding and rectal prolapsed, but development of these symptoms were not detected.

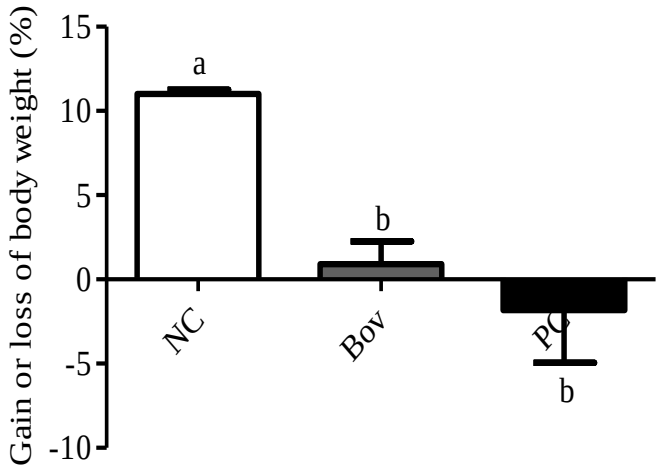


Figure 1: Gain or loss of body weight in BALB/c mice during the experimental period. The gain/loss of weight is shown as percentage of the animals' weight and was calculated comparing the weight at the end of the experiment (day 58) to the weight at the day of the first immunization (day 0). Each bar represents the mean value from six determinations with the standard deviation (SD). Different letters mean significant difference among treatments ($p < 0.05$). NC: negative control group; Bov: bovicin HC5 group; PC: positive control group.

4.4.2. Gastrointestinal permeability

Upon an additional oral administration of PBS, bovicin HC5 or ovalbumin (200 µl) to the animals in the treatment groups, and a subsequent gavage dose of 20 mg β-LG 30 min later, the amount of β-LG was measured in sera obtained 0.5, 1, 2 and 5 h after the β-LG administration.

β-LG was easily identified by the FPLC method developed in this study, and the retention time of β-LG was 10.68 min. It was possible to observe a clear increase at the peak areas, proportional to the increase of the β-LG concentrations in the animal sera.

In all the serum samples from negative control animals, β-LG was not detected. In animals that received bovicin HC5, low levels of β-LG could be detected in sera obtained 5 h after the β-LG administration. In sera obtained from animals that received

ovalbumin, a significant amount of β-LG was detected at 0.5, 1 and 2 h after β-LG administration (3.47 mg ml^{-1}, 3.53 mg ml^{-1} and 12.14 mg ml^{-1}, respectively). After 5 h of administration, any β-LG could be detected in the sera of the animals from the PC group, which indicates that the clearance of this protein required less than 5 h to be complete (Figure 2).

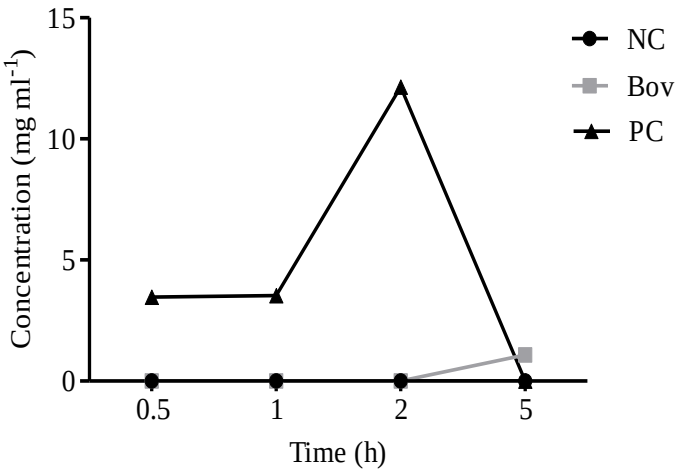


Figure 2: β-lactoglobulin levels in animal sera from the treatment groups. An intragastrically dose of β-LG (20 mg) was administered as a bystander protein to the negative control (NC), bovicin HC5 (Bov) and positive control groups (PC). At the indicated time points following β-LG administration, the levels of β-LG in mice sera were determined by FPLC. The results show an average of the β-LG level detected in four animals of each group. β-LG was not detected in all serum samples from negative control group.

4.4.3. Histological and morphometric analysis

The livers of all the groups showed a preserved lobular architecture, without cellular infiltrates or parenchymal substitutions, and preserved cellularity of parenchyma. Also at the hearts, no alterations were identified (data not shown). It was observed a significant decrease of the total number of spleen cells in the animals treated with bovicin HC5 and ovalbumin, when compared to the negative control group (Figure 3).

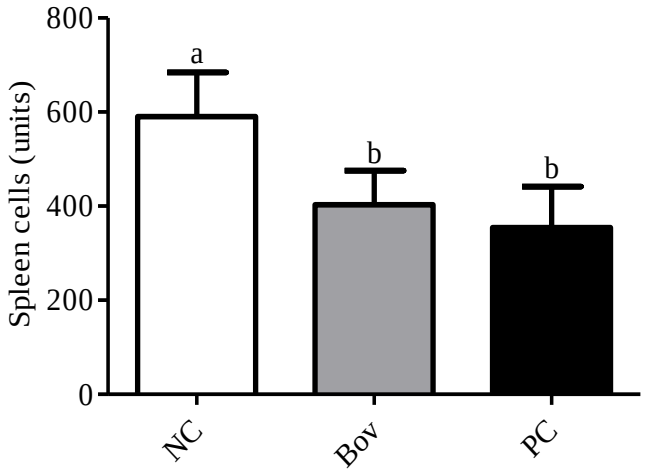


Figure 3: Comparison of the number of splenocytes among the groups analyzed ((NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin). Data were shown as average $\pm$ SD. Different letters mean significant difference among the groups, according to Dunn's test ($p < 0.05$).

To determine if the low percentage of the weight gain/loss detected in the animals from the Bov and PC groups was related to intestinal damage caused by the ingestion of bovicin HC5 or ovalbumin, gut segments from the animals were collected and processed for histological and morphometric analysis.

The small intestine of the negative control group presented a normal aspect, independent of the magnification visualized, and all the intestinal layers remained intact (Figure 4A).

For the group of mice treated with bovicin HC5, the small intestine showed a heterogeneous aspect from animal to animal: mild edema of the lamina propria and alteration of the apical portion of the villi were identified on sequential segments in some mice, and in discrete areas of sequential segments in others (Figure 4B).

Positive control groups developed intestinal inflammation, characterized by inflammatory cell infiltration, tissue destruction, epithelial exulceration and pronounced important edema of the lamina propria. These alterations were homogenous among the animals of the PC group (Figure 4C).

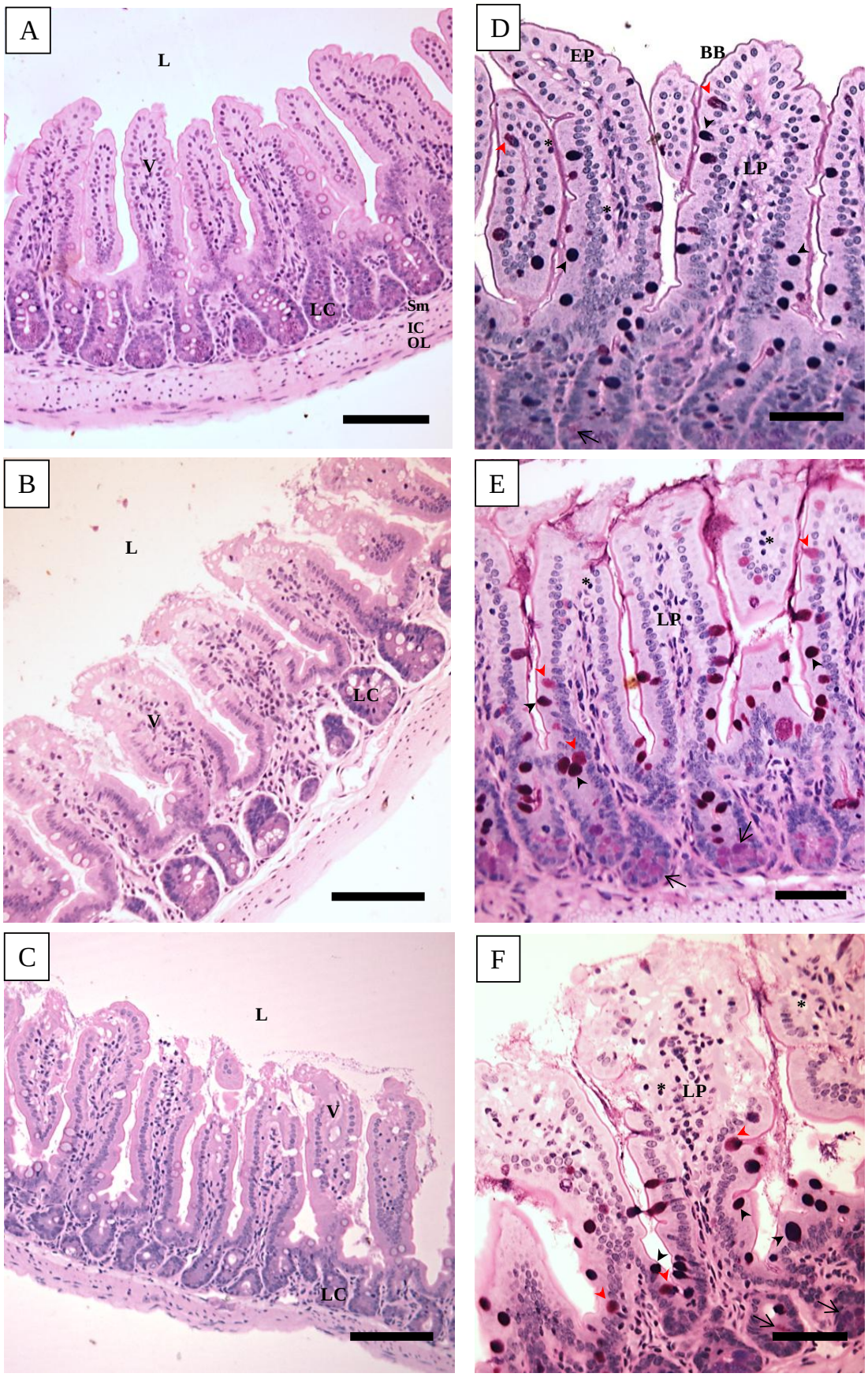


Figure 4: Photomicrographs of histological sections of small intestine of the animal groups studied ((NC), negative control, figures A and D; (Bov) mice treated with

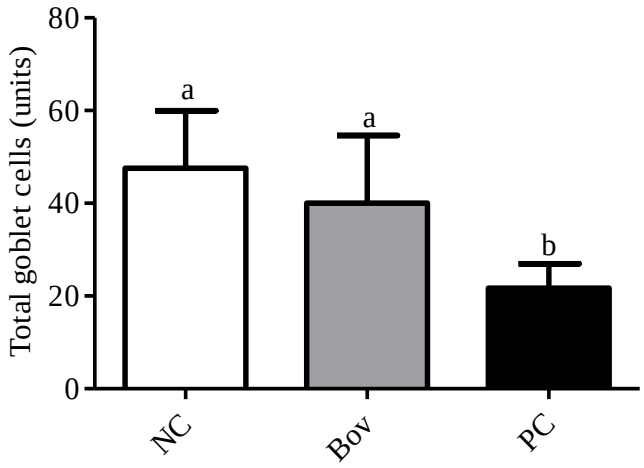
bovicin HC5, figures B and E; (PC) positive control, figures C and F). Jejunum segments were collected and processed for optical microscopy analysis at the end of the experiment. The sections were stained with hematoxylin and eosin (HE; right panel) or PAS/Alcian Blue (left panel). Abbreviations: L: lumen; EP: simple cuboidal epithelium; BB: brush border; V: villum; LP: lamina propria; LC: Lieberkühn crypt; Sm: submucosa; IC: inner circular muscle layer; OL: outer longitudinal muscle layer. The asterisks indicate intraepithelial lymphocytes; simple arrow indicates Paneth cells. Black arrow head indicates goblet cells PAS/AB^+; red arrow head indicates PAS^+ cells. Right panel – Scale bar: 100 µm; Left panel – Scale bar: 50 µm.

Morphometric analysis of the small and large intestine of the animals treated with bovicin HC5 and ovalbumin showed some impairment of the intestinal structure integrity, but the severity of the alterations caused by bovicin HC5 and ovalbumin was clearly different.

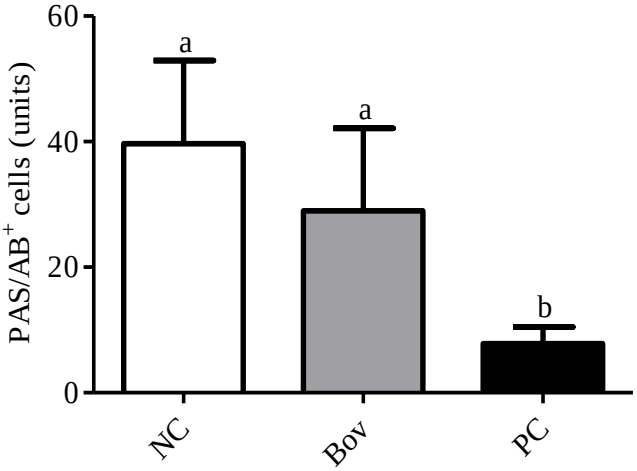
No differences were observed at the number of total goblet cells present in the small intestine of mice treated with bovicin HC5 compared to the negative control group. However, the number of goblet cells in the small intestine of animals treated with ovalbumin was reduced (Figures 4D-F) and statistically different ($p < 0.05$) when compared to the other two groups (Figure 5A). The majority of goblet cells presented in animals of the NC group were PAS/AB^+ cells, which secrete both neutral and acidic mucopolysaccharides. The number of PAS/AB^+ cells did not differ between the NC and Bov groups, but it was significantly reduced in PC group ($p < 0.05$, Figure 5B).

The number of PAS^+ cells, which secrete only neutral mucopolysaccharides, did not differ among the groups (Figure 5C). Cells secreting exclusively acid mucins (AB^+ cells) were not detected.

A



B



C

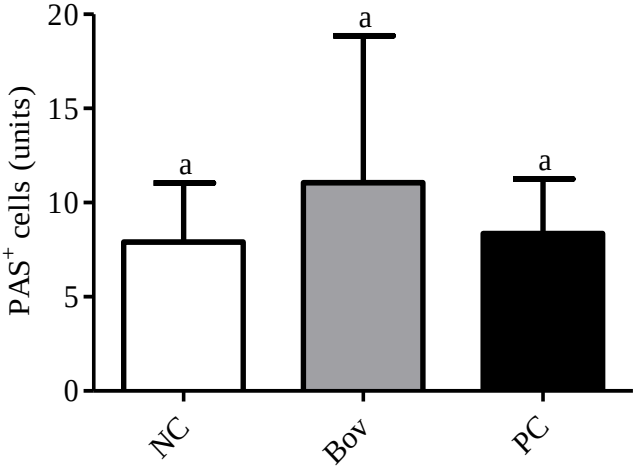
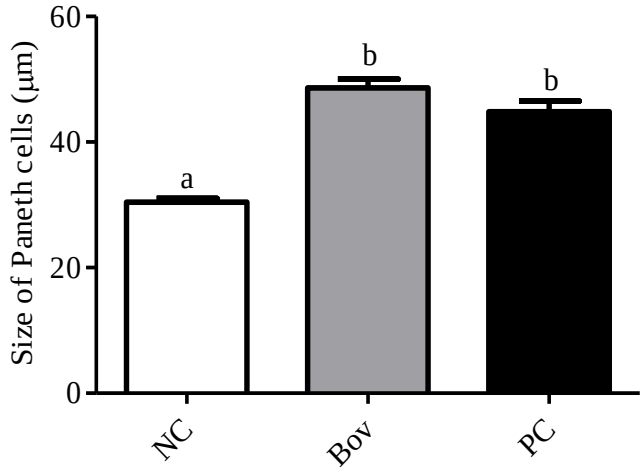


Figure 5: Comparison of the number of total goblet cells and mucopolysaccharides secretion among the experimental groups. (A) total number of cells; (B) PAS/AB$^+$ cells; (C) PAS$^+$ cells. Data were shown as average $\pm$ SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin.

Analyzing the Lieberkühn glands at the small intestine, it was observed an hypertrophy of Paneth cells as well as an increased number of cells in mitosis in the animals treated with bovicin HC5 and ovalbumin, when compared to the negative control group ($p < 0.05$) (Figure 6). No differences were observed between Bov and PC groups.

A



B

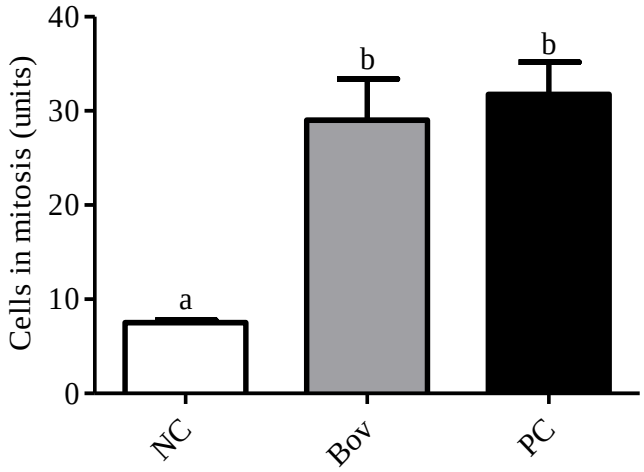


Figure 6: Size of Paneth cells (A) and number of cells in mitosis (B) at the small intestinal crypts of the experimental groups. Data were shown as average $\pm$ SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin.

The jejunum segments of the positive control mice also demonstrated a significant increase ($p < 0.05$) at the counts of mast cells in mucosa and submucosa, when compared to the negative control group and the Bov group (Figure 7).

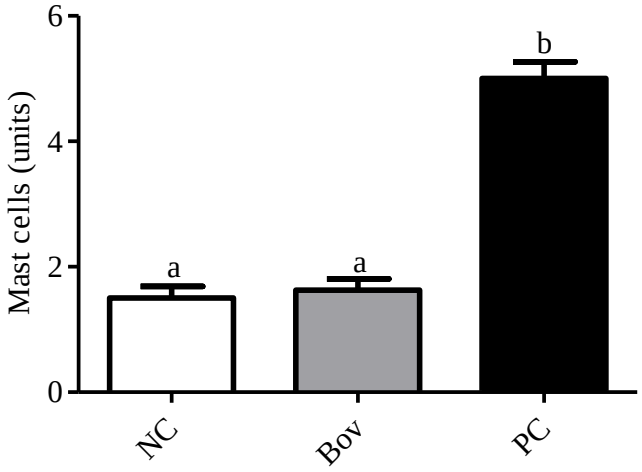
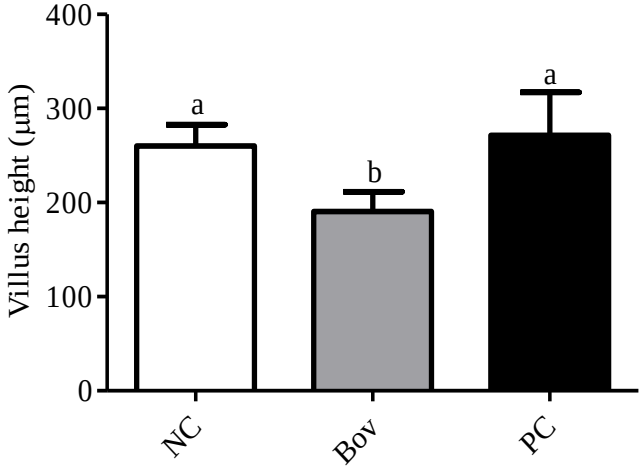


Figure 7: Counts of mast cells in small intestine of the experimental groups. Sections from jejunum segments were stained with toluidine blue/sodium borate (1 %), and the mast cells were counted in the mucosa and submucosa. Data were shown as average $\pm$ SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin.

In the small intestine of animals from the Bov group, significant villous atrophy accompanied by villi enlargement was observed. In the PC group, an increase of the villous diameter was evidenced, although the height of the villi remained the same as compared to the negative control group (Figure 8).

A



B

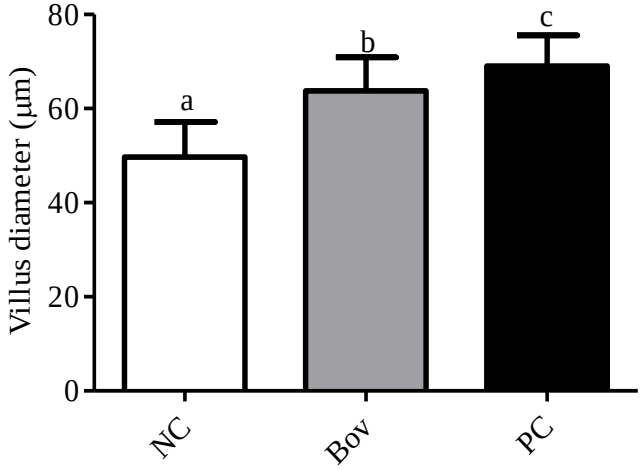


Figure 8: Diameter and height of the small intestinal villi at the experimental groups. Data were shown as average ± SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin.

The large intestine of the negative control group was normal and with a homogenous aspect (Figures 9A and 9B). The effects of the administered compounds were smooth at the large intestine of the animals. No differences on epithelium or at the cellularity were detected for the group that received bovicin HC5 (Figure 9C), while a moderate edema at the lamina propria was detected in animals that received ovalbumin (Figure 9D). A significant reduction at the mucosal thickness was observed among the animals treated with ovalbumin (Figure 10).

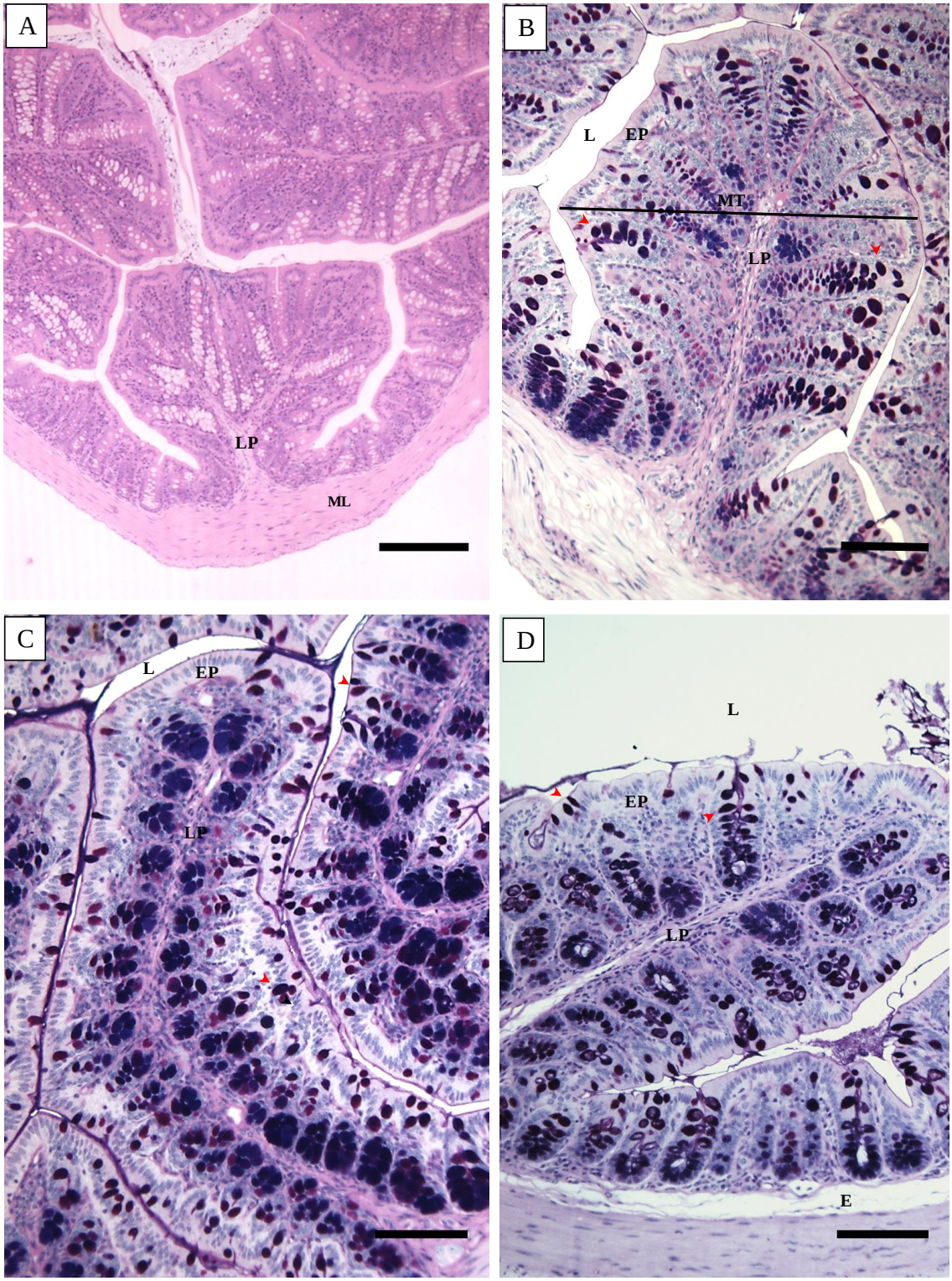


Figure 9: Photomicrograph of histological sections of large intestine of the experimental groups ((NC), negative control group, figures A and B; (Bov) Bovicin HC5 group, figure C; (PC) positive control group, figure D). Jejunum segments were collected and processed for optical microscopy analysis at the end of the experiment. The sections were stained with hematoxylin and eosin (HE; figure A) or PAS/Alcian Blue (figures B-

D). Abbreviations: EP: simple cuboidal epithelium; LP: lamina propria; MT: mucosal thickness; E: edema; MC: muscle layer. Red arrow head indicates goblet cells.
Scale bar = 200 (figure A) or 100 μm (figures B, C and D).

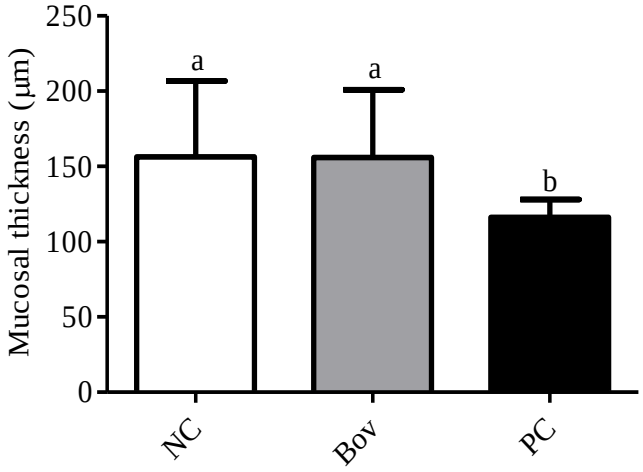
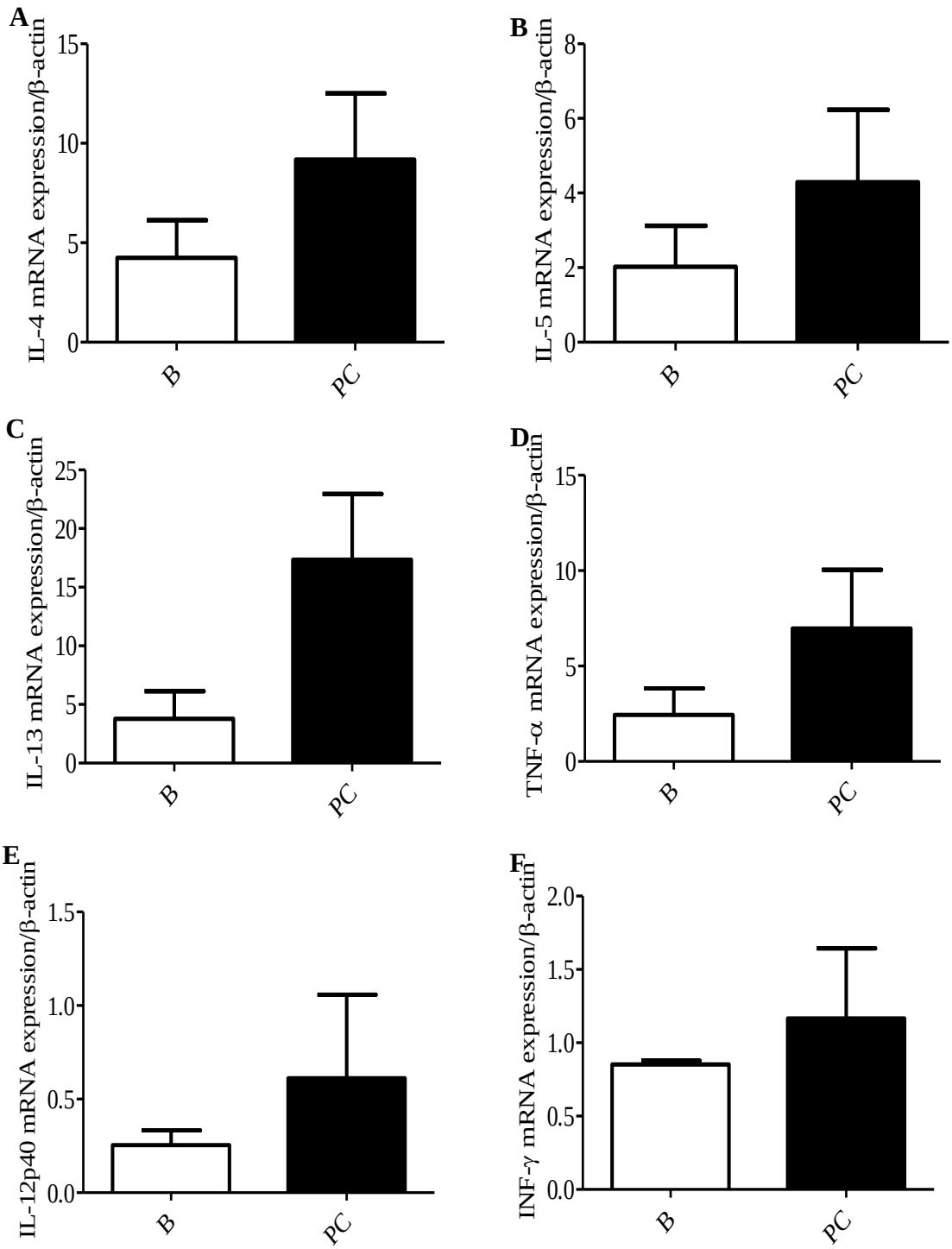


Figure 10: Mucosal thickness of the large intestine of the mice at the experimental groups. Data were shown as average ± SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control; (Bov) mice treated with bovicin HC5; (PC) mice treated with ovalbumin.

4.4.4. Immune parameters

In order to compare the effects of the oral administration of bovicin HC5 and ovalbumin in the modulation of the systemic and local immune response of BALB/c mice, the relative expression of cytokines genes was determined in the spleen and small intestine of the animals. In each set of experiment an aliquot of each target sample was analyzed for β-actin mRNA expression by real-time PCR, in order to normalize for inefficiencies in cDNA synthesis.

Regarding the spleen, the relative expression of the cytokines evaluated did not differ between Bov and PC groups ($p > 0.05$) (Figure 11).


A
IL-4 mRNA expression/β-actin
15
10
5
0
B
PC
B
IL-5 mRNA expression/β-actin
8
6
4
2
0
B
PC
C
IL-13 mRNA expression/β-actin
25
20
15
10
5
0
B
PC
D
TNF-α mRNA expression/β-actin
15
10
5
0
B
PC
E
IL-12p40 mRNA expression/β-actin
1.5
1.0
0.5
0.0
B
PC
F
INF-γ mRNA expression/β-actin
2.0
1.5
1.0
0.5
0.0
B
PC

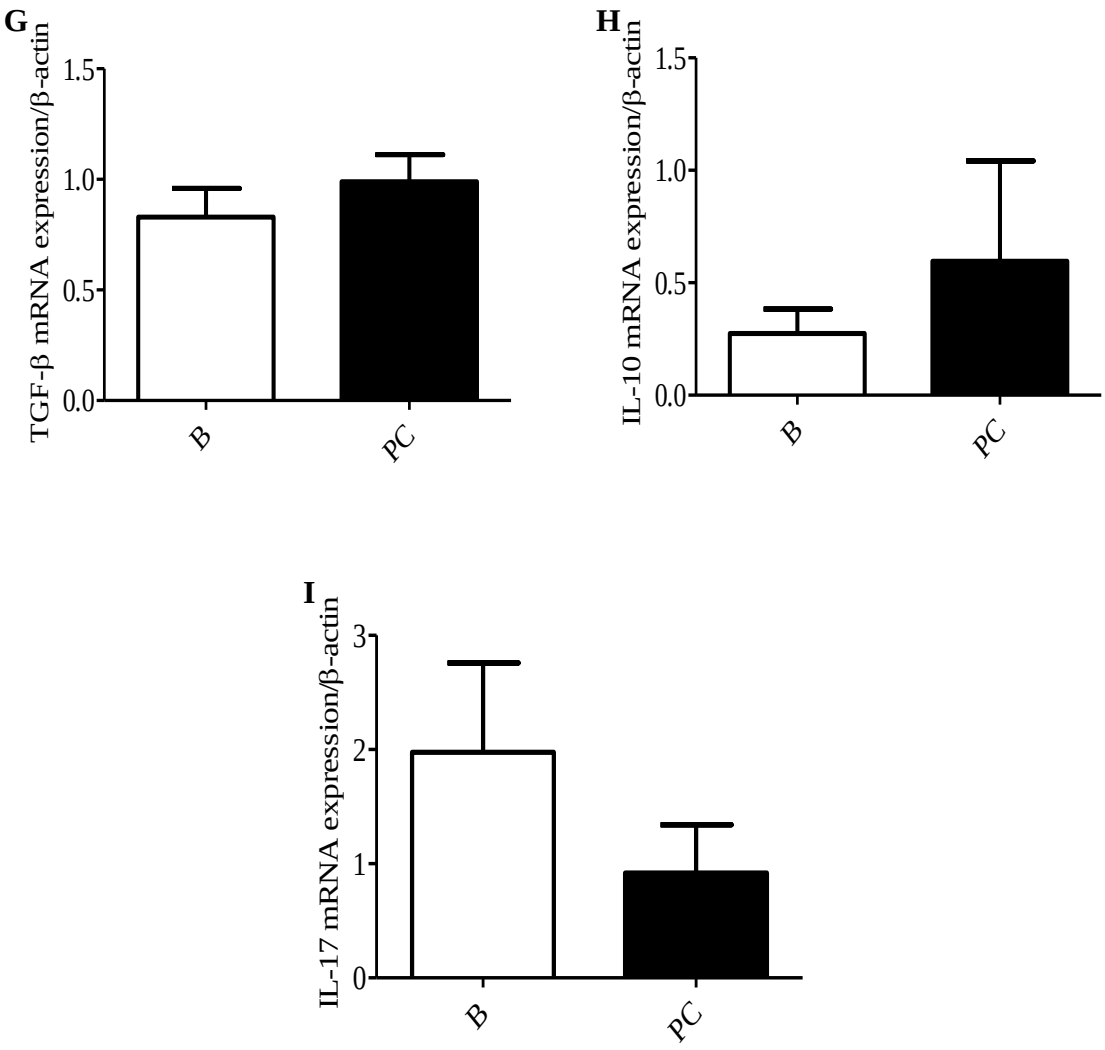
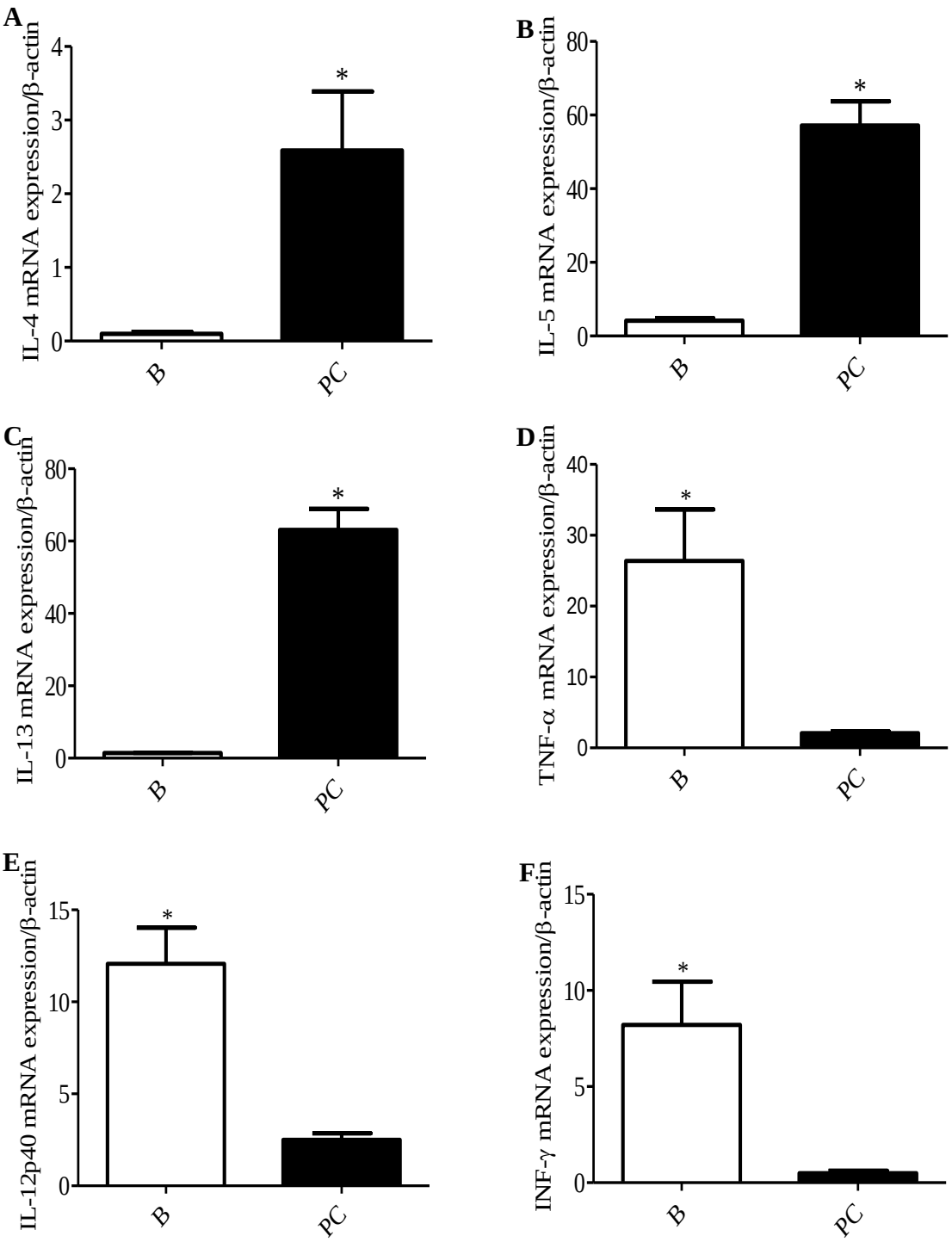


Figure 11: Relative cytokine expression in spleen of five-weeks old female BALB/c mice treated with bovicin HC5 and ovalbumin. IL-4 (A), IL-5 (B), IL-13 (C), TNF-α (D), IL-12 (E), IFN-γ (F), TGF-β (G), IL-10 (H) and IL-17 (I) mRNA was quantified by real time-PCR in spleen collected at the end of the experiment, and calculated by reference to the β-actin in each sample, using the threshold cycle (Ct) method. Results represent the mean value ± SD of data from three mice (values in duplicate), relative to a negative control group.

Comparing the Bov and PC groups, the IL-4, IL-5 and IL-13 mRNA expression was significantly higher in the small intestine of the mice treated with ovalbumin ($p < 0.05$, Figures 12A-C), while the levels of TNF-α, IL-12 and INF-γ messenger RNAs were significantly higher in Bov group (Figures 12D-F). The relative expression of TGF-β, IL-10 and IL-17 did not differ between the groups (Figures 12G-I).


A
IL-4 mRNA expression/β-actin
4
3
2
1
0
*
B
PC
B
IL-5 mRNA expression/β-actin
80
60
40
20
0
*
B
PC
C
IL-13 mRNA expression/β-actin
80
60
40
20
0
*
B
PC
D
TNF-α mRNA expression/β-actin
40
30
20
10
0
*
B
PC
E
IL-12p40 mRNA expression/β-actin
15
10
5
0
*
B
PC
F
INF-γ mRNA expression/β-actin
15
10
5
0
*
B
PC

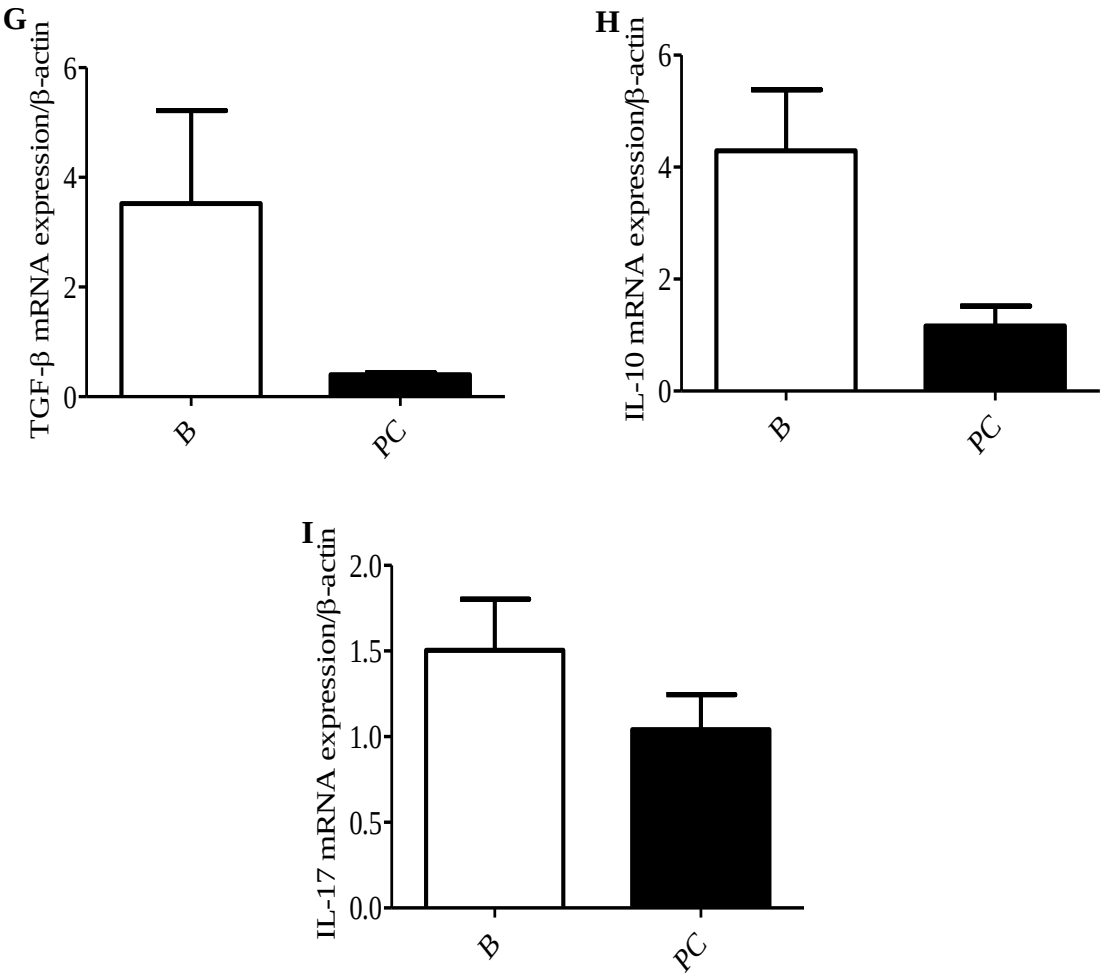


Figure 12: Relative cytokine expression in intestine of five-week old female BALB/c mice treated with bovicin HC5 and ovalbumin. IL-4 (A), IL-5 (B), IL-13 (C), TNF-α (D), IL-12 (E), IFN-γ (F), TGF-β (G), IL-10 (H) and IL-17 (I) mRNA was quantified by real time-PCR in jejunum segments collected at the end of the experiment, and calculated by reference to the β-actin in each sample, using the threshold cycle (Ct) method. Results are demonstrated as the mean value ± SD of data from three mice (values in duplicate), relative to a negative control group. *Significant differences between the relative expression in intestine of mice treated with bovicin HC5 (Bov) and ovalbumin (PC), at $p < 0.05$.

4.5. Discussion

Many antimicrobial peptides produced by bacteria are membrane-active and seem to have little ability to disrupt eukaryotic membranes, probably because of the absence of negatively charged lipids on the cell surface, the lack of a strong membrane potential gradient (internal negative) and the presence of cholesterol (Peschel and Sahl,

2006; Yeaman and Yount, 2003). Because of these characteristics, there is an increasing interest in using bacteriocins as antibiotic substitutes and innate immune modulators (Hancock and Sahl, 2006).

Among the bacteriocins, the lantibiotics have been especially assayed for therapeutic applications. Current trials mainly focus on the use of lantibiotics for topical treatment of bacterial infections (Mookherjee *et al.*, 2007). However, lantibiotics appear to be relatively safe when taken orally (Scott *et al.*, 2007), even though they harbour unusual amino acids and lanthionine rings, which provide certain stability against proteases, including the ones found in the gastrointestinal tract (Hancock and Sahl, 2006).

Lantibiotics can also modulate the immune system, interfering in cytokine expression and leading to selective boosting of innate immunity, both *in vitro* and *in vivo* (Finley and Hancock, 2004; Scott *et al.*, 2007; Liu *et al.*, 2009). On the other hand, antibodies against lantibiotics are very difficult to obtain, probably because these bacteriocins are not captured by the antigen-processing machinery (Hancock and Sahl, 2006).

The modulation of the host immune system induced by bacteriocins is a phenomenon much less understood compared to other peptides or proteins, especially proteins extracted from mushrooms (such as LZ-8 (13 kDa) (Kino *et al.*, 1989), Fip-vvo (15 kDa) (Hsu *et al.*, 1997) and FIP-fve (114 aa)) and host-defense peptides (defensins, cathelicidins, cecropins and magainins) (Yang *et al.*, 2004; Finley and Hancock, 2004).

Nisin, the best-known bacteriocin, is also the most assayed for immunomodulatory activities. Short-term administration of nisin increased the T-lymphocyte population (CD4 and CD8) and decreased the B-lymphocyte cell counts in mice, although these immune effects reported have returned to normal levels after prolonged administration of nisin (de Pablo *et al*., 1999).

Nisin F was able to inhibit the growth of *Staphylococcus aureus* in the respiratory tract of immunocompromised rats (de Kwaadsteniet *et al*., 2009), as well as in the peritoneal cavity, where it remained active for at least 44 h (Brand *et al*., 2010). Another study has shown that nisin F, injected into the peritoneal cavity of mice pre-infected with *S. aureus*, was able to control the infection for 2 h (Brand *et al*., 2011).

In the presence of nisin, neutrophils can evolve a relatively new bacterial killing mechanism, which not only ensures bacterial killing but also restricts spreading of the infection even after the death of the neutrophils. In this mechanism, activated neutrophils produce structures named NETs, whose production is induced by bacterial

LPS and IL-8. Nisin was also found to induce NET formation *in vitro* in a dose-dependent manner (Begde *et al.*, 2011).

To date, it is still contradictory if orally administrated bacteriocins could be significantly absorbed in the intestine, leading to the presence of the bacteriocin in blood and tissues. Given the molecular mass of the bacteriocins belonging to the lantibiotic group (< 5 kDa), absorption and diffusion of intact bacteriocins through the epithelial barrier may not be very efficient. However, the oral administration of bacteriocin E 50–52 in chicken resulted in significant reductions of *Salmonella enteritidis* in the liver and spleen, which suggests that the bacteriocin can enter the systemic system by intestinal absorption (Svetoch *et al*., 2008).

The intestinal mucosa is the first line of host defense, being constantly exposed to a multiplicity of harmful and ineffective antigens. Gut immune system is equipped with many mechanisms that protect mucosal surfaces and prevent tissue injury, such as the brush border glycocalix (that limit the access of micro-organisms, their components and non-digested proteins to the epithelial surface), intraepithelial tight junctions (prevent the access to sub-epithelial tissues), and mucins, antimicrobial peptides and antimicrobial enzymes secreted by the intestinal cells (Mashimo *et al*., 1996).

In normal conditions, both regulator T cells subset (Treg) and IgA are enriched in the intestine: $CD4^+$ $Foxp3^+$ Treg cells and IL-10-producing cells are responsible to regulate innate and adaptive immune responses, and IgA is the first defense against the exposure to orally fed food antigens, thus reinforcing intestinal immune homeostasis and inhibiting the adherence of micro-organism to intestinal cells (Segawa *et al.*, 2008). The induction of intestinal immune response is not easily obtained and the way by which antigens are captured and presented to the immune system is a critical factor that determines if tolerance will occur or if the immune response will be triggered (Perdigón *et al*., 1999; Chirdo *et al.*, 2005).

Proteins orally administrated often induce tolerance and the maintenance of oral tolerance depends on the amount of antigen ingested and the epitopes presented (Élson and Zivory, 1996; Saldanha *et al.*, 2004). The majority of the ingested antigens are degraded, although a few remain intact or are partially degraded, being absorbed in this way. In this condition, the oral tolerance can be overcome and a subsequent immune response can be developed (Dunkley and Husband, 1990).

Sometimes an aggressive immune response against commensal bacteria or non-self peptides, can lead to destructive gut inflammatory disorders, which can be evaluated through food-induced enteropathies in animal models (Sicherer and Leung,

2005). Interactions between non-degraded proteins and gut-associated lymphoid tissue can lead to the breakdown of mucosal tolerance to fed antigens, and consequently to the development of enteropathies. As a result, uncontrolled and hypersensitivity responses are developed, with participation of IgE-producing B cells, T_H2 effector lymphocytes and cytokines, like IL-4 or IL-13, as seen in food-induced allergy (Saldanha *et al*., 2004; Cardoso *et al.*, 2008).

In this study, we evaluated the morphologic and immune effects of the oral administration of bovicin HC5 to BALB/c mice. We used a murine model of food-induced enteropathy to evaluate an intestinal inflammation in which the breakdown in mucosal tolerance was obtained by the oral administration of the food antigen ovalbumin (OVA). OVA has a relative molecular mass of 45 kDa (385 amino acids residues) and four sites of glycosylation. It has some well-established uses, and is a reference protein for both immunological and biochemical studies, being widely used as an antigen for studying allergic diseases in mice (Lloyd *et al.*, 2001).

The model used in this study to induce food enteropathy worked properly, and the non-tolerogenic antigen OVA altered the intestinal architecture and physiology as well as modulated the mucosal immunity by triggering an exacerbated immune response in BALB/c mice. Loss of body weight, influx of mast cells, edema, congestion and cell infiltration in lamina propria, presence of degenerative areas and villous enlargement in small intestine, as well as edema and reduction of mucosal thickness in large intestine were observed in the animals that received ovalbumin.

The oral administration of OVA altered the gastrointestinal physiology of BALB/c mice, inducing an increase in protein permeability, since important changes in the pattern of β-lactoglobulin (β-LG) uptake was observed when compared to the negative control group. High levels of β-LG in the serum of the animals treated with OVA were detected, especially after 2 h after β-LG administration.

After 5 h of β-LG administration, low concentrations of the protein were detected in the sera from animals of the Bov group, when compared to the NC group. This result should not be evaluated as an increase in intestinal permeability, since only 1.08 mg ml^{-1} of β-LG was detected. We did not evaluate the presence of β-LG after this point, and thus it is not clear if β-LG would be still present in the serum of the animals treated with bovicin HC5.

The significant reduction in cellularity in the spleen of mice treated with bovicin HC5 or OVA can be due to the influx of immune cells to the intestine, in an attempt to modulate the immune response established in this site. A significant increase of mast

cells in the mucosa and submucosa of the small intestine was observed in animals from the PC group. Similar results were demonstrated in mice fed OVA diet, in which mast cell degranulation and large amounts of histamine were detected (Nakajima-Adachi *et al.*, 2006).

In normal conditions, the number of mast cells in the intestine is relatively constant, but hyperplasia could be observed in inflammatory reactions or in stages of remodeling /repair of inflammatory or fibrotic disorders (Bischoff and Sellge, 2002). In allergy, independent of the classical role of mast cells in the early stages of the disease, these cells also have an important role in later and chronic stages. In this situation, mast cells interact with and are activated by infiltrated inflammatory cells (eosinophils and lymphocytes) and by resident structural cells (epithelial cells, smooth muscle cells and fibroblasts) (Pawankar *et al*., 2003). Prolonged exposure to OVA followed by oral challenge is known to result in histological changes in the intestine of experimental mice. OVA can also cause mucosal mast cell degranulation with increasing histamine levels in intestine (Hsieh *et al.,* 2003; Saldanha *et al*., 2004; Vaali *et al.*, 2006).

Upon oral administration of bovicin HC5, intestinal inflammation was not observed, although a heterogeneous aspect and alterations in the small intestinal cells have been detected in animals that received bovicin HC5. In Bov group, a destruction of the apical portion of the intestinal villi was detected in some points, as well as an increase of the villous diameter and a reduction of the villous height. In contrast, the OVA administration caused a more prominent increase on the small intestinal villous diameter, an edema and a decrease on mucosal thickness in the large intestine. The degree of impairment of the intestinal mucosa, specifically in the small intestine, could explain the differences observed at the weight gain among the groups throughout the experiment, since these alterations could have influenced the absorption of nutrients in the small intestine.

In agreement with the results obtained in this study, Saldanha *et al*. (2004) demonstrated significant loss of weight in BALB/c mice previously sensitized and challenged with OVA. According to the authors, the weight loss started one week after the oral challenge and remained until the end of the experiment. Moreover, an increase at the vascular permeability of the small intestine, demonstrated by an increased flow out of the Evans blue dye, was also observed in that study.

Bacterial products or components may induce metaplasia, proliferation and hypersecretion of goblet cells (Nell and Grote, 2003). In this study, the pattern of the goblet cells in the small intestine upon oral administration of bovicin HC5 did not differ

from the negative control group, regarding the number of total cells and the secretion of mucopolysaccharides. However, a reduction of the goblet cell counts was observed in the small intestine of the PC group, as well as a reduction in the number of PAS/AB^{+} cells, responsible for the secretion of acidic and neutral mucins. The mucus protects the intestinal wall by limiting the absorption of antigens. Therefore, the hypersecretion of mucopolysaccharides was expected, as a characteristic of allergic inflammation and the result of increased IL-13 expression (Zimmermann *et al*., 2003). However, the reduction of the number of cells responsible for mucus secretion may be not related to the reduction of secretion but to the limited count fields, which were resulted of the partial destruction of villi observed in PC group.

The expression and activity of proteins related to intestinal cell proliferation, differentiation and apoptosis are influenced by the presence of different substances in the gastrointestinal tract (Sergent, 2008). In this study, the intestinal tissue of both Bov and PC groups exhibited epithelium structure alterations, although in different degrees of severity. To better determine the epithelial renewal, features of tissue repair, as hyperplasia of Paneth cells and the presence of epithelial cells in mitotic division, were analyzed.

Paneth cells, together with goblet cells, enterocytes and enteroendocrine cells, represent the principal cell types of the mouse small intestinal epithelium (Wright, 2000). Paneth cells are located adjacent to stem cells, which replenish, by mitotic division, the epithelial cells that die or are lost from the villi. The location of Paneth cells suggests that they play a critical role in defending epithelial cell renewal in the intestine. Besides this function, Paneth cells also play an important role in host intestinal defense mechanisms, contributing to the maintenance of the gastrointestinal barrier, by secreting antimicrobial peptides and other compounds in response to bacteria and bacterial antigens (Ayabe *et al.*, 2000; Keshav, 2006).

Hyperplasia of Paneth cells and increased mitotic activity were observed in both Bov and PC groups, indicating that despite of the loss of villi architecture, the processes of antimicrobial compounds secretion and tissue repair remained activated, in an attempt to counteract the injuries caused by bovicin HC5 and ovalbumin.

To understand the modulation of immune responses upon chronic oral exposure to bovicin HC5, we investigated the relative expression of cytokines in spleen and small intestine of the treated animals. Significant elevation in relative expression of the IL-4, IL-5 and IL-13 was observed in the intestine of animals treated with OVA. A reduction in regulatory cytokines TGF-β and IL-10 relative expression was observed in the

inflamed intestine of the PC group, although the difference was not significant when compared to the Bov group.

OVA might have induced morphologic alterations and modulated intestinal immune response during food-induced enteropathy through a loss of regulatory mechanisms involved in mucosa homeostasis, since low TGF-β and IL-10 mRNA expression in intestinal mucosa was observed (Chung *et al.*, 2005; Chirdo *et al.*, 2005). The high expression of the cytokines IL-4, IL-5 and IL-13 corroborated with the modulation of immunity towards significant T_H2-polarized response (Sampson *et al.*, 2001). The down modulation of regulatory mechanisms, with reduction of TGF-β and IL-10 expression in the intestine, as observed in OVA group, may be involved in the development of food allergy (Cardoso *et al.*, 2008).

In agreement with our results, Goya *et al.* (2003) observed increased mRNA levels of the T_H2 cytokines IL-4, IL-5 and IL-13, as well as decrease of the T_H1 cytokine INF-γ expression, in the lungs of OVA-treated mice, compared with the cytokine levels in control animal lungs.

Paradoxically, induction of oral tolerance has been reported in spleen cells from mice fed the egg diet, and according to the authors, it is possible that the intestinal tissue, after exposure to an egg white diet for 28 days, reflects a state of partial suppression of the injurious T_H2 responses, thereby causing the villous blunting or partial villous atrophy and a tendency to weight recovery (Asai *et al.*, 2002). However, feeding times longer than 28 days did not completely resolve inflammation in all mice (Nakajima-Adachi *et al.*, 2006).

In contrast to the T_H2-polarized response elicited by OVA, higher mRNA expression for the T_H1 cytokines TNF-α, IL-12 and INF-γ were observed in the intestine of bovicin HC5-fed mice. Liu *et al.* (2009) also demonstrated significant induction of IFN-γ after administration of dioscorin, a yam tuber storage protein, for 21-days at a dose of 20 mg/kg/day; moreover, oral dioscorin administrated *in vivo* was able to increase the number of Peyer's patches and the secretion of dioscorin non-specific IgA, and this might have result in higher mucosal immunity.

Human cathelicidin LL-37 modulated the activity of IFN-γ on a variety of cell types, which have important implications in both innate and adaptive immune responses (Nijnik *et al.*, 2009). In the absence of pretreatment with LL-37, the host peptide was able to reduce the synthesis of IFN-γ in the presence of LPS (Nijnik *et al.*, 2009), and, according to the authors, the effects of LL-37 on IFN-γ responses may represent a balancing role in promoting IFN-γ production while down-regulating some of its

effector functions. However, pretreatment with LL-37 induced the IFN-γ production by monocytes, enhancing monocyte-derived dendritic cell functions, as IL-12 secretion and T_H1-polarized co-stimulatory activity (Davidson *et al.*, 2004; Chuang *et al.*, 2009). Similar effects were induced by bovicin HC5 in BALB/c mice, as demonstrated in the present study.

According to the results obtained with other antimicrobial peptides, the oral exposure to bovicin HC5 in the absence of previous sensitization probably would lead to different results, regarding to morphological and immune modulation activities. As demonstrated by Cardoso *et al.* (2007), continuous oral exposure to peanuts induced high levels of IL-10 and TGF-β messenger RNAs, in non-sensitized animals, indicating that tolerance was induced.

In the present work, for the first time, the effects of oral administration of bovicin HC5 to model animals have been described. The results presented confirmed that bovicin HC5, a bacteriocin produced by *S. bovis* HC5, is able to stimulate the gut immune system of BALB/c mice, by influencing the cytokine release through T_H1-polarized response.

However, bovicin HC5 caused morphological alterations in intestine of the animals, with partial destruction of small intestinal cells. This activity might have been caused by the direct action of bovicin HC5 toward epithelial cells, since we have previously demonstrated that bovicin HC5, in higher concentrations, is able to permeabilize membranes in an unspecific way, causing leakage of intracellular compounds (Paiva *et al.*, 2011). However, it is important to note that the impairment of the intestinal cells induced by bovicin HC5 was less pronounced than the effects caused by OVA and did not alter the physiology of the organ, since no alterations in gut permeability were demonstrated.

The relationship between optimal doses and immune response modulation, as well as the changes in intestinal microbial population caused by bovicin HC5 will be substantiated in further studies. As future perspectives, bovicin HC5 could be assayed *in vivo* as the sole anti-microbial agent or in combination with classical antibiotics in different immunostimulatory regimen.

4.6. References

Akiyama, H.; Teshima, R.; Sakushima, J.; Okunuki, H.; Goda, Y.; Sawada, J.; Toyoda, M. Examination of oral sensitization with ovalbumin in *Brown Norway* rats and three strains of mice. Immunology Letters 78, 1–5, 2001.

Asai, K.; Hachimura, S.; Kimura, M.; Toraya, T.; Yamashita, M.; Nakayama, T; Kaminogawa, S. T cell hyporesponsiveness induced by oral administration of ovalbumin is associated with impaired NFAT nuclear translocation and p27kip1 degradation. The Journal of Immunology 169, 4723-4731, 2002.

Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J. A.; Struhl, K. Short Protocols in Molecular Biology. Wiley: New York, 4th edition, 1999. 1931p.

Ayabe, T.; Satchell, D.; Wilson, C.; Parks, W.; Selsted, M.; Ouellette, A. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nature Immunology 1, 113–8, 2000.

Bancroft, J.D.; Stevens, A. Theory and Practice of Histological Techniques. 4th edition, Churchill Livingstone, London , 1996.

Begde, D.; Bundale, S.; Mashitha, P.; Rudra, J.; Nashikkar, N.; Upadhyay, A. Immunomodulatory efficacy of nisin – a bacterial lantibiotic peptide. Journal of Peptide Science 17, 438-444, 2011.

Belguesmia, Y.; Madi, A.; Sperandio, D.; Merieau, A.; Feuilloley, M.; Prévost, H.; Drider, D.; Connil, N. Growing insights into the safety of bacteriocins: the case of enterocin S37. Research in Microbiology 162, 159-163, 2011.

Bischoff, S.C.; Sellge, G. Mast cell hyperplasia: Role of cytokines. International Archives of Allergy and Immunology 127, 118–122, 2002.

Brand, A.M.; de Kwaadsteniet, M.; Dicks, L.M.T. The ability of Nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice. Letters in Applied Microbiology 51, 645–649, 2010.

Brand, A.M..; Smith, R.M.; de Kwaadsteniet, M.; Dicks, L.M.T. Development of a murine model with optimal routes for bacterial infection and treatment, as determined with bioluminescent imaging in C57BL⁄6 mice. Probiotics and Antimicrobial Proteins 3, 125-131, 2011.

Cardoso, C.R.; Teixeira, G.; Provinciatto, P.R.; Godoiz, D.F.; Ferreira, B.R.; Milanezi, C.M.; Ferraz, D.B.; Rossi, M.A.; Cunhaz, F.Q.; Silva, J.S. Modulation of mucosal immunity in a murine model of food-induced intestinal inflammation. Clinical and Experimental Allergy, Experimental Models of Allergic Disease 38, 338–349, 2007.

Cardoso, C.R.; Provinciatto, P.R.; Godoi, D.F.; Vieira, T.S.; Ferreira, B.R.; Teixeira, G.; Rossi, M.A.; Cunha, F.Q.; Silva, J.S. B cells are involved in the modulation of pathogenic gut immune response in food-allergic enteropathy. Clinical and Experimental Immunology 154, 153–161, 2008.

Carson, F.L.; Martin, J.H.; Lynn, J.A. Formalin fixation for electron microscopy: a re-evaluation. American Journal of Clinical Pathology 59, 365-373, 1973.

Chirdo, F.G.; Millington, O.R.; Beacock-Sharp, H.; Mowat, A.M. Immunomodulatory dendritic cells in intestinal lamina propria. European Journal of Immunology 35, 1831–1840, 2005.

Chuang, C. M.; Monie, A.; Wu, A.; Mao, C.P.; Hung, C.F. Treatment with LL-37 peptide enhances antitumor effects induced by CpG oligodeoxynucleotides against ovarian cancer. Human Gene Therapy 20, 303–313, 2009.

Chung, Y.; Lee, S.H.; Kim, D.H.; Kang, C.Y. Complementary role of CD41 CD251 regulatory T cells and TGF-beta in oral tolerance. Journal of Leukocyte Biology 77, 906–913, 2005.

Davidson, D.J.; Currie, A.J.; Reid, G.S.; Bowdish, D.M.; MacDonald, K.L.; Ma, R.C.; Hancock, R.E.W.; Speert, D.P. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. The Journal of Immunology 172, 1146–1156, 2004.

de Kwaadsteniet, M.; ten Doeschate, K.; Dicks, L.M.T. Nisin F in the treatment of respiratory tract infections caused by *Staphylococcus aureus*. Letters in Applied Microbiology 48, 65–70, 2009.

de Pablo, M.A.; Gafotio, J.J.; Gallego, A.; Ortega, E.; Gálvez, A.M.; Alvarez de Cienfuegos López, G. Evaluation of immunomodulatory effects of nisin containing diets on mice. FEMS Immunology and Medical Microbiology 24**,** 35–42, 1999.

Delves-Broughton J. Nisin as a food preservative. Food Australia 57, 525-527, 2005.
Dunkley, M.L.; Husband, A.J. Routes of priming and challenge for IgA antibody-containing cell responses in the intestine. Immunology Letters 26, 165-170, 1990.

Élson, C.H.; Zivory, J. Oral tolerance: A commentary. In: Kagnoff, M.; Kiyono, H. (eds) Essentials of mucosal immunology. Academic Press, Inc., San Diego, CA, p. 543-554, 1996.

Finlay, B.B.; Hancock, R.E. Can innate immunity be enhanced to treat microbial infections? Nature Reviews Microbiology 2, 497–504, 2004.

Gálvez, A.; López, R.L.; Abriouel, H.; Valdivia, E.; Ben Omar, N. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Critical Reviews in Biotechnology 28, 125-152, 2008.

Goya, I.; Villares, R.; Zaballos, A.; Gutiérrez, J.; Kremer, L.; Gonzalo, J.A.; Varona, R.; Carramolino, L.; Serrano, A.; Pallares, P.; Criado, L.M.; Kolbeck, R.; Torres, M.; Coyle, A.J.; Gutiérrez-Ramos, J.C.; Martínez, C.; Márquez, G. Absence of CCR8 does not impair the response to ovalbumin-induced allergic airway disease. The Journal of Immunology 170, 2138-2146, 2003.

Hancock, R.E.W.; Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24, 1551-1557, 2006.

Hsieh, K.Y.; Tsai, C.C.; Wu, C.H.; Lin, R.H. Epicutaneous exposure to protein antigen and food allergy. Clinical & Experimental Allergy 33, 1067–75, 2003.

Hsieh, K.Y.; Hsu, C.I.; Lin, J.K.; Tsai, C.C.; Lin, R.H. Oral administration of an edible-mushroom-derived protein inhibits the development of food-allergic reactions in mice. Clinical & Experimental Allergy 33, 1595–1602, 2003.

Hsu, H.C.; Hsu, C.I.; Lin, R.H.; Kao, C.L.; Lin, J.Y. Fip-vvo, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*. Biochemical Journal 323, 557–565, 1997.

Keshav, S. Paneth cells: leukocyte-like mediators of innate immunity in the intestine. Journal of Leukocyte Biology 80, 500-508, 2006.

Kino, K.; Yamashita, A.; Yamaoka, K.; Watanabe, J.; Tanaka, S.; Ko, K.; Shimizu, K.; Tsunoo, H. Isolation and characterization of a new immunomodulatory protein, ling zhi-8 (LZ-8), from *Ganoderma lucidium*. The Journal of Biological Chemistry 264, 472-478, 1989.

Knippels, L.M.J.; Houben, G.F.; Spanhaak, S.; Penninks, A.H. An oral sensitization model in *Brown Norway* rats to screen for potential allergenicity of food proteins. Methods 19, 78-82, 1999.

Liu, Y.W.; Liu, J.C.; Huang, C.Y.; Wang, C.K.; Shang, H.F.; Hou, W.C. Effects of oral administration of yam tuber storage protein, dioscorin, to BALB/c mice for 21-days on immune responses. Journal of Agricultural and Food Chemistry 57, 9274–9279, 2009.

Lloyd, C.M.; Gonzalo, J.A.; Coyle, A.J.; Gutierrez-Ramos, J.C. Mouse models of allergic airway disease. Advances in Immunology 77, 263, 2001.

Maher, S.; McClean, S. Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. Biochemical Pharmacology 71, 1289-1298, 2006.

Malo, C.; Morin, C.L. Establishment of an animal model of ovalbumin sensitised mouse to study protein induced enteropathy. Gut 27, 1298-1305, 1986.

Mantovani, H.C.; Hu, H.; Worobo, R.W.; Russell, J.B. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology 148, 3347-3352, 2002.

Mashimo, H.; Wu, D.C.; Podolsky, D.K.; Fishman, M.C. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. Science 274, 262-265, 1996.

Mookherjee, N.; Rehaume, L.M.; Hancock, R.E.W. Cathelicidins and functional analogues as antisepsis molecules. Expert Opinion on Therapeutic Targets 11, 993–1004, 2007.

Nakajima-Adachi, H.; Ebihara, A.; Kikuchi, A.; Ishida, T.; Sasaki, K.; Hirano, K.; Watanabe, H.; Asai, K.; Takahashi, Y.; Kanamori, Y.; Shimojo, N.; Matsuda, H.; Kohno, Y.; Hachimura, S.; Kaminogawa, S. Food antigen causes T_H2-dependent enteropathy followed by tissue repair in T-cell receptor transgenic mice. Journal of Allergy and Clinical Immunology 117, 1125-1132, 2006.

Nell, M.J.; Grote, J.J. Effects of bacterial toxins on air-exposed cultured human respiratory sinus epithelium. Annals of Otology, Rhinology and Laryngology 112, 461–468, 2003.

Nijnik, A.; Pistolic, J.; Wyatt, A.; Tam, S.; Hancock, R.E.W. Human cathelicidin peptide LL-37 modulates the effects of IFN-γ on APCs. Journal of Immunology 183, 5788-5798, 2009.

Paiva, A.D.; Breukink, E.; Mantovani, H.C. The role of Lipid II and membrane thickness in the mechanism of action of the lantibiotic bovicin HC5. Antimicrobial Agents and Chemotherapy, 2011. *In press.*

Paiva, A.D.; de Paula, S.O.; Baracat-Pereira, M.C.; Breukink, E.; Mantovani, H.C. Assessment of the *in vitro* cytotoxicity of the lantibiotics bovicin HC5 and nisin on eukaryotic cells and model membranes. Microbiology, 2011. *Under review.*

Parisien, A.; Allain, B.; Zhang, J.; Mandeville, R.; Lan, C.Q. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. Journal of Applied Microbiology 104, 1-13, 2008.

Pawankar, R.; Yamagishi, S.; Takizawa, R.; Yagi, T. Mast cell-IgE and mas cell-structural cell interactions in allergic airway disease. Current Drug Targets Inflammation & Allergy 2, 303-312, 2003.

Perdigón, G.; Vintiñi, E.; Alvarez, S.; Medina, M.; Medici, M. Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. Journal of Dairy Science 82, 1108-1114, 1999.

Peschel, A.; Sahl, H.G. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature Reviews Microbiology 4, 529–536, 2006.

Saldanha, J.C.S; Gargiulo, D.L.; Silva, S.S.; Carmo-Pinto, F.H.; Andrade, M.C.; Alvarez-Leite, J.I.; Teixeira, M.M.; Cara, D.C. A model of chronic IgE mediated food allergy in ovalbumin-sensitized mice. Brazilian Journal of Medical and Biological Research 37, 809–816, 2004.

Sampson, H.A.; Sicherer, S.H.; Bimbaum, A.H. AGA technical review on the evaluation of food allergy in gastrointestinal disorders. American Gastroenterological Association. Gastroenterology 120, 1026-1040, 2001.

Scott, M.G.; Dullaghan, E.; Mookherjee, N.; Glavas, N.; Waldbrook, M.; Thompson, A.; Wang, A.; Lee, K.; Doria, S.; Hamill, P.; Yu, J.J.; Li, Y.; Donini, O.; Guarna, M.M.; Finlay, B.B.; North, J.R.; Hancock, R.E.W. An anti-infective peptide that selectively modulates the innate immune response. Nature Biotechnology 25, 465–472, 2007.

Shuichi Segawa, S.; Nakakita, Y.; Takata, Y.; Wakita, Y.; Kaneko, T.; Kaneda, H.; Watari, J.; Yasui, H. Effect of oral administration of heat-killed *Lactobacillus brevis*

SBC8803 on total and ovalbumin-specific immunoglobulin E production through the improvement of T_H1/T_H2 balance. International Journal of Food Microbiology 121, 1–10, 2008.

Sergent, T.T.; Ribonnet, L.; Kolosova, A.; Garsou, S.; Schaut, A.; de Saeger, S.; Peteghem, C.V.; Larodelle, Y.; Puseemier, L.; Scheneider, Y. Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. Food and Chemical Toxicology 46, 813-841, 2008.

Sicherer, S.H.; Leung, D.Y. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. Journal of Allergy and Clinical Immunology 116, 153–63, 2005.

Svetoch, E.A.; Eruslanov, B.V.; Perelygin, V.V.; Mitsevich, E.V.; Mitsevich, I.P.; Borzenkov, V.N.; Levchuk, V.P.; Svetoch, O.E.; Kovalev, Y.N.; Stepanshin, Y.G.; Siragusa, G.R.; Seal, B.S.; Stern, N.J. Diverse antimicrobial killing by *Enterococcus faecium* E 50-52 bacteriocin. Journal of Agricultural and Food Chemistry 56, 1942–1948, 2008.

Toke, O. Antimicrobial peptides: new candidates in the fight against bacterial infections. Biopolymers 80, 717-735, 2005.

Vaali, K.; Puumalainen, T.J.; Lehto, M.; Wolff, H.; Rita, H.; Alenius, H.; Palosuo, T. Murine model of food allergy after epicutaneous sensitization: role of mucosal mast cell protease-1. Scandinavian Journal of Gastroenterology 41, 1405–1413, 2006.

Vaucher, R.A.; Teixeira, M.L.; Brandelli, A. Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells. Current Microbiology 60, 1-5, 2010.

Wright, N.A. Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. International Journal of Experimental Pathology 81, 117-143, 2000.

Yang, D.; Biragyn, A.; Hoover, D.M.; Lubkowski, J.; Oppenheim, J.J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annual Review of Immunology 22, 181–215, 2004.

Yeaman, M.R.; Yount, N.Y. Mechanisms of antimicrobial peptide action and resistance. Pharmacological Reviews 55, 27–55, 2003.

Zimmermann, N.; Hershey, G.K.; Foster, P.S.; Rothenberg, M.E. Chemokines in asthma: cooperative interaction between chemokines and IL-13. Journal of Allergy and Clinical Immunology 111, 227-42, 2003.

CHAPTER 5

Effects of the oral administration of *Streptococcus bovis* HC5 on BALB/c mice.

5.1. Abstract

Streptococcus bovis HC5 is a bacteriocin-producing strain that inhibits many gram-positive bacteria, including commensal and pathogenic bacteria. Although *S. bovis* HC5 appears to be a bacterium commonly found in the bovine rumen, its effects on other animal models with simple monogastric digestive system is much less understood. In this study, we evaluated the effects of the oral administration of viable and heat-killed *S. bovis* HC5 cells to BALB/c mice, in order to assess the immunomodulatory properties of *S. bovis* HC5. The administration of *S. bovis* HC5 for 58 days to BALB/c mice resulted in important histological and morphological alterations on intestine, and also in a cellularity reduction at the spleen. No alterations at heart and liver were detected. The oral administration of *S. bovis* HC5 also influenced cytokine production in the intestine, and the immune-mediated activity differed between live and heat-killed cells. The relative expression of IL-12 and INF-γ was significantly higher in the small intestine of mice that had been treated with viable *S. bovis* HC5 cells, while an increase in IL-5, IL-13 and TNF-α expression was detected in mice treated with heat-killed *S. bovis* HC5 cells. This study indicated that the oral administration of *S. bovis* HC5 resulted in histological and morphometric alterations in the intestine, and there was a distinguishable difference in the immunostimulatory activity of live and heat-killed *S. bovis* HC5 cells. Future studies are needed to examine the interactions between *S. bovis* HC5 and the mammalian cells and other bacteria found in the intestine.

5.2. Introduction

Lactic acid bacteria (LAB) are one of the best studied groups of microorganisms, and many of them are known to produce antimicrobial substances (de Vuyst and Vandamme, 1994). Besides their antimicrobial activity, several strains of LAB are also able to enhance innate and adaptive immunity, a feature that has been demonstrated over the years, in animal models (Nagafuchi *et al*., 1999; Gill and Rutherfurd, 2001) and human trials (Chiang *et al*., 2000; Parra *et al*., 2004; Valeur *et al*., 2004). However, the presence of immunomodulatory properties and the pattern of immunomodulation depend on the bacterial species involved, the individual strain (Nagafuchi *et al*., 1999), the use of viable or killed bacteria, and the bacterial dose (Gill and Rutherfurd, 2001; Bujalance *et al.*, 2007).

Streptococcus is a genus of diverse spherical gram-positive lactic acid bacteria. Some species of Streptococci have occasionally been associated with pathogenicity, and implicated in diseases as sore throat, pneumonia, meningitis, endocarditis, necrotyzing fasciitis, ruminal acidosis and bloat. On the other hand, many strains are non-pathogenic and occur as natural commensal bacteria in different habitats, including the skin, the oral cavity and the respiratory, gastrointestinal, and urogenital tracts of humans and animals (Holt, 1994).

Streptococcus bovis, a member of the group D of Lancefield (Lancefield, 1933), is one of the best characterized gastrointestinal Streptococci in both humans and animals. In ruminants, *S. bovis* is normally present at populations ranging from 10^4 to 10^7 colony forming units (CFU)/g of ruminal contents (Nagaraja and Titgemeyer 2006), but it can outgrow other ruminal bacteria under optimal growth conditions (Morovsky*et al.*, 1998; Russell and Rychlik, 2001). When cattle are fed starch-based diets, *S. bovis* shift to homolactic fermentation and produce large amounts of lactate, causing ruminal acidosis (Hungate, 1966).

Streptococcus bovis HC5 was isolated from bovine rumen (Mantovani *et al.*, 2001), and this bacterium produces the lantibiotic bovicin HC5, a bacteriocin with broad antibacterial spectrum of activity (Mantovani *et al.*, 2002). *S. bovis* HC5 could inhibit many ruminal bacteria (Mantovani *et al*., 2001), and it appears that *S. bovis* HC5 or bovicin HC5 have potential to be used for manipulation of the ruminal fermentation (Mantovani *et al.*, 2002).

Virtually no information is available regarding the immunostimulating properties of *S. bovis* strains on the gastrointestinal tract of animal hosts. The aim of the

present study was to investigate the effects of the oral administration of viable and heat-killed *S. bovis* HC5 cells on cytokine production and the integrity of the gastrointestinal tract of BALB/c mice.

5.3. Experimental procedures

5.3.1. *Streptococcus bovis* HC5, media and growth conditions

Streptococcus bovis HC5 was cultivated under anaerobic conditions, at 39 ºC, in basal medium containing (per liter): 0.292 g K_2HPO_4; 0.292 g KH_2PO_4; 0.48 g $(NH_4)_2SO_4$; 0.48 g NaCl; 0.1 g $MgSO_4.7H_2O$; 0.064 g $CaCl_2.2H_2O$; 0.5 g cystein hydrochloride; 4 g Na_2CO_3; 0.1 g trypticase; 0.5 g yeast extract. Glucose was added as carbon source (4 g l^{-1}).

The cells were grown until mid-log phase (OD_{600nm}= 0.7 or 10^9 CFU ml^{-1}), collected by centrifugation (1,742 *g*, 15 min, 5 ^oC), and then washed twice with sterile phosphate buffer saline (PBS, 10 mmol L^{-1} phosphate buffer, 150 mmol L^{-1} NaCl, pH 7.2). Heat-killed *S. bovis* HC5 were prepared by autoclaving (121 ^oC, 20 min) mid-log phase cultures (prepared as described above). After treatment, *S. bovis* cells were suspended in PBS, and stored at - 20 °C, until use.

5.3.2. Animals

Five-week-old female BALB/c mice weighing 18±1 g were provided by the animal breeding colony of the Federal University of Viçosa. The animals were randomly divided into three experimental groups, containing 6 animals each: Group 1, mice given PBS (negative control, NC group); Group 2, mice given viable cells of *S. bovis* HC5 (V group); Group 3, mice given heat-killed *S. bovis* HC5 (HK group).

The mice were housed in an animal room maintained at 24±2 °C, with a light/dark cycle of 12 h and a relative humidity of 55±15% during the experiment and for 10 days prior to initiate the study. The mice were fed a standard laboratory rodent chow (Purina®) and water *ad libitum* over the experiment. All procedures were conducted in accordance with the Guidelines for Animal Experiments adopted by the Ethical Committee in Animal Research of the Federal University of Viçosa.

The animals were immunised as described by Malo and Morin (1986) with modifications. Initially, the mice were subcutaneously immunized with the respective streptococci cells (100 µl of a culture containing 1 x 10^{10} CFU ml^{-1}) in alum (50 µl of a 20 mg ml^{-1} alum hydroxide solution in sterile saline buffer) (first immunization, day 0);

after three weeks, the mice were subcutaneously boosted with the bacterial cells, but without adjuvant (second immunization, day 21). The group 1-mice were immunized with sterile PBS (10 mM, pH 7.2), using the same conditions described above.

The bacterial cells (100 µl of a culture containing 1 x 10^{10} CFU ml^{-1}) or PBS (100 µl) were administered without the use of adjuvant, by daily gavages, using *18-gauge*stainless steel feeding needles. The oral administration started one week after the second sensitization injection (day 28) and continued for the full experimental period (day 58).The mice were weighted weekly and monitored daily, regarding general appearance and adverse reactions at the immunizations sites.

Blood samples were collected at the beginning and at the end of the experiment (days 0 and 58), from the orbital plexus under light ether anesthesia. The samples were kept at room temperature for 2 hours, and the sera were centrifuged at 12,000 x *g* for 5 min (Eppendorf®, Centrifuge 5415C, Hamburg, Germany), at room temperature (Ausubel *et al*., 1999). The samples were stored at - 20 °C until use.

5.3.3. Gut permeability

The possible changes in gut permeability were determined by the uptake of β-lactoglobulin following challenge, as described by Knippels *et al*. (1999), with some modifications. After the experimental period (day 58), animals sensitized with *S. bovis* HC5 (viable and heat-killed cells) and control animals were orally challenged with 200 µl of the respective samples (*S. bovis* HC5 (viable and heat-killed cells) or PBS). After thirty minutes, the animals received an additional intragastrically dose of β-lactoglobulin (β-LG, 90 % of purity, 0.2 ml of a 100 mg ml^{-1} solution in tap water; obtained from Sigma Chemicals Co., St. Louis, MO), and blood samples were collected from the orbital plexus under light ether anesthesia, after 0.5, 1, 2 and 5 h of the β-LG administration. The blood samples were processed as described and stored at - 20 °C until use.

Sera were used for the quantification of β-LG by FPLC, using a cationic change column (Mono Q; 5 µm; 150 by 6 mm [inner diameter]).The column was equilibrated with buffer A (20 mM Tris in ultrapure water) and the β-LG was eluted using a linear gradient of 25 to 50 % buffer B (20 mM Tris, 1 M NaCl, in ultrapure water), 22 °C, and at a flow rate of 1 ml min^{-1}.The absorbance was monitored at 220 and 280 nm.

In order to determine the concentration of β-LG in animal sera, a calibration curve was prepared, using solutions with known concentrations (0; 6.25; 12.5; 25.0;

50.0 mg ml^{-1}), mixed to pre-immune serum of the animals from each group. The standard curve was constructed by plotting the peak areas related to β-lactoglobulin against the concentration of standard solutions used.

Serum samples before β-LG administration were used as negative control. Analyses were performed in duplicate and the chromatographic profiles obtained were compared to the calibration curve, in order to determine the β-LG levels in serum samples.

5.3.4. Histological and morphometric analysis

After 58 days of experiment, the animals were sacrificed by cervical dislocation. The organs, heart, liver, spleen and gut, of the all mice were aseptically removed, washed in PBS buffer and fixed in Carson formalin solution (Carson *et al.*, 1973), for 24 hours, at room temperature. The fixed organs were sectioned, dehydrated in ethanol (70º, 80º, 95º and absolute ethanol, during 30 min each), and embedded in resin (Historesin®, Leica). The fragments were incubated at 37 ºC, for 24 hours.

Transverse and longitudinal histological sections were obtained by microtome. Semi-serial cuts, with a thickness of 3 µm and interval between cuts of 30 µm, were obtained. The slides were stained with toluidine blue/sodium borate (1 %), hematoxylin and eosin (HE), Alcian Blue (pH 2.5) combined with periodic acid-Schiff (PAS) (Bancroft and Stevens (1996), with modifications), depending on the histological analysis that would be performed.

Sections stained with HE were used for morphologic analysis. To each animal, twenty fields of longitudinal gut sections stained with toluidine blue/sodium borate (1 %) or HE with increases of 10x were randomly selected in order to determine the villi height (from the basal region, just above the crypt, to the top of the villi), villi width (taking the average of three points, located in the upper, middle and basal region of the villi), and mucosal thickness.

Sections stained with PAS were used for visualization of goblet cells and the determination of mucopolysaccharides (acidic, in blue; neutral, in dark red; acidic/neutral, in dark purple). Ten fields of 353 x 265 µm with increases of 20x were randomly selected, and the goblet cells PAS^+ and AB^+ were counted for the analysis of the presence of different mucins.

Sections stained with toluidine blue/sodium borate (1 %) were used to detect mast cells. An area equivalent to 20 jejunum villi (mucosa and submucosa), in each animal, was evaluated. Data were reported as number of cells per field.

Images of histological sections were captured with the light microscope Olympus AX 60, coupled to a micro camera. The morphometric analyzes were performed with the image analysis program Image Pro Plus 4.0 for Windows (Media Cybernetics). The results were shown as the mean value ± standard deviation.

5.3.5. Analysis of relative gene expression by real-time PCR

Jejunum segments and the whole spleen (100 mg of tissue) were aseptically removed from the animals, washed in sterile PBS, and individually manipulated.

The spleen was processed with 1 ml of saline (0.85 %) for cell extraction. The cells were transferred to micro centrifuge tubes and kept on ice for up to 1 h; the cells were centrifuged (7500 x *g*, 5 min) and treated with 1 ml erythrocytes lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 2 mM EDTA), for 10 min on ice. The splenocytes were centrifuged again and the supernatant was discarded. The cells were frozen in liquid nitrogen and stored at -80 ºC until use.

A jejunum segment of 6 cm was removed and washed three times with saline (0.85 %), for removal of waste. The segments were transferred to micro centrifuge tubes and kept on ice for up to 1 h. The organs were frozen in liquid nitrogen and stored at -80 ºC until use.

The mRNA was extracted from jejunum segments and spleen using the Tri Reagent (Sigma®), following the protocol recommended by the manufacturer. Tissue samples were homogenized with 1 ml Tri Reagent, and the samples were incubated at room temperature for 5 min. Then, 0.2 ml chloroform was added and the samples were vigorously shaken for 15 s; the samples were kept at room temperature for 15 min and centrifuged (12000 x *g*, 15 min, 4 ºC). The RNA containing aqueous phase was collected and transferred to a new and sterile tube; 50 µl of isopropanol were then added and the samples were incubated at room temperature for 5 min; the samples were centrifuged (12000 x *g*, 15 min, 4 ºC) and the supernatant was transferred to another new and sterile tube. After that, 450 µl of isopropanol were added and the samples were kept at room temperature for 10 min.

Following, the samples were centrifuged (12000 x *g*, 10 min, 4 ºC) and the supernatant was discarded. The precipitate was washed by adding 1 ml ethanol (75 %) and suspended in vortex; the sample was centrifuged (7500 x *g*, 5 min, 4 ºC) and 1 ml ethanol was added to the *pellet*; the samples were stored *overnight* at - 80 ºC. The RNA samples was dried at room temperature, suspended in 40 µl of DEPC water, and maintained in water bath at 37 ºC, for 15 min, to facilitate the dissolution of RNA

samples. An aliquot of 5 µl was used to obtain the concentration of RNA per µl in the samples, using a Genesis 10S UV-VIS Spectrophotometer (Thermo Scientific, Ridgefield Court, Asheville, USA).

Complementary DNA (cDNA) was synthesized through a reverse transcription reaction (M-MuLV reverse transcriptase, Promega). Real-time PCR relative quantification of mRNA analyses were performed on the Gene Amp®5700 Sequence Detection System Version 1.3 (Applied Biosystems) using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantification of amplicons. The standard PCR conditions were 95°C for 10 min, 40 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, followed by the standard denaturation curve. The sequences of murine primers were designed using the Primer Express software (Applied Biosystems) and the nucleotide sequences present in the Gen Bank data base (the primers sequences are depicted in Table 1). PCR conditions for each target were optimized with regard to primer concentration, absence of primer dimer formation, and efficiency of amplification of target genes and housekeeping gene control. In each reaction, 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 450 nM specific primers, and 2.5 ng of cDNA were used.

Threshold for positivity of real-time PCR was determined based on negative controls. The results were demonstrated as mRNA expression of the test groups, relative to negative control group. Instructions from Applied Biosystems User's Bulletin #2 (P/N 4303859) were used to calculate the relative level of gene expression, by reference to the β-actin in each sample, using the cycle threshold (Ct) method. Briefly, Ct value was calculated by determining the point at which the exponential increase in signal (fluorescence) exceeds a somewhat arbitrary signal level (usually 10 times the standard deviation of the baseline). The mean Ct values from duplicate measurements were used to calculate expression of the target gene, with normalization to an internal control (β-actin), and then compared with the target–internal control in the control animals (negative control group) to calculate fold increase expression, using the expression 2^DCt, according to the User's Bulletin. Negative controls without RNA and without reverse transcriptase were also performed. Results are shown as mean values ± standard deviation (n=3).

Table 1: Sequences of sense (S) and antisense (AS) *primers* used for real time-PCR analysis.

Primers	**Sequences**
β-actin S	5’ AGC TGC GTT TTA CAC CCT TT 3’
β-actin AS	5’ AAG CCA TGC CAA TGT TGT CT 3’
IL-10 S	5’ TGG ACA ACA TAC TGC TAA CC 3’
IL-10 AS	5’ GGA TCA TTT CCG ATA AGG CT 3’
IL-4 S	5’ CTG ACG GCA CAG AGC TAT TGA 3’
IL-4 AS	5’ TAT GCG AAG CAC CTT GGA AGC 3’
IL-5 S	5’ GAG GTT ACA GAC ATG CAC CAT T 3’
IL-5 AS	5’ TCA GTT GGT AAC ATG CAC AAA G 3’
IL-13 S	5’ ACC AAC ATC TCC AAT TGC AA 3’
IL-13 AS	5’ ATG CAA TAT CCT CTG GGT CC 3’
TNF-α S	5’ TGT GCT CAG AGC TTT CAA CAA 3’
TNF-α AS	5’ CTT GAT GGT GGT GCA TGA GA 3’
IL-12 p40 S	5’ AGC ACC AGC TTC TTC ATC AGG 3’
IL-12 p40 AS	5’ GCG CTG GAT TCG AAC AAA G 3’
IFN-γ S	5’ GCA TCT TGG CTT TGC AGC T 3’
IFN-γ AS	5’ CCT TTT TCG CCT TGC TGT TG 3’
TGF-β S	5’ GCT GAA CCA AGG AGA CGG AAT 3’
TGF-β AS	5’ GCT GAT CCC GTT GAT TTC CA 3’
IL-17 S	5’ GCT CCA GAA GGC CCT CAG A 3’
IL-17 AS	5’ CTT TCC CTC CGC ATT GAC A 3’

5.3.6. Statistical analysis

The results were initially evaluated by one-way analysis of variance. For histological and morphometric data, when differences among groups were identified, the Dunn`s a multiple comparison test or Student`s t test was conducted to examine significant differences among the treatments; for interleukin gene expression, the groups were compared by t test. A probability value of less than 0.05 was considered statistically significant.

All the comparisons were performed using the GraphPad Prism 5 software.

5.4. Results

Daily oral administration of viable and heat-killed *S. bovis* HC5 cells (10^{10} CFU ml^{-1}, 100 µl final volume) to BALB/c mice started at day 28 and continued uninterruptedly for 30 days, until the animals were sacrificed (day 58).

5.4.1. Weight gain

The weight gain of the animals belonging to the experimental groups (negative control (NC), viable cells of *S. bovis* HC5 (V) and heat-killed *S. bovis* HC5 (HK)) was weekly monitored after the first immunization (day 0). At the beginning of the experiment, there was no significant difference among the average initial weight of the mice (18.51, 18.0 and 18.18 g to NC, V and HK groups, respectively).

Among the mice of the negative control group, the average weight ranged from 18.51 $\pm$ 0.35 g (day 0) to 20.8 $\pm$ 0.31 g (day 58) during the experiment, which means a weight gain of 11.01 %. The percent of weight gain of mice belonging to the V and NK groups during the experiment period was 10.2 % and 7.24 %, respectively, and did not differ among the groups tested ($p > 0.05$) (Figure 1).

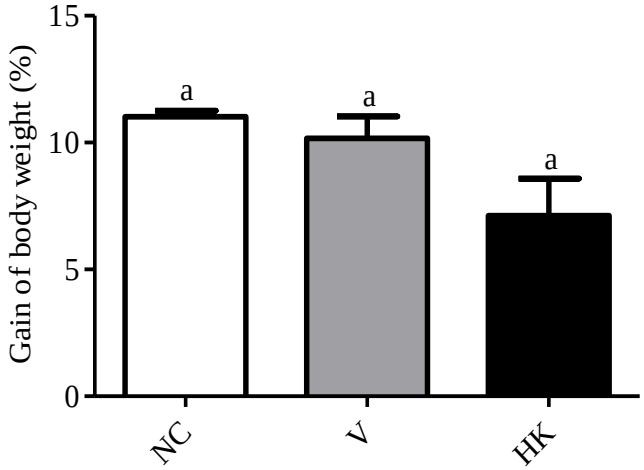


Figure 1: Effect of the oral administration of *S. bovis* HC5 on percent weight gain of BALB/c mice. The weight of the animals is visualized as percentage of the animals' weight, which was calculated comparing the weight at the end of the experiment (day 58) to the weight at the day of the first immunization (day 0). Each bar represents the mean value from six determinations with the standard deviation (SD). Different letters mean significant difference among treatments ($p < 0.05$). (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells.

5.4.2. Gastrointestinal permeability

Upon an additional oral administration of PBS or *S. bovis* HC5 cells (200 µl) to the animals in the treatment groups, and a subsequent gavage dose of β-LG 30 min later, the amount of β-LG was measured in sera obtained 0.5, 1, 2 and 5 h after the β-LG administration.

β-LG was easily identified by the FPLC method developed in this study, and the retention time of β-LG was 10.68 min. It was possible to observe a clear increase at the peak areas, proportional to the increase of the β-LG concentrations in the animal sera.

No β-LG could be detected in serum samples obtained before β-LG administration or in samples from negative control animals upon β-LG administration. In V group, β-LG was detected only in sera obtained 0.5 h after the β-LG administration (2 mg ml^{-1}). In sera obtained from animals that received heat-killed *S. bovis* HC5 cells, a significant amount of β-LG was detectable at 0.5, 1 and 2 h after β-LG administration (4.52 mg ml^{-1}, 4.51 mg ml^{-1} and 6.0 mg ml^{-1}, respectively). After 5 h of administration, β-LG could not be detected in the sera from the animals of all tested groups (Figure 2).

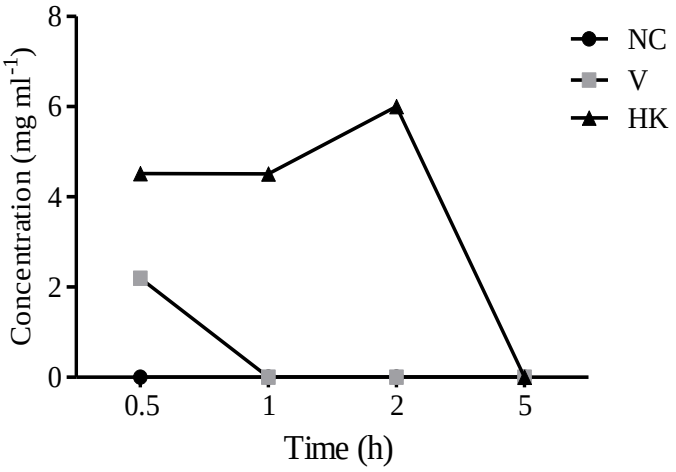


Figure 2: β-lactoglobulin levels in animal sera from the treatment groups. An intragastrically dose of β-LG (20 mg) was administered as a bystander protein to the negative control group and the groups that received *S. bovis* HC5 (viable and heat-killed cells). At the indicated time points following β-LG administration, the levels of β-LG in mice sera were determined by FPLC. The results show an average of the β-LG level detected in four animals of each group.β-LG was not detected in all serum samples from negative control group. (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells (HK).

5.4.3. Histological and morphometric analysis

The livers showed a preserved lobular architecture, without cellular infiltrates or parenchymal substitutions, and preserved cellularity of parenchyma. No significant alterations were identified in the heart of the animals tested (data not shown). However, a significant decrease in total number of cells at the spleen of the animals treated with viable and heat-killed *S. bovis* HC5 cells was observed, when compared to the negative control group (Figure 3).

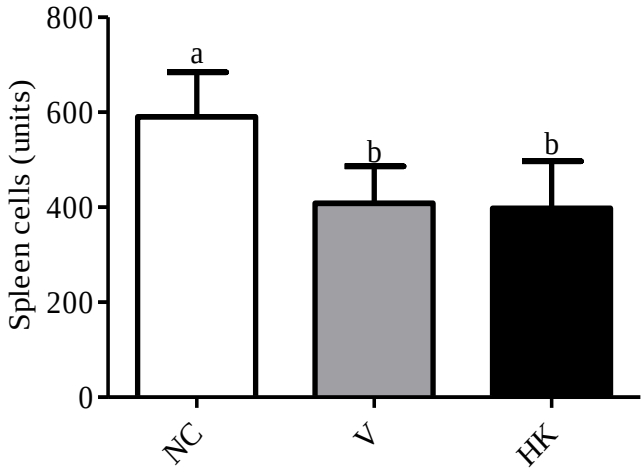


Figure 3: Number of spleen cells among the experimental groups. Data were shown as average ± SD. Different letters mean significance difference among the groups, according to Dunn's test ($p < 0.05$). (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells.

The small intestine of the negative control group presented a normal aspect, independent of the magnification visualized, and all the intestinal layers remained intact (Figure 4A). For the group of animals treated with viable *S. bovis* HC5 cells, the small intestine showed homogenous aspect among the animals, and a presence of eosinophils in the lamina propria was more evident; there were small areas with degenerative alterations of the epithelium and reduction of the brush border (Figure 4B). For the group treated with heat-killed *S. bovis* HC5 cells, epithelial degenerative changes, reduction of the brush border, cell infiltration, moderate edema and congestion of the lamina propria were detected (Figure 4C).

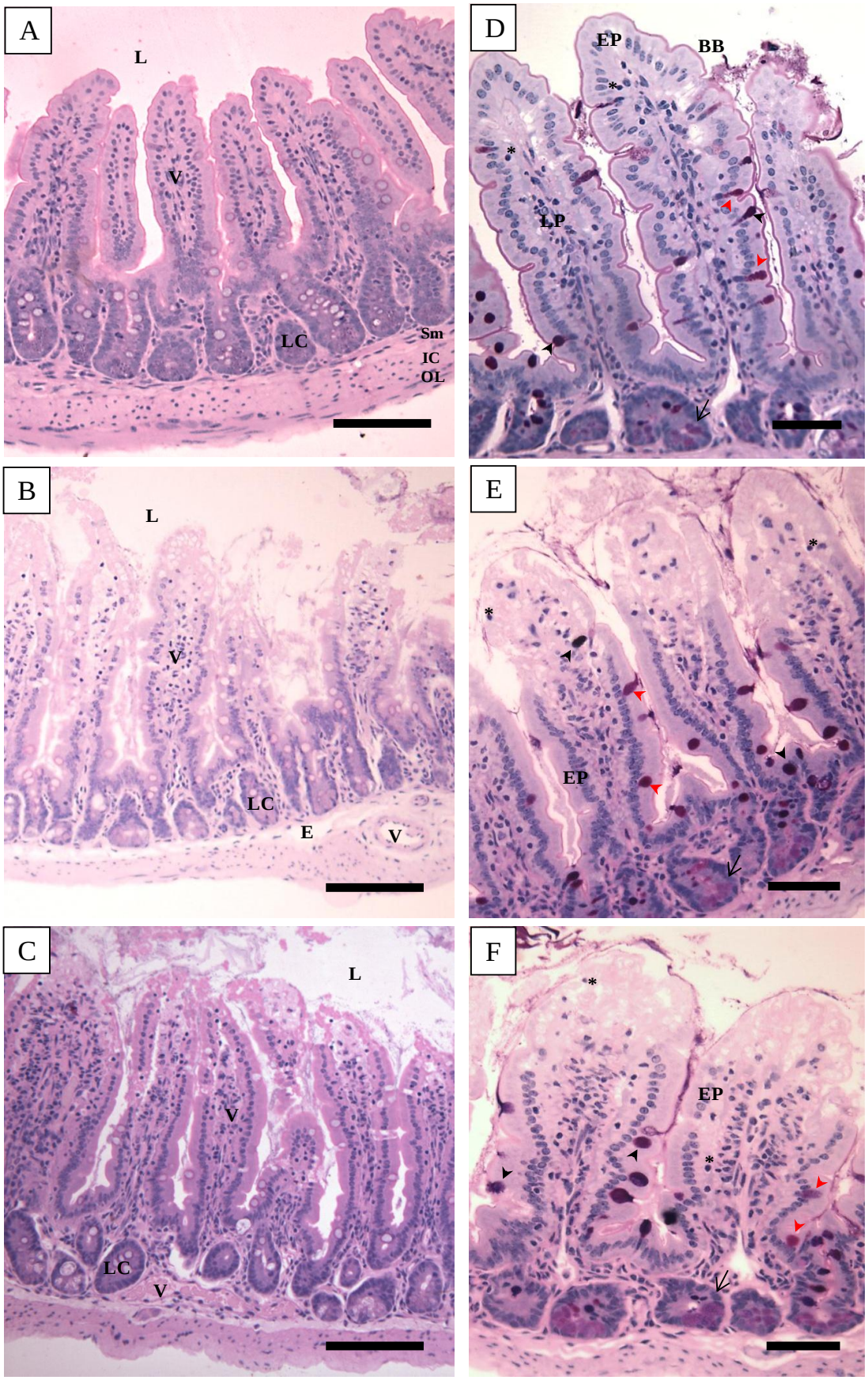


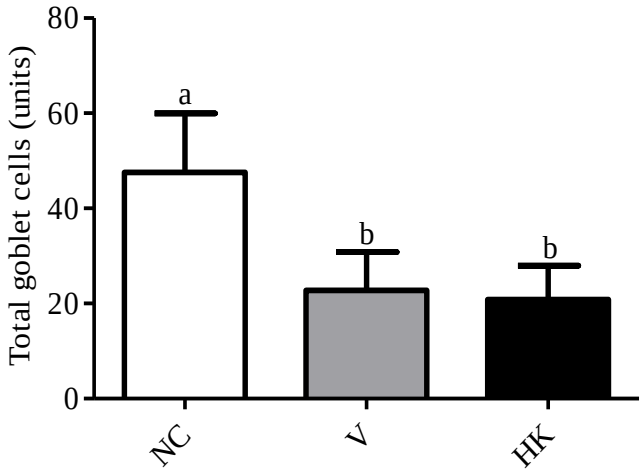
Figure 4: Photomicrographs of histological sections of small intestine of the animal groups studied ((NC), negative control group, figures A and D; (V) mice treated with

viable *S. bovis* HC5 cells, figures B and E; (HK) mice treated with heat-killed *S. bovis* HC5 cells, figures C and F). Jejunum segments were collected and processed for optical microscopy analysis at the end of the experiment. The sections were stained with hematoxylin and eosin (HE; right panel) or PAS/Alcian Blue (left panel). Abbreviations: L: lumen; EP: simple cuboidal epithelium; BB: brush border; V: villum; LP: lamina propria; LC: Lieberkühn crypt; E: edema; V: blood vessel; Sm: submucosa; IC: inner circular muscle layer; OL: outer longitudinal muscle layer. The asterisks indicate intraepithelial lymphocytes; simple arrow indicates Paneth cells. Black arrow head indicates goblet cells PAS/AB^+; red arrow head indicates PAS^+ cells.
Right panel – Scale bar: 100 µm; Left panel – Scale bar: 50 µm.

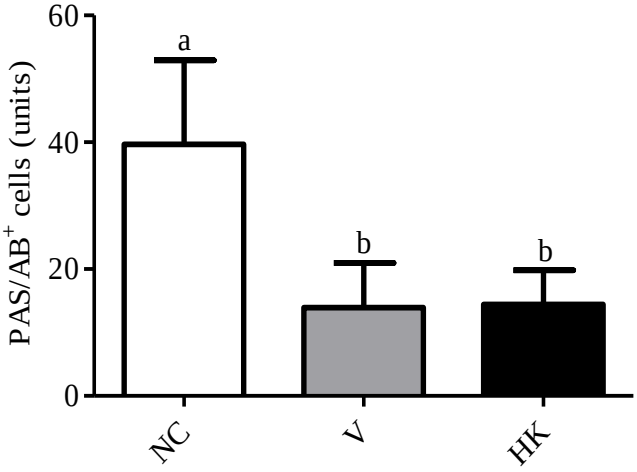
The number of total goblet cells present in small intestine of the mice treated with *S. bovis* HC5 (viable or heat-killed cells) was reduced and statistically different when compared to the number of cells encountered in the small intestine of the negative control group (Figures 4D-F and 5A). The majority of goblet cells presented in animals of the negative control group were PAS/AB+ cells, which secrete both neutral and acidic mucopolysaccharides (83 % of the total number of goblet cells). The PAS/AB^+ cells were reduced in V and HK groups (61.25 % and 69.44 %, respectively), with significant differences ($p < 0.05$) (Figure 5B).

PAS^+ cells, which secrete only neutral mucopolysaccharides, represented a small fraction of the total number of goblet cells in all the groups (17.0 %, 38.75 % and 30.55 %, for NC, V and HK groups). However, there was a significant difference in the number of PAS^+ cells between the groups that received *S. bovis* HC5 ($p < 0.05$) (Figure 5C). Cells secreting exclusively acid mucins (AB^+ cells) were not detected in this study.

A



B



C

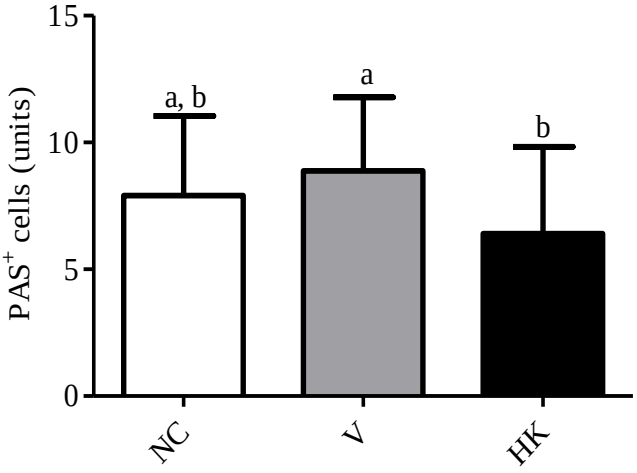
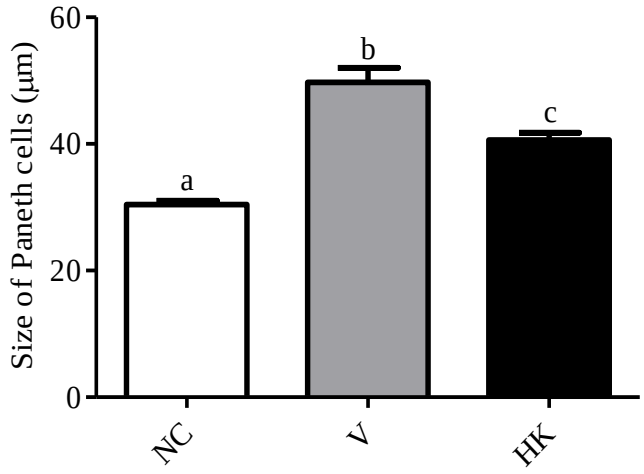


Figure 5: Comparison of the number of total goblet cells and mucin production among the experimental groups. (A) total number of cells; (B) PAS/AB$^+$ cells; (C) PAS$^+$ cells. Data were shown as average $\pm$ SD. Different letters mean significance difference among groups, according to Dunn's test ($p<0.05$). (NC) negative control; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells (HK).

At the small intestinal crypts, it was observed a significant hypertrophy of Paneth cells in animals treated with *S. bovis* HC5, and this increase was more pronounced in the treatment with viable cells (Figure 6A). Also a significant increase in the number of cells in mitosis was observed in both V and HK groups when compared to the negative control group; in this case, the viability of the bacteria did not influence the results (Figure 6B).

A



B

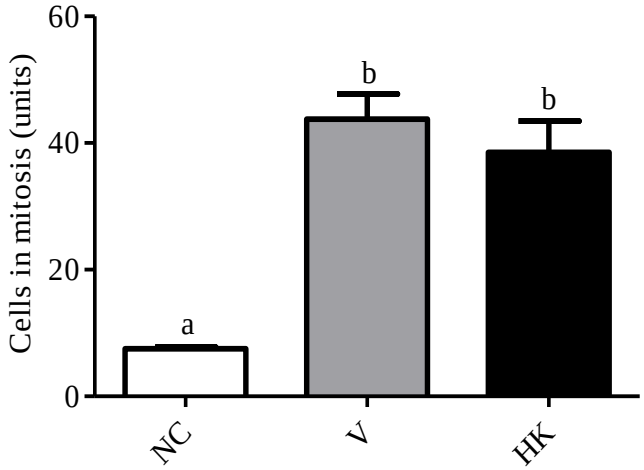
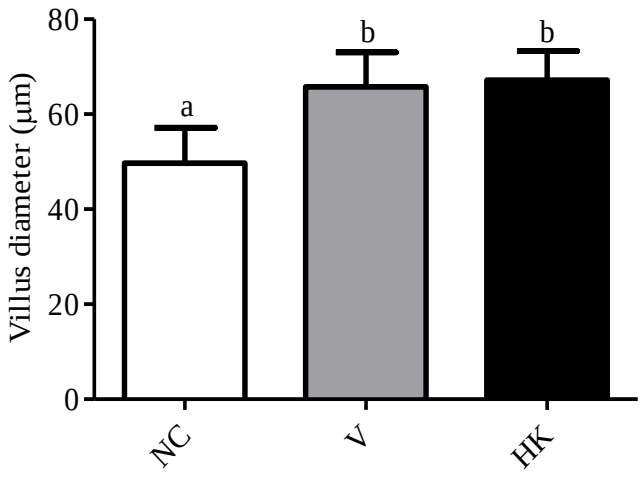


Figure 6: Size of Paneth cells (A) and number of cells in mitosis (B) at the small intestinal crypts of the experimental groups. Data were shown as average ± SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells (HK).

At the small intestine, changes in the architecture of the villi were evidenced when the animals were treated with *S. bovis* HC5. A significant increase of the diameter of the villi was observed, independent of the viability of the bacteria (Figure 7A). However, a significant decrease in villous height was detected only at the V group (Figure 7B).

A



B

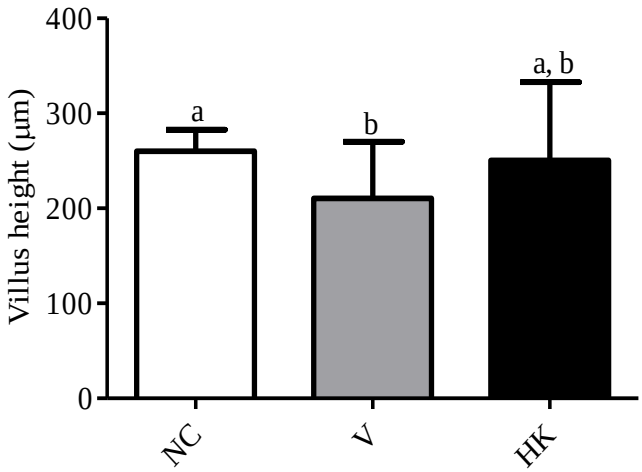


Figure 7: Diameter and height of the small intestinal villi at the experimental groups. Data were shown as average ± SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells (HK).

The large intestine of the negative control group was normal and with a homogenous aspect (Figure 8A). The epithelium and cellularity of the large intestine were not affected by *S. bovis* HC5, but a moderate edema at the lamina propria was observed in both V and HK group (Figures 8A and B). A significant reduction at the mucosal thickness was also observed among the animals treated with *S. bovis* HC5 (Figure 9).

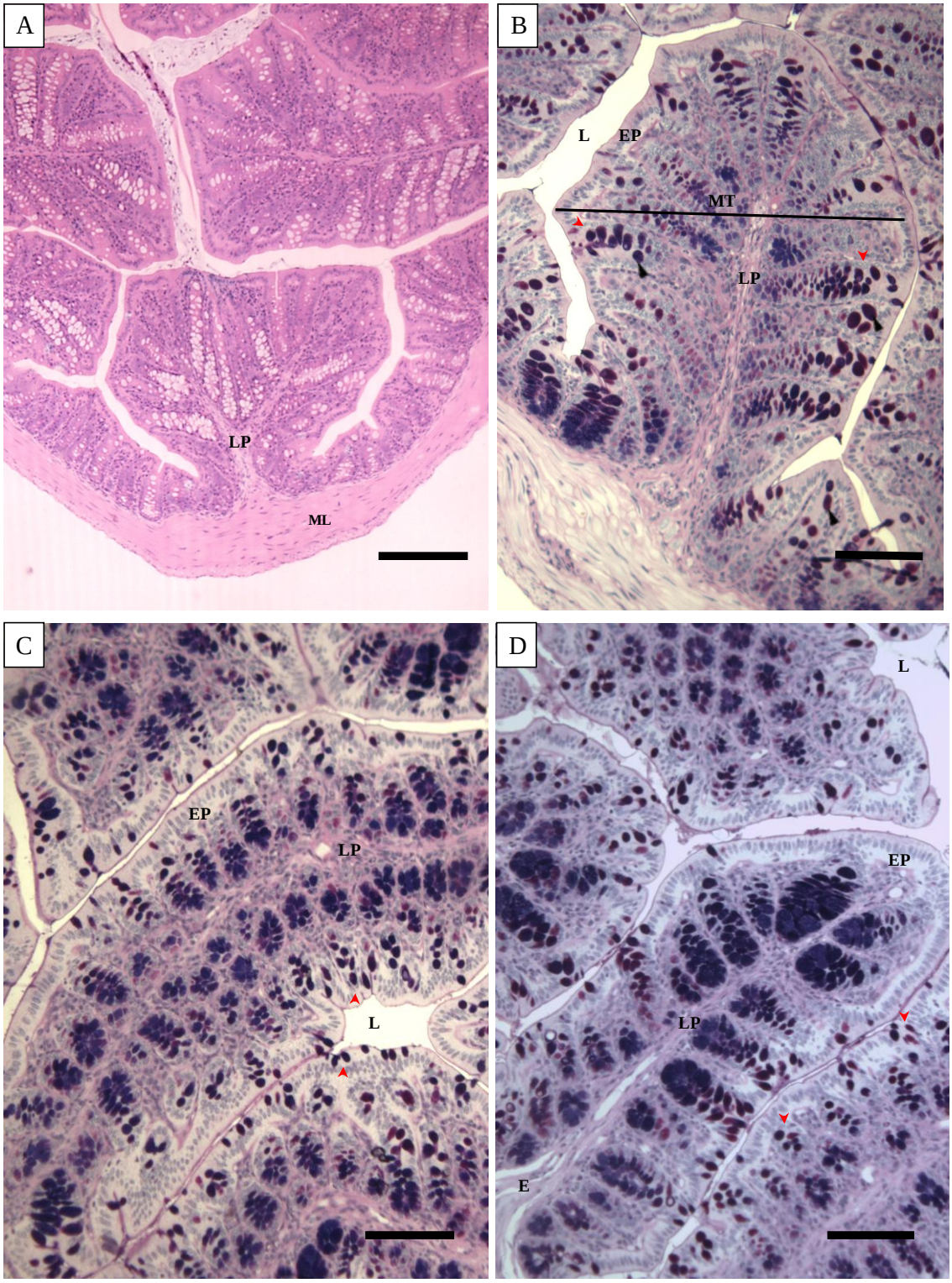


Figure 8: Photomicrograph of histological sections of large intestine of the experimental groups studied ((NC), negative control group, figures A and B; (V) mice treated with viable *S. bovis* HC5 cells, figure C; (HK) mice treated with heat-killed *S. bovis* HC5 cells, figure D). Jejunum segments were collected and processed for optical microscopy analysis at the end of the experiment. The sections were stained with hematoxylin and eosin (HE; figure A) or PAS/Alcian Blue (figures B-D). Abbreviations: EP: simple

cuboidal epithelium; LP: lamina propria; MT: mucosal thickness; E: edema; MC: muscle layer. Red arrow head indicates goblet cells. Scale bar = 200 (figure A) or 100 µm (figures B, C and D).

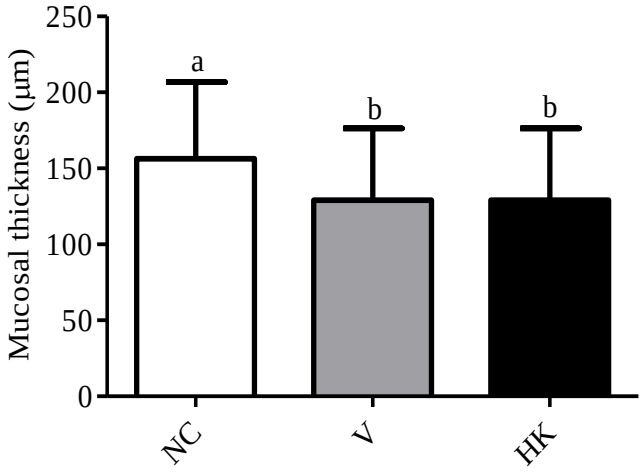
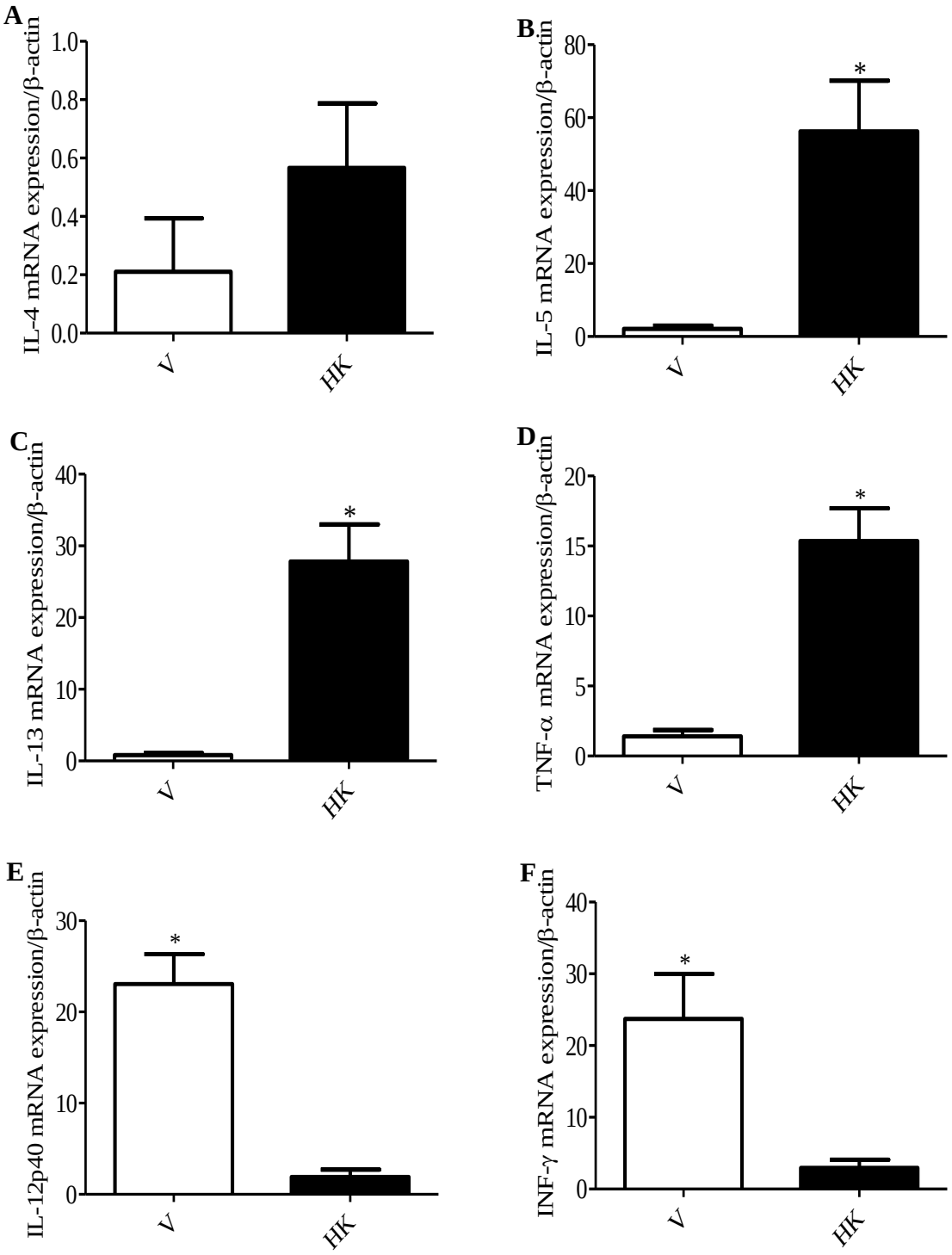


Figure 9: Mucosal thickness of the large intestine of the mice at the experimental groups. Data were shown as average ± SD. Different letters mean significant difference among groups, according to Dunn's test ($p < 0.05$). (NC) negative control group; (V) mice treated with viable *S. bovis* HC5 cells; (HK) mice treated with heat-killed *S. bovis* HC5 cells (HK).

5.4.4. Immune parameters

To investigate the effects of *S. bovis* HC5-oral administration in the modulation of the immune response of BALB/c mice, the relative expression of cytokines genes was characterized in small intestine and spleen of the animals. In each experimental setup an aliquot of each target sample was analyzed by real-time PCR for β-actin mRNA expression, in order to normalize for inefficiencies in cDNA synthesis.

Comparing the groups that received *S. bovis* HC5 cells, the IL-12 and INF-γ mRNA expression was significantly higher in the small intestine of the mice treated with viable *S. bovis* HC5 cells ($p < 0.05$, Figures 10E and 8F), while the relative expression of IL-5, IL-13 and TNF-α was significantly increased in the small intestine of mice treated with heat-killed *S. bovis* HC5 cells (Figures 10B, 10C and 10D). The mRNA levels of the regulatory cytokines TGF-β and IL-10 were basically the same in the groups tested, and the expression of IL-4 and IL-17, although higher in the group treated with heat-killed *S. bovis* HC5, did not differ between the groups (Figures 10A, 10E, 10G-I).


A
IL-4 mRNA expression/β-actin
1.0
0.8
0.6
0.4
0.2
0.0
V
HK
B
IL-5 mRNA expression/β-actin
80
60
40
20
0
*
V
HK
C
IL-13 mRNA expression/β-actin
40
30
20
10
0
*
V
HK
D
TNF-α mRNA expression/β-actin
20
15
10
5
0
*
V
HK
E
IL-12p40 mRNA expression/β-actin
30
20
10
0
*
V
HK
F
INF-γ mRNA expression/β-actin
40
30
20
10
0
*
V
HK

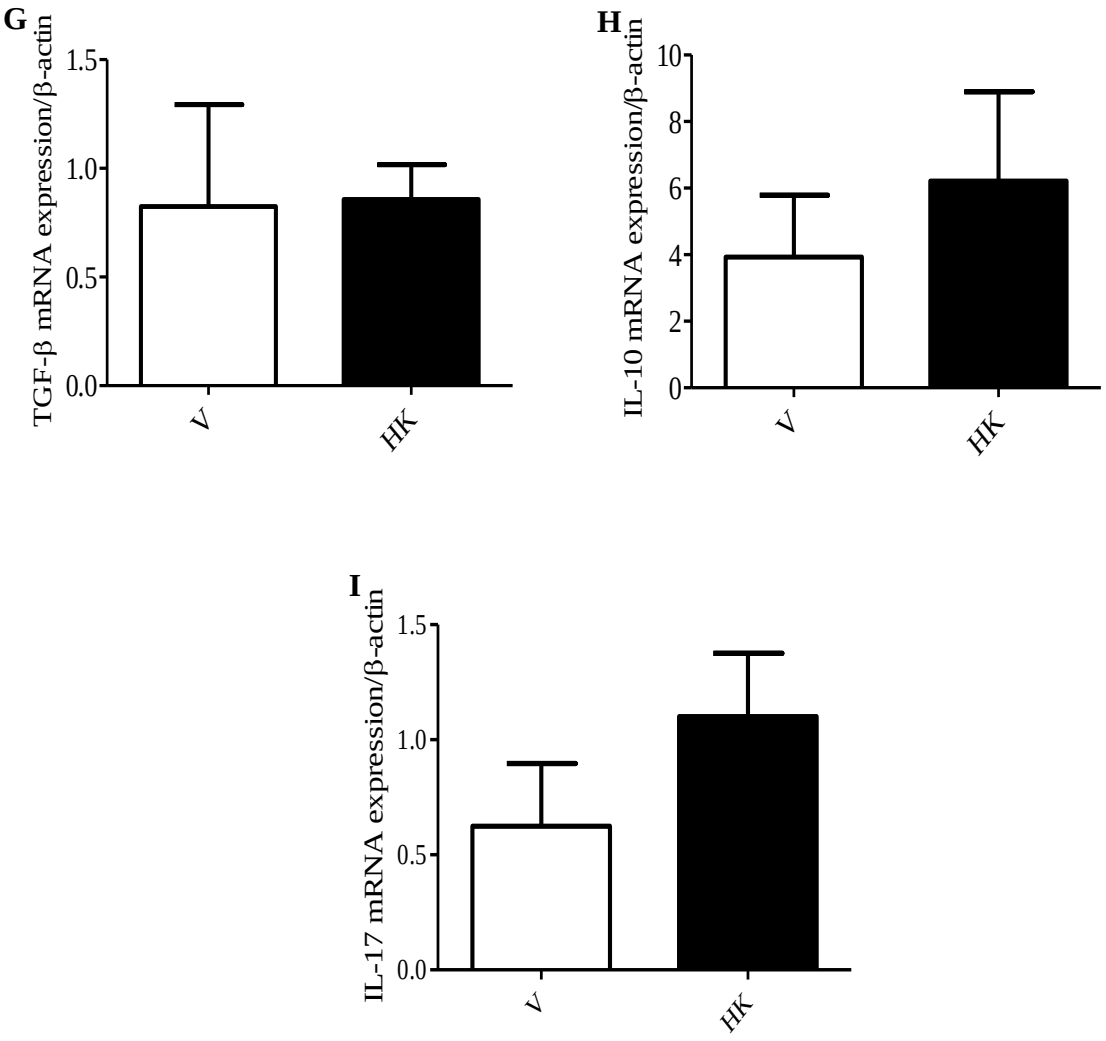
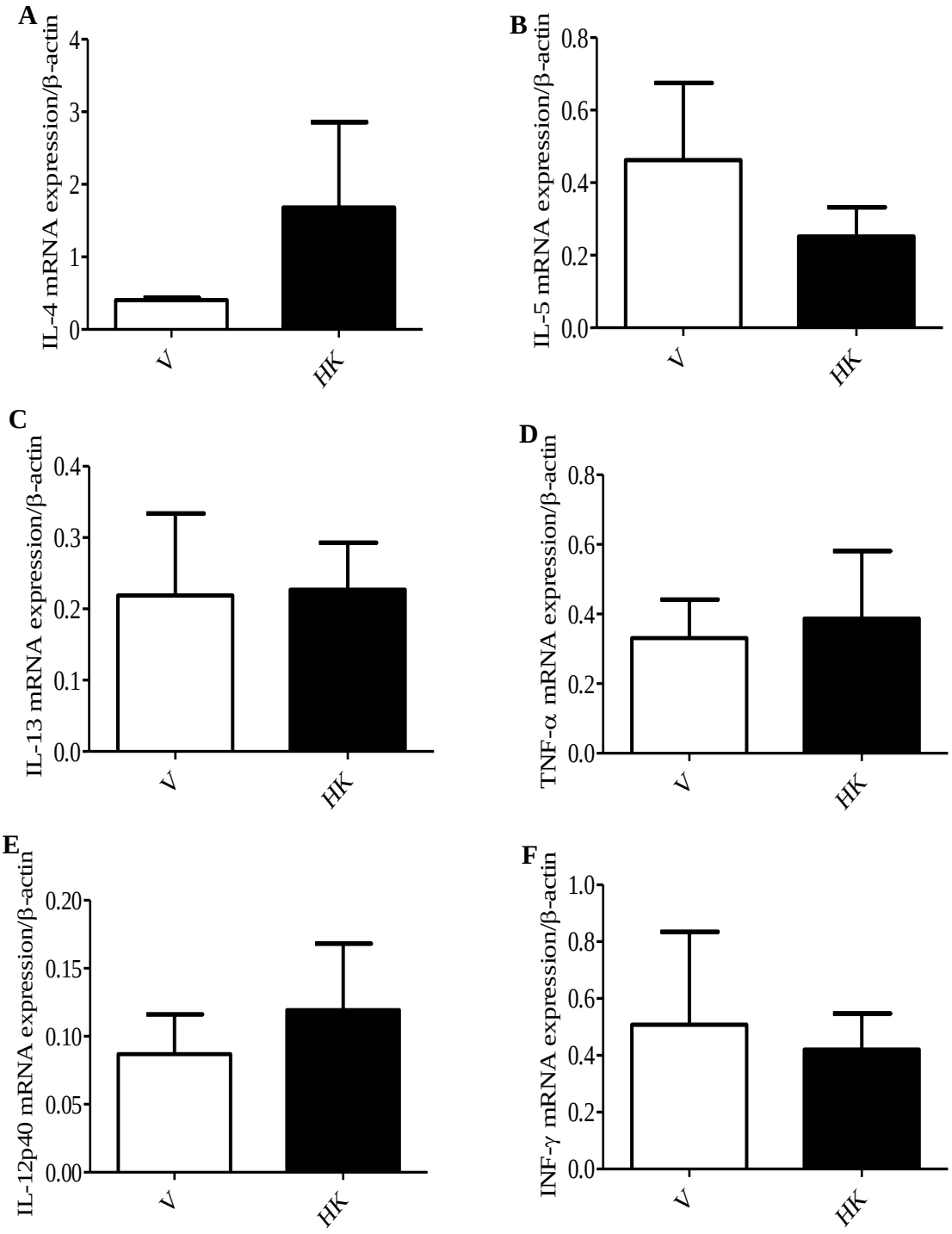


Figure 10: Cytokine production (IL-4, IL-5, IL-13, TNF-α, IL-12, IFN-γ, TGF-β, IL-10, IL-17) in small intestine of five-weeks old female BALB/c mice that received *S. bovis* HC5 cells. Segments of jejunum were collected on day 58 of the experiment and mRNA was extracted. The relative expression of the interleukin genes determined by real time-PCR was calculated in reference to the β-actin in each sample. Results are shown as the mean value ± SD of data from three mice (values in duplicate), relative to a negative control group. *Significant differences between the relative expression on spleen from mice treated with viable *S. bovis* HC5 cells (V) and treated with heat-killed *S. bovis* HC5 cells (HK), at $p < 0.05$.

No differences in mRNA expression of the cytokines in spleen were found between the groups of animals treated with viable or heat-killed *S. bovis* HC5 cells (Figure 11).


A
IL-4 mRNA expression/β-actin
0
1
2
3
4
V
HK
B
IL-5 mRNA expression/β-actin
0.0
0.2
0.4
0.6
0.8
V
HK
C
IL-13 mRNA expression/β-actin
0.0
0.1
0.2
0.3
0.4
V
HK
D
TNF-α mRNA expression/β-actin
0.0
0.2
0.4
0.6
0.8
V
HK
E
IL-12p40 mRNA expression/β-actin
0.00
0.05
0.10
0.15
0.20
V
HK
F
INF-γ mRNA expression/β-actin
0.0
0.2
0.4
0.6
0.8
1.0
V
HK

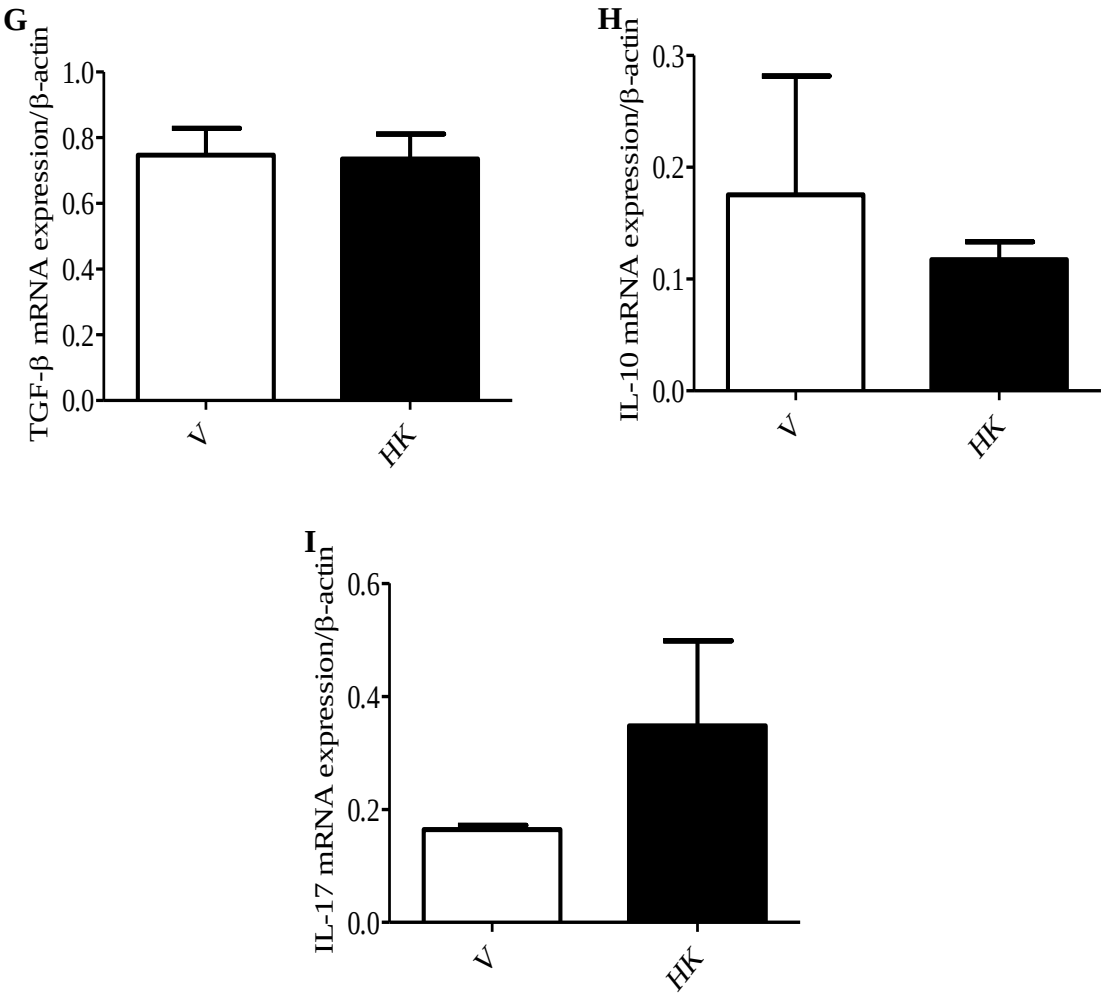


Figure 11: Cytokine production (IL-4, IL-5, IL-13, TNF-α, IL-12, IFN-γ, TGF-β, IL-10, IL-17) in spleen of five-weeks old female BALB/c mice that received *S. bovis* HC5 cells. Spleen was collected on day 58 of the experiment and mRNA was extracted. The relative expression of the interleukin genes determined by real time-PCR was calculated in reference to the β-actin in each sample. Results are shown as the mean value ± SD of data from three mice (values in duplicate), relative to a negative control group. *Significant differences between the relative expression on spleen from mice treated with viable *S. bovis* HC5 cells (V) and treated with heat-killed *S. bovis* HC5 cells (HK), at $p < 0.05$.

5.5. Discussion

In the last years, several bacterial strains have been described as capable of eliciting immunomodulatory functions, increasing the interest in the effects of commensal enteric bacteria on local and systemic immunity of the animal host (Hart *et al.*, 2004; Foligne *et al.*, 2007). Several lactic acid bacteria, as *Lactobacillus* strains, have been reported to stimulate cells of the innate immune system *in vitro* (Perdigón *et al.*, 2001; Lammers *et al.*, 2003).

In vitro experiments have been used to predict the survival of the bacteria through the gastrointestinal passage *in vivo*. The experimental conditions are critical and sometimes differ from what can be expected *in vivo,* so the reliability of such models in predicting the *in vivo* situation is questionable. Moreover, *in vitro* tests do not really assure the capacity of bacteria to colonize the gut (Ibnou-Zekri *et al*., 2003; Foligne *et al.*, 2006).

According to Bujalance *et al.* (2007), the persistent colonization by a bacterial strain is not a necessary requirement to exert immunomodulatory effects, since these effects could be achieved when a continuously administration of a desired bacterium that is able to transiently survive in the gastrointestinal tract is performed.

The effects of the administration of *Streptococcus bovis* HC5 on animal host have not been reported until now. In the present study, we determined the morphological and immune effects upon intragastrically administration of *S. bovis* HC5 in BALB/c mice. Although some *in vivo* experiments had shown similar immune-stimulatory effects to genetically engineered and non-viable microorganisms when compared to the effects of live microorganisms (Maeda *et al.*, 2009), we decide to evaluate viable and also heat-killed cells of *S. bovis* HC5, in order to determine the effects of the bacterial viability on the *in vivo* model adopted.

The treatment with *S. bovis* HC5 did not influence the weight gain of the mice, since no differences were observed compared to the negative control group. When the effects of *S. bovis* HC5 cells on gastrointestinal physiology of BALB/c mice were evaluated, an influence of cell viability was observed on the gut permeability. Upon oral challenge with heat-killed *S. bovis* HC5 cells, the gut permeability to proteins was increased, as evidenced by an increased uptake of the bystander protein β-LG when compared to the negative control group. After 0.5 h of β-LG administration, an increased passage of β-LG through the gastrointestinal barrier was observed in animals treated with viable *S. bovis* HC5 cells, but the β-LG passage was still 50 % lower when

compared to the animals treated with heat-killed *S. bovis* HC5 cells. After 5 h of administration, the clearance of β-LG was completed and β-LG could not be detected in the sera of the animals from all the tested groups.

Saunders *et al.* (1994) showed that stress may impair the intestinal barrier function. As our oral challenges were performed by gavage dosing, the animals might have been stressed by handling, thus resulting in an increased permeability.

In addition to the gut permeability, the possible occurrence of systemic and local effects upon an oral administration of *S. bovis* HC5 cells were investigated, by monitoring the histological and morphological changes on the organs and the differential expression of cytokines in spleen and small intestine of treated animals.

Histological alterations were not observed in the liver and the heart of the animals treated with *S. bovis* HC5. However, if compared to the control group, a significant reduction on the total number of splenocytes was observed when the animals were treated with viable and heat killed *S. bovis* HC5 cells. This may be due to the migration of immune cells to the intestine, where the main alterations were observed.

The histological alterations caused by *S. bovis* HC5 at the small intestine was basically the same in mice that received viable or heat-killed cells, and degenerative alterations, edema and congestion were observed in almost all the animals evaluated. An edema was also observed in the large intestine in both groups. Morphometric analysis of the small and large intestine of the animals treated with *S. bovis* HC5 showed some impairment of intestinal structure integrity.

Goblet cells are exocrine unicellular glands specialized in mucus secretion, and they secrete the protective layer of mucus on the intestinal epithelial tissue. Extrinsic factors in contact with the intestinal epithelium may alter the number of goblet cells by releasing of nucleotide triphosphates, which trigger the secretion of goblet cells (Góes and Taboga, 2005). Moreover, the degree of goblet cell secretory activity, as well as the mucin content, can be affected by the exposure to exogenous substances, especially bacteria, in order to protect the intestinal wall and to limit the absorption of antigens (Zimmermann *et al*., 2003).

In this study, a different pattern of goblet cells in the small intestine was observed upon oral administration of *S. bovis* HC5, and a reduction of the number of total cells and the number of PAS/AB^+ cells were observed. This situation could not be due to the reduction of secretion but could be explained by the limited count fields, since part of the villi was destructed in V and HK groups. An increase of goblet cells producing acid mucins (AB^+ cells) should be expected in response to the exposure to

microorganisms, since the acidic mucin is more difficult to be degraded (Deplancke *et al*, 2002). However, cells secreting exclusively acid mucins were not observed in this study.

The administration of *S. bovis* HC5 caused the flattening of the villi in the small intestine and the atrophy of the mucosal thickness in the large intestine. A more prominent reduction of the villous height was detected in animals treated with viable *S. bovis* HC5 cells. These alterations were probably caused by the direct effect of the bacteria or their components on the intestinal epithelial cells. Similar results were obtained when *Lactobacillus acidophilus* UFV-H2b20 were administrated to mice (Neumann *et al*., 1998).

In general, substances present in the gastrointestinal tract influence the expression and activity of key proteins involved in the regulation of cell proliferation, differentiation and apoptosis (Sergent, 2008). An increase of intestinal cell death occurred as a result of the administration of *S. bovis* HC5, but an increased cell turnover was also observed. The process of epithelial renewal was highly visible in the small intestines of the animals treated with *S. bovis* HC5, and thus Paneth cell and the presence of epithelial cells in mitotic division, which are considered important factors in this process, were analyzed.

Paneth cells are situated just below the intestinal stem cells in the intestinal glands. This location acts as a protection of the stem cells, which is essential for long-term maintenance of the intestinal epithelium, since the mitotic division of stem cells serve to constantly replenish epithelial intestinal cells (Góes and Taboga, 2005). Besides their role in defending the epithelial cell renewal, Paneth cells are characterized by the production of compounds, such as antimicrobial proteins (α-defensins), lysozyme, TNF-α and phospholipase A2 (Ganz, 2000; Ayabe *et al.*, 2000). In addition to functioning as direct antimicrobial compounds through bacterial-membrane permeabilization, antimicrobial proteins can function as opsonins, chemokines and modulators of host-cell cytokine production, which regulate innate immune response against extracellular microbial infection (Kolls *et al.*, 2008).

Bacteria and bacterial antigens (PAMPs), such as lipopolysaccharide, muramyl dipeptide and lipid A, are recognized via pattern-recognition receptors (PRRs), as the toll-like receptors (TLR), causing the increase of the production of cytokines by cells of the innate immune system (dendritic cells, macrophages and epithelial cells), which in turns triggers the secretion of anti-microbial compounds produced by intestinal mucosal

cells into the lumen of the intestinal gland, contributing to the maintenance of the gastrointestinal barrier (Kolls *et al.*, 2008).

A hyperplasia of Paneth cells and also a mitotic activity increased were observed in both V and HK groups, indicating that despite of the loss of villi architecture, the processes of antimicrobial compounds secretion and tissue repair remained activated, in an attempt to counteract the injuries caused by *S. bovis* HC5 cells. Again, the effects on the Paneth cells and mitotic division were more prominent in the mice that received viable cells.

To determine if the events triggered by chronic exposure to *S. bovis* HC5 cells orally administered were antigen-specific and restricted to the intestinal mucosa or whether they were part of a systemic response, the expression of cytokines was analyzed at the intestine and the spleen of the BALB/c mice. Despite of the slight differences observed at the histological and morphometric changes on intestine of the animals treated with viable and heat-killed cells of *S. bovis* HC5, the immune response of the animals was more affected by the viability of the bacteria. Several local immune-mediated effects could be observed on jejunum of the BALB/c mice, although systemic immune-mediated effects have not been detected.

Some LAB can affect the host`s systemic and mucosal immunity (Maassen *et al*., 2000), although the intensity and characteristics of these immunostimulatory activities can vary among species and even among strains, being affected by the growth phase of the bacterium, the animal model adopted, and the doses administrated, which makes it difficult to generalize the experimental results obtained (Perdigón *et al.*, 1999; Maassen *et al*., 2000; Maassen *et al*., 2003; Sashihara *et al.*, 2007). Moreover, the autolysis of bacterial cells (Sashihara *et al.*, 2007), the structural modification or degradation of the effective components from viable and heat-killed bacterial cells affect the immune-stimulatory effects induced by bacteria, and have been described as an important issue for recognition by the phagocytes (Segawa *et al.*, 2008).

According to Vintiñi *et al*. (2000), the ecological niche of the micro-organism under evaluation is also an important characteristic and may be determinant for the immunological effect in the gut. In general, LAB are able to interact with the mucosal immune system associated with the intestine (gut associated lymphoid tissue – GALT) in two different stimulation pathways: through M cells localized at Peyer's patches or through the intestinal epithelial cells (Perdigón *et al*., 2000). The interaction of LAB with M cells induce an increase in the number of IgA-producing cells in the intestine and an increase in the migration of $CD4^+$ T cells to the lamina propria, triggering an

specific immune response against their epitopes. Interaction of LAB with intestinal epithelial cells did not induce an increase in IgA-producing cells and the clonal expansion of T cells residing in the lamina propria is not observed; this type of interaction is not related to the processing of LAB as antigens and induces an inflammatory and non-specific response (Hershberg and Mayer, 2000). The interaction through M cells was observed for *Lactobacillus casei*, *Lactobacillus plantarum* and *Streptococcus salivarus* ssp. *thermophilus*, in a dose dependent manner (Perdigón *et al.*, 1999).

We have shown that viable cells of *S. bovis* HC5 were a potent inducer of T_H1-type cytokines IL-12 and INF-γ by immune-competent cells in the intestinal mucosa, which is consistent with the processing of *S. bovis* HC5 through M cells. On the other hand, heat-killed *S. bovis* HC5 cells induced the production of the pro-inflammatory cytokine TNF-α, and also type 2 cytokine, IL-5 and IL-13, and this immune response may be resulted from the direct interaction of the bacterial antigens with intestinal epithelial cells.

Certain LAB induce the production of T_H1-type cytokines, such as IL-12 and IFN-γ, and shift a T_H2-dominant condition to a T_H1-dominant condition (Pochard *et al*., 2002). TLRs present on the cell surface, especially TLR2, may play important roles in recognizing peptidoglycan in bacteria for subsequent induction of innate immunity, thereby leading to T_H1 immunity (Akira and Takeda, 2004). Nod2, present in the cytoplasm as peptidoglycan-recognizing factor, senses peptidoglycan degradation products containing the muramyl dipeptide, which is common in both gram-positive and gram-negative bacteria (Inohara *et al*., 2003; Girardin *et al*., 2003). In addition, lipoteichoic acid, which is the predominant surface glycolipid of gram-positive bacteria, has been reported to induce IL-12 production through a CD-14 mediated pathway, similar to IL-12 induction by gram-negative LPS (Cleveland *et al*., 1996).

The inhibitory effect of the production of T_H2-type cytokines induced by LAB requires the presence of the antigen-presenting cells (APC) residing in Peyer's patches or lamina propria of the small intestine. This APC mediated effect may be due to the phagocytosis of bacteria, which is mainly associated with a Th1 response, or to the involvement of Toll-like receptors, which, once stimulated, can lead to the expression of $T_H1$cytokines. In addition, APCs stimulated by LAB produce large amounts of IL-12, that contribute to the induction of Th1 response by the activation of STAT-4 (signal transducer and activator of transcription), known to promote the direct expression of IFN-γ (Pochard *et al.*, 2002).

Some components of the membranes from gram-positive bacteria can stimulate a non-specific immune response, by causing acute inflammation, and also stimulate the synthesis of phagocytosis activating cytokines, as IL-6, and pro-inflammatory cytokines, as TNF-α (Miettinen *et al.*, 2000).

In agreement with our results, the administration of the halophilic LAB *Tetragenococcus halophilus* Th221 suppressed T_H2 immunity and promoted Th1 immunity, inducing the IL-12 production (Masuda *et al.*, 2008), and a heat-killed *Lactobacillus plantarum* L-137, a strain isolated from fermented food, was also a potent inducer of TNF-α (Murosaki *et al.*, 2000). Mohamadzadeh *et al.* (2005) has also shown the effectiveness of viable cells of *Lactobacillus* on T_H1 response, in which activation of human dendritic cells by *Lactobacillus* skews T cells toward T_H1 polarization. *Lactobacillus gasseri* OLL2809 stimulated the production of IL-12 (p70) by murine splenocytes, and suppressed serum antigen-specific IgE levels via the T_H1/T_H2 balance (Sashihara *et al*., 2007).

However, Maeda *et al.* (2009) did not detect pro-inflammatory cytokines in the serum of the C57BL/6 mice after oral administration of heat-killed *Lactobacillus plantarum* L-137. According to Pochard *et al*. (2002), in the presence of both viable as non-viable bacterial cells of *Lactobacillus plantarum* NCIMB8826, *Lactococcus lactis* MG1363, *Lactococcus lactis* ATCC393 and *Lactobacillus rhamnosus* GG, an increased secretion of INF-γ and a reduction in the synthesis of IL-4 and IL-5 were observed in mononuclear cells from healthy and allergic patients. In a similar way, Kalliomaki *et al*. (2001) reported that after oral supplementation with *Lactobacillus rhamnosus* GG, children with atopic dermatitis and allergy to cow's milk exhibited a transient increase in the production of INF-γ and a reduction in the production of IL-4.

The factors that dictate whether an infection will trigger a T_H1- or T_H2-type response are not fully understood, but the response generated does play an important role in the clearance of different pathogens and in the maintenance of immunity at mucosal sites. Generally, T_H1 and T_H2 responses are designed to eliminate different types of pathogens: T_H1-type cytokines tend to produce pro-inflammatory responses that are more effective for killing intracellular pathogens, while T_H2 responses are more effective against extracellular bacteria, parasites and toxins. To avoid uncontrolled tissue damage (caused by an excessive T_H1 response) the animal host should produce a well balanced T_H1 and T_H2 response.

The intestinal immune system has developed a degree of tolerance to the presence of commensal bacteria antigens under steady-state conditions. This selective

tolerance is lost during the introduction of pathogenic bacteria or during changes in the commensal flora, which may tip the T_H1/T_H2 balance towards T_H17-cell development (Kolls *et al.*, 2008). In this study, although the relative expression of IL-17 has been increased in animals treated with heat-killed *S. bovis* HC5, no significant differences were observed among the groups that received *S. bovis* HC5 cells.

In conclusion, the histological and morphometric alterations observed at the intestine of the animals suggest a major local effect of the *S. bovis* HC5 administration. The oral administration of *S. bovis* HC5 also influenced the cytokine`s production pattern at the intestine, and there was a distinguishable difference in the immunostimulatory activity of live and heat-killed *S. bovis* HC5 cells. The results obtained in this study could vary depending on the bacterial dose administrated or the experimental time upon evaluation. The mechanisms by which *S. bovis* HC5 interact with intestinal cells and stimulate immune cells, as well as the possible effects of this bacterium on the microbial community composition in the intestine, will be the subject of future studies.

5.6. References

Akira, S.; Takeda, K. Toll-like receptor signaling. Nature Reviews Immunology 4, 499-511, 2004.

Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J. A.; Struhl, K. Short Protocols in Molecular Biology. Wiley: New York, 4th edition, 1999. 1931p.

Ayabe, T.; Satchell, D.P.; Wilson, C.L.; Parks, W.C.; Selsted, M.E.; Ouellette, A.J. Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. Nature Immunology 1, 113-118, 2000.

Bancroft, J.D.; Stevens, A. Theory and Practice of Histological Techniques. 4th edition, Churchill Livingstone, London, 1996.

Bujalance, C.; Moreno, E.; Jimenez-Valera, M.; Ruiz-Bravo, A. A probiotic strain of *Lactobacillus plantarum* stimulates lymphocyte responses in immunologically intact

and immunocompromised mice. International Journal of Food Microbiology 113, 28–34, 2007.

Carson, F.L.; Martin, J.H.; Lynn, J.A. Formalin fixation for electron microscopy: a re-evaluation. American Journal of Clinical Pathology 59, 365-373, 1973.

Chiang, Y.J.; Kolw, H.K.; Brown, K.; Naramura, M.; Fukuhara, S.; Hu, R.J.; Jang, I.K.; Gutkind, J.S.; Shevach, E.; Gu, H. CbI-b regulates the CD28 dependence of T-cell activation. Nature 403, 216-220, 2000.

Cleveland, M.G.; Gorham, J.D.; Murphy, T.L.; Tuomanen, E.; Murphy, K.M. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14- 364 dependent pathway. Infection and Immunity 64, 1906–12, 1996.

Deplancke, B.; Vidal, O.; Ganessunker, D.; Donovan, S.M.; Mackie, R.I.; Gaskins, H.R. Selective growth of mucolytic bacteria including *Clostridium perfringens* in a neonatal piglet model of total parenteral nutrition. The American Journal of Clinical Nutrition 76, 1117-1125, 2002.

de Vuyst, L.; Vandamme, E.J. Bacteriocins of lactic acid bacteria. Londres: Blackie Academic & Professional, 1994. 539p.

Foligne, B.; Nutten, S.; Grangette, C.; Dennin, V.; Goudercourt, D.; Poiret, S.; Dewulf, J.; Brassart, D.; Mercenier, A.; Pot, B. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World Journal of Gastroenterology 13, 236-243, 2007.

Foligne, B.; Nutten, S.; Steidler, L.; Dennin, V.; Goudercourt, D.; Mercenier, A.; Pot, B. Recommendations for improved use of the murine TNBS-induced colitis model in evaluating antiinflammatory properties of lactic acid bacteria: technical and microbiological aspects. Digestive Diseases and Sciences 51, 394-404, 2006.

Ganz, T. Paneth cells – guardians of the gut hatchery. Nature Immunology 1, 99-100, 2000.

Gill, H.S.; Rutherfurd, K.J. Immune enhancement conferred by oral delivery of *Lactobacillus rhamnosus* HN001 in different milk-based substrates. Journal of Dairy Research 68, 611-616, 2001.

Girardin, S.E.; Boneca, I.G.; Carneiro, L.A.M.; Antignac, A.; Jéhanno, M.; Viala, J.; Tedin, K.; Taha, M.K.; Labigne, A.; Zathringer, U.; Coyle, A.J.; DiStefano, P.S.; Bertin, J.; Sansonetti, P.J.; Philpott, D.J. Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Science 300, 1584-1587, 2003.

Góes, R.M.; Taboga, S.R. Células caliciformes. In: Carvalho, H.F.; Collares-Buzato, C.B. Células: uma abordagem multidisciplinar. Barueri, Manole, p.163- 173, 465 p., 2005.

Hart, A.L.; Lammers, K.; Brigidi, P.; Vitali, B.; Rizzello, F.; Gionchetti, P.; Campieri, M.; Kamm, M.A.; Knight, S.C.; Stagg, A.J. Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53, 1602-1609, 2004.

Hershberg, R.M.; Mayer, L.F. Antigen processing and presentation by intestinal epithelial cells - polarity and complexity. Immunology Today 21, 123−128, 2000.

Holt, J.G. Genus *Streptococcus*. In: Bergy DH, Holt JG, Krieg NR, Sneath PH, editors. Bergey's manual of determinative bacteriology. Baltimore, MD: Lippincott Williams & Wilkins; 1994.

Hungate, R.E. The rumen and its microbes. New York: Academic Press, 1966. 533 p.

Ibnou-Zekri, N.; Blum, S.; Schiffrin, E.J.; von der Weid, T. Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties in vitro. Infection and Immunity 71, 428–436, 2003.

Inohara, N.; Ogura, Y.; Fontalba, A.; Gutierrez, O.; Pons, F.; Crespo, J.; Fukase, K.; Inamura, S.; Kusumoto, S.; Hashimoto, M. et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. The Journal of Biological Chemistry 278, 5509–5512, 2003.

Kalliomaki, M.; Salminen, S.; Arvilommi, H.; Kero, P.; Koskinen, P.; Isolauri, E. Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. Lancet 357, 1076–1079, 2001.

Knippels, L.M.J.; Houben, G.F.; Spanhaak, S.; Penninks, A.H. An Oral Sensitization Model in Brown Norway Rats to Screen for Potential Allergenicity of Food Proteins. Methods, v. 19, 78-82, 1999.

Kolls, J.K.; McCray Jr., P.B.; Chan, Y.R. Cytokine-mediated regulation of antimicrobial proteins. Nature Reviews Immunology 8, 829-835, 2008.

Lammers, K.M.; Brigidi, P.; Vitali, B.; Gionchetti, P.; Rizzello, F.; Caramelli, E.; Matteuzzi, D.; Campieri, M. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. FEMS Immunology and Medical Microbiology 38, 165-172, 2003.

Lancefield, R.C. A serological differentiation of human and other groups of hemolytic Streptococci. The Journal of Experimental Medicine 57, 571–95, 1933.

Maassen, C.B.M.; Boersma, W.J.A; van Holten-Neelen, C.; Claassen, E.; Laman, J.D. Growth phase of orally administered *Lactobacillus strains* differentially affects IgG1/IgG2a ratio for soluble antigens: implications for vaccine development. Vaccine 21, 2751–2757, 2003.

Maassen, C.B.M.; van Holten-Neelen, C.; Balk, F.; den Bak-Glashouwer, M.J.H.; Leer, R.J.; Laman, J.D.; Boersma,W.J.A.; Glassen, E. Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus strains*. Vaccine 18, 2613–2623, 2000.

Maeda, N.; Nakamura, R.; Hirose, Y.; Murosaki, S.; Yamamoto, Y.; Kase, T.; Yoshikai, Y. Oral administration of heat-killed *Lactobacillus plantarum* L-137 enhances protection against influenza virus infection by stimulation of type I interferon production in mice. International Immunopharmacology 9, 1122-1125, 2009.

Malo, C.; Morin, L. Establishment of an animal model of ovalbumin sensitised mouse to study protein induced enteropathy. Gut 27, 1298-1305, 1986.

Mantovani, H.C.; Hu, H.; Worobo, R.W.; Russell, J.B. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology 148, 3347-3352, 2002.

Mantovani, H.C.; Kam, D.K.; Ha, J.K.; Russell, J.B. The antibacterial activity and sensitivity of *Streptococcus bovis* strains isolated from the rumen of cattle. FEMS Microbiology Ecology 37, 223-229, 2001.

Masuda, S.; Yamaguchi, H.; Kurokawa, T.; Shirakami, T.; Tsuji, R.F.; Nishimura, I. Immunomodulatory effect of halophilic lactic acid bacterium *Tetragenococcus halophilus* Th221 from soy sauce moromi grown in high-salt medium. International Journal of Food Microbiology 121, 245–252, 2008.

Miettinen, M.; Lehtonen, A.; Julkunen, I.; Matikainen, S. *Lactobacilli* and *streptococci* activate NF-kB and STAT signaling pathways in human macrophages. The Journal of Immunology 164, 3733–3740, 2000.

Mohamadzadeh, M.; Olson, S.; Kalina, W.V.; Ruthel, G.; Demmin, G.L.; Warfield, K.L. et al. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. PNAS 102, 2880–5, 2005.

Morovsky, M.; Pristas, P.; Czikkova, S.; Javorsky, P. A bacteriocin-mediated antagonism by *Enterococcus faecium* BC25 against ruminal *Streptococcus bovis*. Microbiological Research 153, 277–281, 1998.

Murosaki, S.; Muroyama, K.; Yamamoto, Y.; Yoshikai, Y. Antitumor effect of heat killed *Lactobacillus plantarum* L-137 through restoration of impaired interleukin-12 production in tumor-bearing mice. Cancer Immunology and Immunotherapy 49, 157–164, 2000.

Nagafuchi, S.; Takahashi, T.; Yajima, T.; Kuwata, T.; Hirayama, K.; Itoh, K. Strain dependency of the immunopotentiating activity of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Bioscience, Biotechnology, and Biochemistry 63, 474–479, 1999.

Nagaraja, T.G.; Titgemeyer, E.C. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. Journal of Dairy Science 90, E17–38, 2006.

Neumann, E.; Oliveira, M.A.P.; Cabral, C.M.; Moura, L.N.; Nicoli, J.R.; Vieira, E.C.; Cara, D.C.; Podoprigora, G.I.; Vieira, L.Q. Monoassociation with *Lactobacillus acidophilus* UFV H2b20 stimulates the immune defense mechanisms of germfree mice. Brazilian Journal of Medical and Biological Research 31, 1565-1573, 1998.

Parra, M.D.; Martinez de Moretin, B.E.; Cobo, J.M.; Mateos, A.; Martinez, J.A. Daily ingestion of fermented milk containing *Lactobacillus casei* DN114001 improves innate-defense capacity in healthy middle-aged people. Journal of Physiology and Biochemistry 60, 85–91, 2004.

Perdigón, G.; Vintiñi, E.; Alvarez, S.; Medina, M.; Medici, M. Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. Journal of Dairy Science 82, 1108-1114, 1999.

Perdigón, G.; Holgado, A.P.R. Mechanisms involved in the immunostimulation by lactic acid bacteria. In: Fuller, R.; Perdigón, G. Probiotics 3: Immunomodulation by the gut microflora and probiotics. Dordrecht: Kluwer Academic, 2000. p. 213-233.

Perdigón, G.; Fuller, R.; Raya, R. Lactic Acid Bacteria and their effect on the immune system. Current Issues in Intestinal Microbiology 2**,** 27-42, 2001.

Pochard, P.; Gosset, P.; Grangette, C.; Andre, C.; Tonnel, A.B.; Pestel, J.; Mercenier, A. Lactic acid bacteria inhibit T_H2 cytokine production by mononuclear cells from allergic patients. Journal of Allergy and Clinical Immunology 110, 617-623, 2002.

Russell, J.B.; Rychlik, J.L. Factors that alter rumen microbial ecology. Science 292, 1119–22, 2001.

Sashihara, T.; Sueki, N.; Furuichi, K.; Ikegami, S. Effect of growth conditions of *Lactobacillus gasseri* OLL2809 on the immunostimulatory activity for production of interleukin-12 (p70) by murine splenocytes. International Journal of Food Microbiology 120, 274–281, 2007.

Saunders, P.R.; Kosecka, U.; McKay, D.M.; Perdue, M.H. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. American Journal of Physiology - Gastrointestinal and Liver Physiology, 267, G794–G799, 1994.

Segawa, S.; Nakakita, Y.; Takata, Y.; Wakita, Y.; Kaneko, T.; Kaneda, H.; Watari, J.; Yasui, H. Effect of oral administration of heat-killed Lactobacillus brevis SBC8803 on total and ovalbumin-specific immunoglobulin E production through the improvement of T_H1/T_H2 balance. International Journal of Food Microbiology 121, 1–10, 2008.

Sergent, T.T.; Ribonnet, L.; Kolosova, A.; Garsou, S.; Schaut, A.; de Saeger, S.; Peteghem, C.V.; Larodelle, Y.; Puseemier, L.; Scheneider,Y. Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. Food and Chemical Toxicology 46, 813-841, 2008.

Valeur, N.; Engel, P.; Carbajal, N.; Connolly, E.; Ladefoged, K. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Applied and Environmental Microbiology 70, 1176-1181, 2004.

Vintiñi, E.; Alvarez, S.; Medina, M.; Medici, M.V.; de Budeguer, M.; Perdigón, G. Gut mucosal immunostimulation by lactic acid bacteria. Biocell 23, 223-232, 2000.

Zimmermann, N.; Hershey, G.K.; Foster, P.S.; Rothenberg, M.E. Chemokines in asthma: cooperative interaction between chemokines and IL-13. Journal of Allergy and Clinical Immunology 111, 227-42, 2003.

GENERAL CONCLUSIONS

Lipid II, the essential cell wall precursor, was detected as the specific target for bovicin HC5 in sensitive cell membranes and it was used by bovicin HC5 in the pore-formation process. The pore-forming activity of bovicin HC5 was membrane-thickness dependent, being observed only in model membranes composed of phospholipids with 14 carbons or shorter acyl chains. In such thin membranes, higher concentrations of bovicin HC5 were able to disrupt the membrane, independent on the presence of Lipid II. Bovicin HC5 was capable to recruit some Lipid II molecules into a pore-like structure, an activity that consequently could lead to the inhibition of bacterial cell wall biosynthesis.

Upon interaction with Lipid II, bovicin HC5 inserted into the membrane, with its C-terminus deeper inserted, and adopted a perpendicular orientation with respect to the membrane surface. The interaction with Lipid II occurred even in the absence of pore formation and bovicin HC5 did not lose its affinity for Lipid II in acidic conditions. Moreover, the structure of bovicin HC5 was significantly altered after binding to Lipid II and more pronounced alterations were observed in acidic conditions.

After oral administration to BALB/c mice, bovicin HC5 caused morphological alterations in the small intestine of the animals, probably caused by an unspecific permeabilizing effect of this bacteriocin toward intestinal cells, although no differences in gut permeability have been detected. Additionally, bovicin HC5 was able to stimulate the gut immune system, influencing the cytokine release through T_H1-polarized response, by increasing the relative expression of TNF-α, IL-12 and INF-γ.

Streptococcus bovis HC5 also induced local effects after oral administration to BALB/c mice, since alterations occurred only at intestinal level and translocation could was not observed. Important impairment of small and large intestine was detected in animals treated with viable and heat-killed *S. bovis* HC5 cells. The immunostimulatory

activity varied between viable and heat-killed cells of *S. bovis* HC5, since an increase of T_H1-type cytokines IL-12 and INF-γ was detected in animals that received viable *S. bovis* HC5 cells, while heat-killed *S. bovis* HC5 cells induced an increased production of TNF-α, IL-5 and IL-13 in the small intestine.