USE OF BACILLUS AMYLOLIQUEFACIENS FOR CLOSTRIDIUM PERFRINGENS AND CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE

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List of abbreviations

AAD = antibiotic associated diarrhea

ACN = acetonitril

ADP = adenosine diphosphate

A.U. = arbitrary units

IgA = immunoglobulin A

Asp = aspartic acid

Asx = aspartic acid or asparagine

BC30 = *Bacillus coagulans* GBI-30

BHI = brain hearth infusion broth

BI/027/NAP1 = restriction enzyme analysis type BI/ribotype 027/North American Field Pulse type 1

bp = basepairs

CA-CDI = community acquired *Clostridium difficile* infection

CDAD = *Clostridium difficile* associated disease

CDI = *Clostridium difficile* infection

CDT = *Clostridium difficile* binary toxin

CDTa = enzymatic component *Clostridium difficile* binary toxin

CDTb = binding component *Clostridium difficile* binary toxin

CFSN = cell free supernatant

cfu = colony forming units

CID = collision induced dissociation

Cwp66, Cwp84 = cell wall protein 66, cell wall protein 84

DNA = deoxyribonucleic acid

DPPH = 2,2-diphenyl-1-picrylhydrazyl

ELISA = enzyme-linked immunosorbent assay

EMA = European Medicine Agency

ESI = electrospray ionisation

Fbp68 = fibronectin binding protein 68

FDA = US Food and Drug Administration

FliC = *Clostridium difficile* flagellin

FliD = *Clostridium difficile* flagellar cap protein

FMT = fecal microbiota transplantation

GlcNAc = N-acetylglucosamine

Glu = glutamic acid

GTP = guanosine triphosphata

gyrA, gyrB = gyrase A, gyrase B

HBSS = Hank's balance salt solution

HCl = hydrogen chloride

HPLC = high performance liquid chromatography

IL-6, IL-8 = interleukin-6, interleukin-8

InsP6 = inositol hexakiphosphate

IPEC = intestinal porcine epithelial cells

ITS = insulin – transferrin – selenium

kb = kilobase

KH_2PO_4 = mono potassium phosphate

MAP = mitogen activated protein

MeOH = methanol

MIC = minimal inhibitory concentration

$MgSO_4.7H_2O$ = magnesium sulphate heptahydrate

MOLP = medium optimized for lipopeptide production

MRS = MRS broth

MRSA = methicillin resistant *Staphylococcus aureus*

MS = mass spectrometry

NaCl = sodium chloride

Na_2HPO_4 = disodium phosphate

NaOH = sodium hydroxide

NE = necrotic enteritis

OD = optical density

PaLoc = pathogenicity locus

PCP = peptidyl carrier protein

PCR = polymerase chain reaction

QPS = qualified presumption of safety

RBP = receptor binding protein

RCM = reinforced clostridial medium

RNA = ribonucleic acid

SDS-PAGE = sodium dodecyl sulphate poly acrylamide gel electrophoresis

SEM = standard error on the mean

SlpA = surface layer protein A

TcdA = *Clostridium difficile* toxin A

TcdB = *Clostridium difficile* toxin B

TFA = trifluoroacetic acid

Thr = threonine

TLR = toll like receptor

TSB = tryptone soya broth

Tyr = tyrosine

TNF-α = tumor necrosis factor alpha

U = units

VRE = vancomycin resistant *Enterococcus*

INTRODUCTION

CLOSTRIDIUM DIFFICILE

1 THE ORGANISM

In 1935, Hall and O'Toole isolated a novel anaerobic bacterium from the stool of healthy infants. They named it *Bacillus difficilis* referring to the difficulties they encountered in its isolation and culture. It was later renamed as *Clostridium difficile*.[1] It was, however, not until 1978 that *C. difficile* was recognized as a human pathogen associated with severe disease.[2] In the past decade however, it has emerged rapidly as one of the most important healthcare associated pathogens and is associated with prolonged treatment and hospital stay leading to increased healthcare costs.[3, 4]

1.1 GENERAL CHARACTERISTICS

C. difficile is a Gram-positive, toxin-producing, rod-shaped bacterium that grows in strictly anaerobic conditions. Vegetative cells are 2-8 µm long and 0.5 µm wide.[1, 2] The bacterium is highly motile due to the presence of peritrichous flagellae.[5] Under unfavorable conditions such as nutrient deprivation or exposure to high temperatures, it is able to form highly resistant spores which can survive for months. *C. difficile* can be part of the normal intestinal microbiota without causing disease. Up to 70% of all newborns and 3% of adults are asymptomatically colonized with *C. difficile* and are a potential source for the spread of the pathogen.[6, 7] *C. difficile* is heterotrophic since it requires the presence of 6 amino acids (cysteine, isoleucine, leucine, proline, tryptophan and valine) for optimal growth.[8] A recent report however, recognizes the bacterium as the first autotrophic bacterial pathogen since it was able to grow with CO_2 and H_2 as sole carbon and energy source. This great metabolic flexibility could be a major asset in the pathogenicity of *C. difficile*.[9]

1.2 GENETICS

C. difficile belongs to the Clostridiaceae and the genus *Clostridium*. The genus *Clostridium* is a very heterogeneous group of bacteria that do not form a phylogenetically coherent group. It is subdivided in different clusters based on gene or protein sequences. *C. difficile* is one of the pathogens belonging to cluster XI.[10, 11] *C. difficile* strains are subdivided in different types based on whole genome DNA sequencing. Strain typing is important to detect and understand changes in epidemiology and to identify epidemic hospital outbreaks. Different typing techniques have been used including toxinotyping based on changes in the pathogenicity locus, ribotyping based on

ribosomal DNA, restriction enzyme analysis or pulsed field gel electrophoresis, both based on whole genome DNA.[12-15]

The first whole genome sequence was obtained in 2006 from *C. difficile* strain 630. This virulent, multi-drug resistant strain was isolated from a Swiss patient with severe pseudomembranous colitis. The genome consisted of a circular chromosome of 4 290 252 bp and a plasmid of 7881 bp. Up to 11% of the genome consisted of mobile genetic elements, mainly conjugative transposons, which might play an important role in the acquisition of genes involved in e.g. antimicrobial resistance and virulence.[16]

1.3 VIRULENCE FACTORS

1.3.1 TOXIN A AND B

PATHOGENICITY LOCUS

The two best characterized virulence factors of *C. difficile* are toxin A (TcdA) and toxin B (TcdB). Different studies indicate the importance of these two toxins since clinical isolates that lack both toxins are nonpathogenic to humans and animals. Both toxins are encoded by genes localized on a 19.6 kb large pathogenicity locus (PaLoc) (Figure 1). This locus furthermore contains genes encoding a positive regulator of toxin expression (TcdR), a negative regulator (TcdC) and a bacteriophage related pore forming protein (TcdE). Toxin synthesis is growth-phase dependent and at its maximum in the stationary growth phase. Toxin expression is regulated by different environmental signals including nutrient depletion, carbon source and antibiotics. *TcdR* encodes an alternative sigma factor for RNA polymerase that specifically triggers the transcription of *tcdA* and *tcdB* genes. Moreover, TcdR expression is autoregulated and an initial small increase in its expression will result in a rapid accumulation of TcdR.[17] During the exponential growth phase, TcdC is expressed. TcdC is a small acidic protein that acts as a negative regulator of toxin expression. It is an anti-sigma factor that prevents interaction between TcdR-RNA polymerase and the toxin promoters. Epidemic *C. difficile* 027 strains appear to have a mutation in *tcdC* resulting in a higher toxin production which appears to be in part the reason for their hypervirulence.[18] *TcdE* encodes a small hydrophobic protein and is required for efficient release of the toxins. Moreover, it seems to protect the *C. difficile* cells themselves from cell lysis or membrane permeability during toxin secretion.[19]

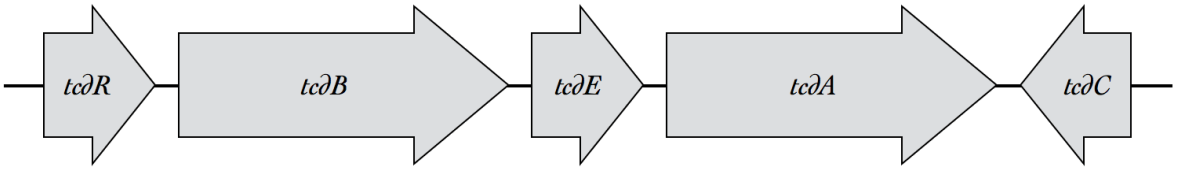

Figure 1. Composition of the PaLoc region. *TcdA* and *tcdB* encode the two most important *C. difficile* virulence factors: toxin A and toxin B, respectively. Toxin expression is regulated by a positive regulator encoded by *tcdR* and a negative regulator encoded by *tcdC*. *TcdE* is related to a pore forming protein produced by bacteriophages.

STRUCTURE

Toxin A and B are members of the large clostridial toxins family. Toxin A has a molecular size of 308 kDa and toxin B has a mass of 269.6 kDa. Both toxins are the prototypes of the clostridial glycosylating toxins. Their structure is based on the ABCD model and contains a biologically active domain (A), a receptor binding domain (B), an autocatalytic domain for toxin processing (C) and a translocation domain (D).[20] (Figure 2)

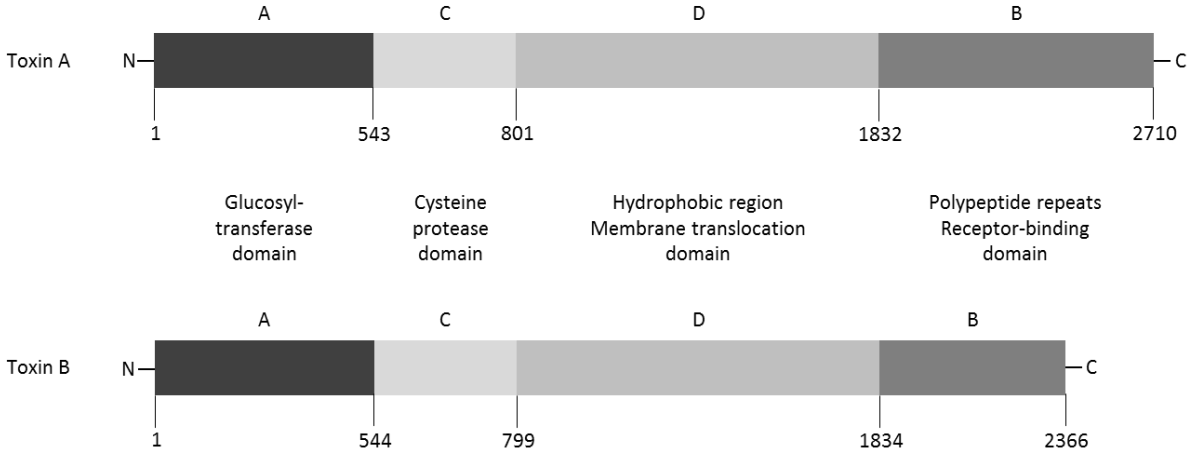

Figure 2. **Structure of toxins A and B.** Toxin A consists of 2710 amino acids and has a molecular size of 308 kDa. Toxin B is made up of 2366 amino acids and has a mass of 269.6 kDa. Both toxins contain four domains based on the ABCD model. The N-terminal glucosyltransferase domain A comprises the biological activity of the toxins. The cysteine protease domain C is the autocatalytical site necessary for processing of the toxin. Domain D contains a hydrophobic region that is necessary for membrane translocation. The C-terminal domain contains polypeptide repeats involved in receptor binding.

MECHANISM OF ACTION

The C-terminal region of both toxins is involved in receptor binding and contains combined repetitive oligopeptides that consist of multiple short and long amino acid repeats. These amino acid repeats form motifs that bind sugar moieties on the surface of the host cells. It still remains unclear what the exact binding ligands on the host cells are. TcdA has been shown to bind α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAc but this sugar cannot be found on human cells.[21] TcdA is also able to interact with the disaccharide β-Gal-(1,4)-β-GlcNAc which is found on human I, X and Y blood antigens and glycosphingolipids but it is not clear if these serve as ligands in the human colon as well.[22] Sucrose-isomaltase has been identified as a receptor in the rabbit ileum but this protein is not found on target cells in the human colon.[23] For TcdB, the situation is even less clear. TcdB is able to invade different cell types suggesting a common receptor but it still remains unidentified.[24]

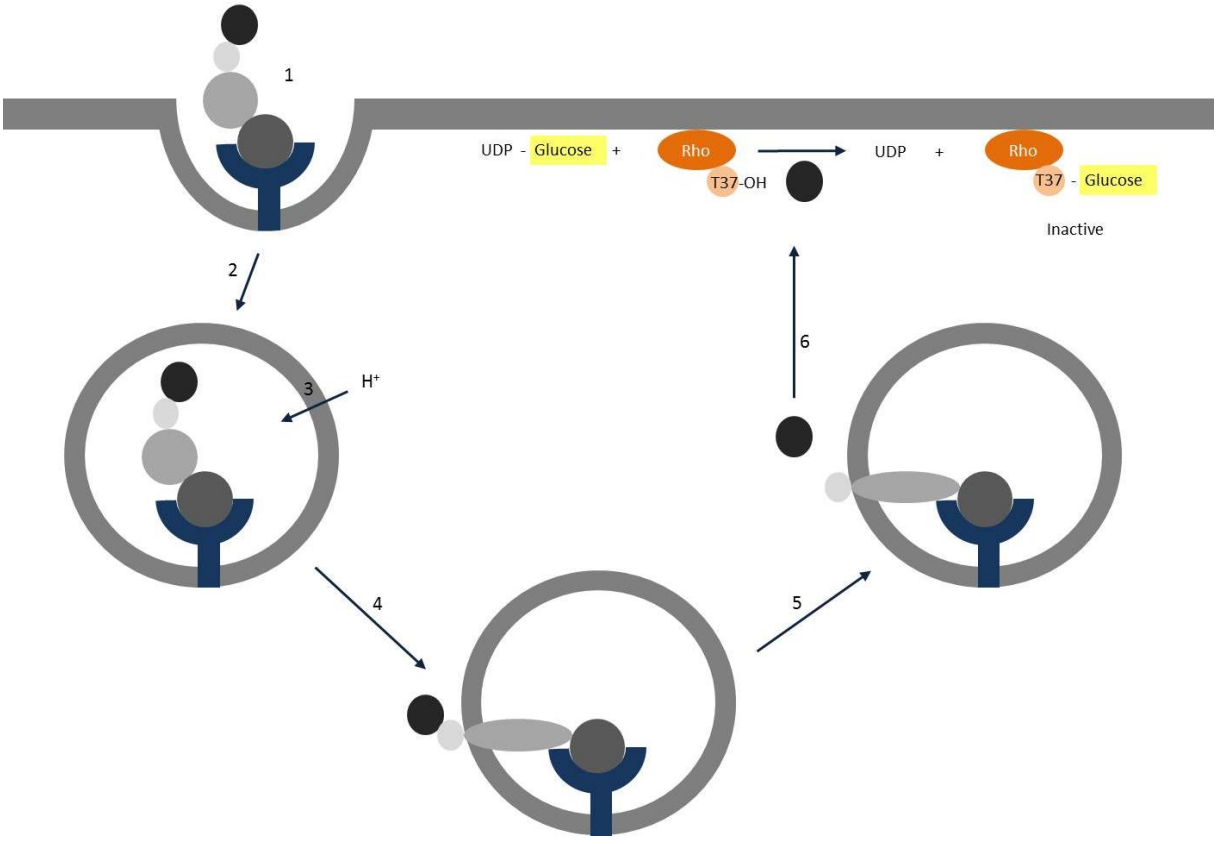

Figure 3. Mechanisms of intoxication. The toxins bind with a receptor on the surface of the enterocytes (1). The complex is internalized by endocytosis (2). Acidification of the endosome (3) induces a conformational change externalizing the autocatalytic and glucosyltransferase domain (4). The glucosyltransferase domain is released into the cytosol (5) and inactivates Rho GTPases (6).

After receptor binding, the whole complex is internalized by clathrin mediated endocytosis.[25] Acidification of the endosome induces a conformational change exposing the hydrophobic region in the translocation domain enabling insertion into the endosomal membrane. Hereby, the autocatalytic and glucosyltransferase domain are delivered into the cytosol.[26] Release of the enzymatic glucosyltransferase domain is dependent on cellular inositol hexakiphosphate (InsP6).[27] InsP6 might play an important role in stabilization of toxin conformation essential for proper proteolytic activity and/or cleavage.[20] Once cleaved, the enzymatic glucosyltransferase domain is released into the cytosol and inactivates Rho GTPases (Rho, Ras and Cdc42) by monoglucosylation of Thr.[37, 20, 28, 29] (Figure 3) Rho GTPases are involved in many cellular processes including the regulation of the actin cytoskeleton. Inactivation of RhoGTPases by TcdA and TcdB causes a disruption of cell-cell junctions resulting in increased epithelial permeability and luminal fluid accumulation associated with CDI. Moreover, the toxins can induce cell rounding and cell death by apoptosis and necrosis.[30, 31] Both toxins also trigger the release of proinflammatory cytokines from immune and epithelial cells including TNF-α and IL-8. IL-8 is important in the pathogenesis of CDI since it is involved in the recruitment and activation of neutrophils which are typically present in high amount at the site of inflammation.[32]

IMPORTANCE OF TCDA AND TCDB IN DISEASE

In the 1980s, researchers postulated that TcdA was the main virulence factor responsible for CDI. Results of animal studies indicated that administration of purified TcdA to hamsters caused disease while administration of TcdB alone did not. However, challenge of hamsters with TcdB and a low concentration of TcdA was able to induce disease symptoms. Co-administration of TcdA and TcdB even resulted in more severe disease. Based on these results, they concluded that both toxins worked synergistically with TcdA being responsible for initial intestinal damage allowing TcdB to exert its cytotoxic activity.[33] TcdA was associated with extensive tissue damage and fluid accumulation in the intestinal tract and was named enterotoxin while TcdB was named cytotoxin due to its 1000-fold more potent cytotoxic activity against cultured cells.[34, 35] The discovery of pathogenic strains that only produce TcdB and not TcdA ($TcdA^-TcdB^+$) forced researchers to adjust their initial findings. Pituch et al. reported that up to 11% of all their clinical isolates were $TcdA^-TcdB^+$.[36] In addition, no TcdB-negative strains ($TcdA^+TcdB^-$) that only produce TcdA have yet been isolated. Lyras et al. provide evidence that TcdB is essential for virulence of *C. difficile* since results of *in vitro* and *in vivo* tests prove that TcdA mutants were still as virulent as the wild type strain while TcdB mutants were associated with a significantly attenuated virulence phenotype.[37] However, Kuehne et al. indicated the importance of both toxins since a TcdB mutant producing only TcdA was indeed able to

induce disease after challenge of hamsters. These contradictory results indicate the necessity of further research to definitively define the exact role of both toxins.[35]

1.3.2 BINARY TOXIN

In addition to toxins A and B, a third toxin has been identified known as the binary toxin CDT. Up to 23% of all *C. difficile* strains produce this additional toxin including the hypervirulent strain 027.[38] CDT is an actin-specific ADP-ribosyltransferase that consists of two subunits: CDTa, the enzymatic component, and CDTb, the binding component, that are encoded by the genes *cdtA* and *cdtB* respectively, located on a 4.3 kb locus on the *C. difficile* chromosome, known as the CDT locus. It additionally contains *cdtR* that encodes a response regulator that controls the transcription of *cdtA* and *cdtB*.[39, 40] CDTb is responsible for receptor binding and translocation of the enzymatic component CDTa into the cytoplasm of the host cell. Lipolysis-stimulated lipoprotein receptor was identified as the host cell receptor for CDT and is highly expressed in the gut.[41] Once in the cytoplasm, CDTa catalyzes the ADP-ribosylation of monomeric actin, thereby interfering with the polymerization of actin and inducing depolymerization of actin filaments. Actin is involved in many cellular functions including establishment of cell morphology. Inhibition of actin polymerization and depolymerization causes a complete disruption of the actin cytoskeleton and loss of cell integrity.[42] Moreover, it induces the formation of microtubule based protrusions on the surface of epithelial cells with an increased adherence of clostridial cells to the intestinal epithelium as a result.[43] Binary toxin positive strains have been associated with a higher mortality rate, more severe diarrhea and relapses. They are more likely community associated, indicating that it has indeed a contribution to the pathogenicity of the infecting strain.[44-47] In addition, a recent report describes the isolation of *C. difficile* strains from patients with diarrhea due to CDI which are positive for binary toxin but negative for toxins A and B.[48]

1.3.3 OTHER VIRULENCE FACTORS

Not only the above described toxins are involved in the pathogenicity of *C. difficile*. Numerous studies have highlighted a number of other factors that have been associated with virulence. Successful colonization of *C. difficile* starts with the attachment of the strain to intestinal host cells. Different colonization factors have been described including flagellin FliC and flagellar cap protein FliD, fibronectin binding protein Fbp68, cell wall proteins Cwp66 and Cwp84, surface layer protein SlpA and a heat shock protein GrOEL.[49-54]

2 CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE IN HUMANS

C. difficile causes a spectrum of disease with varying severity ranging from asymptomatic carriers over mild, moderate or severe diarrhea to a fulminant and possibly life threatening pseudomembranous colitis.[55] Macroscopic observations after endoscopy typically reveal characteristic white/yellow plaques spread throughout the colon mucosa that might explain the malabsorption and observed diarrhea. Typical microscopic features associated with *C. difficile* infections (CDI) are a marked mucosal and submucosal edema and the presence of neutrophils. Pseudomembranous colitis is characterized by 'volcanic eruptions' that contain an overload of fibrin, mucin, neutrophils and deteriorated mucosa.[56, 57]

2.1 RISK FACTORS

The most important risk factors associated with CDI are antibiotic exposure, advanced age and hospitalization. The intestinal microbiota plays an important role in protecting the host against invading harmful microorganisms, a mechanism which is known as colonization resistance. Disturbances in this complex ecosystem makes the host susceptible for gastrointestinal infections. In the case of CDI, the disruption of the intestinal microbiota is mainly associated with the consumption of antibiotics. Antibiotic administration has a drastic effect on the composition of the gut microbiota and is associated with a decreased bacterial diversity. The induced changes are antibiotic- and host-specific. If antibiotic therapy is stopped, the gut microbiota is able to partially recover but some effects are persistent.[58]

A large study conducted by Wiström et al. indicated that up to 12% of patients receiving antibiotic therapy develop diarrhea.[59] Other studies even report incidences of diarrhea in up to 30% of patients receiving antibiotics.[60, 61] Although Tedesco et al. stated in 1974 that patients receiving clindamycin developed diarrhea in 21% of cases and pseudomembranous colitis in 10% of cases for various reasons, it was not until 1978 that *C. difficile* was identified as the major cause of antibiotic associated pseudomembranous colitis.[62, 63] A subsequent reduction in the use of clindamycin was associated with a reduced risk of clindamycin associated colitis. The frequent use of second and third generation cephalosporins in the 1980s-1990s was associated with a high risk for developing CDI which lasts until today.[64] Fluoroquinolones were first introduced as antimicrobials in 1988 but only in 2001 was the association made with an increased risk for CDI.[65] The hypervirulent ribotype 027, responsible for major hospital outbreaks of CDI in North America and Europe, was found to be fluoroquinolone resistant.[66, 67] Table 1 gives an overview of the risk associated with different antibiotics.

Table 1. Antibiotics: Risk of *C. difficile* associated diarrhea [68]

High risk	Moderate risk	Low risk
Cephalosporins (3rd generation)	Narrow-spectrum	Sulfonamides
Clindamycin	Cephalosporins	Nitrofurantoin
Fluoroquinolones	Macrolides	Mandelamine
Broad-spectrum penicillins	Trimethoprim-sufamethoxazole	Vancomycin (IV)
	Carbapenems	Metronidazole
		Aminoglycosides
		Rifampicin

The two other major risk factors are hospitalization and advanced age with CDI being most commonly diagnosed in hospitalized patients older than 65 years.[67, 69, 70] A comprehensive survey by Karlström et al. clearly indicated a dramatic increase in the incidence of CDI in patients after the age of 60 years.[71] Since aging is associated with an impaired immune function, it might be possible that older patients do not develop an efficient immune response against *C. difficile* toxins as compared to younger individuals but this remains to be confirmed.[72] The hospital setting brings together a number of factors that make it an ideal environment for *C. difficile* to survive and cause infections including the frequent use of antibiotics, close contact with infected patients and the easy spread of disinfectant-resistant spores to other patients by health care workers. Other risk factors that have been associated with CDI are underlying disease, nasogastric tubing, use of gastric acid suppressants and the use of anticancer drugs.[73-76]

2.2 INFECTION CYCLE

C. difficile can be found throughout the environment, especially in the soil. It can be found in the human gut as part of the indigenous gut microbiota in up to 3% of adults without causing disease. A colonization rate as high as 70% can be found in newborns without causing disease. This might have several causes including the absence of toxin receptors on enterocytes or downstream signaling pathways in the immature gut mucosa, competition with the maturing infant gut microbiota and maternally acquired protective factors.[6, 7] *C. difficile* spores are commonly found in the healthcare facilities on different surfaces in the environment (e.g. floor, toilet, bathroom,…) and medical equipment (thermometer, blood pressure monitor,…). Spores can be spread by direct person-to-person contact with infected patients or indirectly by the hands of healthcare workers.[77, 78] *C. difficile* is transmitted through the fecal-oral route. Upon oral ingestion of *C. difficile*, vegetative cells get killed in the stomach but spores can survive the acidity of the stomach and pass into the small bowel.

Once in the small intestine, the spores germinate to vegetative cells under the influence of bile salts. Disruption of the gut microbiota abolishes the colonization resistance that confers protection against pathogens giving *C. difficile* the chance to persist in the large bowel. *C. difficile* colonizes the colon and produces several virulence factors that are associated with disease. The two best characterized virulence factors are toxins A and B. Toxin production induces the synthesis of TNF-α and pro-inflammatory interleukins leading to an increased vascular permeability, recruitment of neutrophils and monocytes, loss of epithelial integrity and epithelial cell apoptosis. Local production of hydrolytic enzymes causes degradation of connective tissue which leads to colitis, pseudomembrane formation and watery diarrhea (Figure 4). Some strains produce an additional toxin, the binary toxin CDT but its precise role in disease development is not yet well understood. Thus, three major events are associated with pathogenicity of *C. difficile*: alteration of the indigenous gut microbiota, intestinal colonization with toxigenic *C. difficile* and multiplication of the strain associated with toxin production.[79]

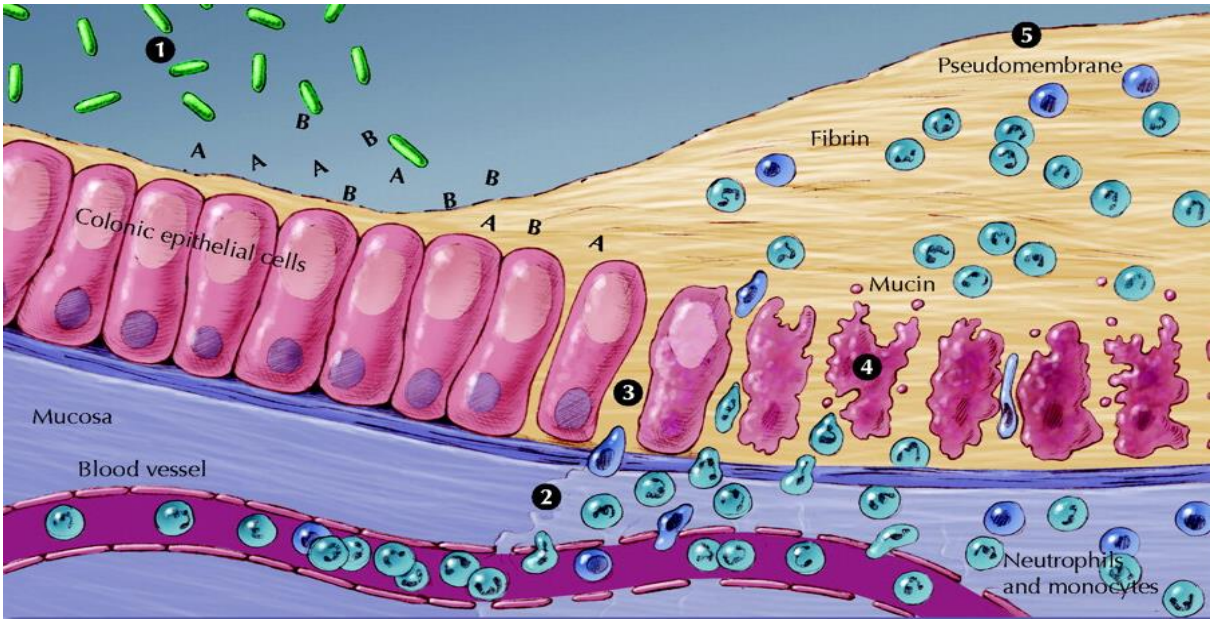

Figure 4. *C. difficile* pathogenesis. *C. difficile* vegetative cells colonize the colon and produce several virulence factors (1). Toxins A and B induce the production of tumor necrosis factor-α and proinflammatory interleukins leading to increased vascular permeability, recruitment of neutrophils and monocytes (2), loss of epithelial cell junctions (3) and apoptosis of epithelial cells (4). Production of hydrolytic enzymes causes degradation of connective tissue which eventually leads to colitis, pseudomembrane formation (5) and watery diarrhea.[79]

2.3 CHANGING EPIDEMIOLOGY

C. difficile is one of the leading causes of health care associated infections. Until the late 1990s, incidences of CDI remained relatively stable. In the past decade however, the epidemiology changed dramatically worldwide with different publications reporting increasing incidence and severity. Over the past five years, *C.difficile* has even replaced methicillin resistant *Staphylococcus aureus* (MRSA) as the most common cause of healthcare associated infections in the United States.[80] CDI incidence increased from 3.82 per 1000 hospital discharges in 2000 to 8.75 per 1000 hospital discharges in 2008 and is diagnosed in 0.9% of all hospital admissions with a mortality rate of 9.1%.[81] In Belgium, *C. difficile* is responsible for 4.2% of all healthcare associated bacterial infections. CDI incidences in Belgium have been rising since 1998 but stabilized at high level since 2004. In 2009 and 2010, a small decrease was noted after which the mean incidence remained stable at high level for the past 4 years. In 2014, a mean incidence of 1.49 and 0.86 cases per 1000 admissions was calculated for all CDI episodes and hospital-acquired CDI episodes respectively.[82-84]

As stated above, antibiotic exposure, advanced age and hospitalization are the most important risk factors for CDI. *C. difficile* is, however, more and more isolated from people lacking these traditional risk factors. Community-acquired cases (CA-CDI) are being reported more frequently. CDI is defined as community acquired if symptoms occur in the community, within 48 hours after hospitalization or 12 weeks post-hospitalization.[85] In the United Kingdom, incidences increased from 0 to 18 cases per 100 000 persons per year between 1994 and 2004.[86] In Sweden, 22-28% of all cases of CDI were community acquired.[71, 87] From 2007 to 2011, 36-38% of all cases of CDI in Belgium are not hospital acquired. As opposed to data from other countries, there is currently no evidence that CA-CDI cases in Belgium are rising.[83] Moreover, Fellmeth et al. showed that more than half of all CA-CDI cases are not associated with the standard risk factors including advanced age and antibiotic exposure.[88] Patients suffering from CA-CDI appear to be younger and a higher proportion of females is affected as compared to healthcare associated CDI. The disease appears to be less severe and relapses are less likely. Moreover, a substantial part of the infections is not associated with antibiotic exposure.[89, 90]

A number of different reasons can explain the recent rise in morbidity and mortality due to CDI including advances in technology leading to better diagnostic techniques and better surveillance programs with obligatory reporting of cases. One of the main reasons, however, is the emergence of a new hypervirulent clone called BI/027/NAP1 (ribotype 027). This strain overproduces the virulence toxins A and B and binary toxin. It was first associated with an epidemic outbreak in 2002 in southern Canada where an unusual increase in the incidence and severity of CDI was detected.[66, 91] This

epidemic strain spreads rapidly through Northern America and Europe.[92, 93] It was isolated for the first time in Belgium in 2005 and was responsible for a rise in mortality. Nowadays, a decline is noted in the number of infections due to ribotype 027 but it is being replaced by other virulent strains. For example, ribotype 106 is now one of the most commonly found strains in the United Kingdom.[94] In Belgium, ribotypes 002, 014, 020 and 078 are now more frequently isolated than ribotype 027.[95]

2.4 RECURRENT INFECTIONS

One of the major problems with CDI is the high rate of recurrent infections. Recurrent infections can be defined as complete resolution of symptoms followed by reoccurrence of the disease. A first episode of recurrence occurs in 15-35% of patients but up to 65% of these patients will suffer from additional recurrences. Recurrent infections can be caused by either reinfection with a new strain or relapse with the original infecting strain. Reinfection is most likely caused by germination of endogenous *C. difficile* spores after antibiotic treatment is stopped. For reinfections, spores in the environment and contact with other *C. difficile* patients are the most important sources of infection. Relapse and reinfection is thought to occur at approximately the same rate.[79, 96, 97] Risk factors associated with recurrent disease include age higher than 65 years, additional antibiotic therapy after CDI treatment, increased severity of underlying disease and a low immune response against *C. difficile* toxin A.[98] Evidence exists that an adequate antibody response to *C. difficile* toxin A during an initial episode of CDI is associated with protection against recurrence.[98]

2.5 CURRENT TREATMENT STRATEGY

2.5.1 ANTIBIOTIC THERAPY

Since CDI is the consequence of antibiotic consumption, an important initial step in the treatment of the disease is the discontinuation of the inciting antibiotic as soon as possible. Different studies show that simultaneous use of antibiotics for reasons other than CDI is associated with prolonged diarrhea and an increased risk of recurrent CDI. If ongoing antibiotic therapy is however necessary for treatment of the primary infection, it is recommended to choose antibiotics that are less frequently associated with the development of CDI.[99, 100] Additional treatment measures also include administration of fluids and electrolytes, avoidance of anti-motility drugs and revised use of proton pump inhibitors.[101]

In mild cases of CDI, discontinuation of the inciting antibiotic might be sufficient to resolve disease. Most patients will however need additional antibiotic therapy. Metronidazole and vancomycin are the two main antibiotics used to treat CDI. Disease severity determines which antibiotic should be used. Mild-to-moderate CDI is treated with metronidazole while vancomycin is the drug of choice for

severe or complicated disease.[99, 100] Several studies indicate that metronidazole is not inferior to vancomycin for the treatment of mild-to-moderate disease.[102, 103] One of the major advantages of metronidazole is its low cost. Moreover, it is believed that metronidazole is less associated with the theoretical risk of acquisition of vancomycin resistant enterococci (VRE). There is however no valid evidence to support this theory since both antibiotics have been associated with the overgrowth of VRE during treatment for CDI.[104]

For severe or complicated CDI, vancomycin has proven to be more effective and is the drug of choice.[105] For patients who develop fulminant CDI that fails to respond to antibiotic treatment and who progress to systemic toxicity, peritonitis, toxic megacolon or bowel perforation, emergency colectomy can be live-saving.[106] First recurrences are normally treated with the same antibiotic as the initial episode after reassessment of disease severity. Since metronidazole is fully absorbed by the gastrointestinal tract, long duration therapy might lead to serious adverse effects. Therefore, second and further recurrences should be treated with vancomycin.[99-101]

In 2011, the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA) approved the use of fidaxomicin to treat CDI.[107] Fidaxomicin is a macrolide antibiotic that exerts its antibacterial activity by inhibiting RNA synthesis. Results of clinical trials indicate that fidaxomicin is as effective as vancomycin for the treatment of CDI.[108, 109] Fidaxomicin has a narrow spectrum of activity and has thus a limited impact on the composition of the indigenous microbiota.[110] Moreover, it is associated with a reduced risk for relapse and inhibits *C. difficile* sporulation.[111] Although no resistance has yet been reported, a *C. difficile* strain with an elevated MIC value has been isolated from a patient with CDI.[112] Unfortunately, the use of fidaxomicin has not yet become common practice due to its high cost. The cost of treatment with fidaxomicin is estimated at approximately $296 per day which is more than double of that of vancomycin being $139 per day.[113] Moreover, fidaxomicin does not show superiority over standard antibiotic therapy for the treatment of hypervirulent strains.[108, 109]

2.5.2 NON-ANTIBIOTIC TREATMENT

Fecal microbiota transplantation (FMT) is defined as the administration of a stool sample containing distal gut microbiota from a healthy donor to a patient with a disease caused by an alteration of his/her normal gut microbiota.[114] The last few years, FMT has gained more and more attention for the treatment of recurrent CDI in order to restore the disturbed intestinal microbiota caused by repeated antibiotic treatment.[115] The use of FMT as a first line treatment for patients suffering from recurrent CDI is now recommended in the practice guidelines from both the American College of Gastroenterology and the European Society of Clinical Microbiology and Infectious Diseases.[99-101]

Results of a recent randomized controlled clinical trial indicated that FMT was superior to conventional antibiotic treatment.[116] Moreover, the treatment appears to be well tolerated by patients since no severe side effects have yet been identified. Possible long term adverse effects however are yet unknown and require further research. As compared to the high cost of vancomycin therapy, FMT has the advantage of being inexpensive.[115]

2.6 INFECTION CONTROL PROGRAMS

The increased incidence of CDI has led to the development of several guidelines in an attempt to control CDI. Since 2007, Belgian hospitals are obliged to report nosocomial cases of CDI. Elaboration of a good surveillance program can help to detect a rise in the incidence of CDI and identify possible epidemic outbreaks of the pathogen. Infection control programs including proper personnel education can aid in the early detection of CDI leading to earlier treatment and proper infection control practices.

Infection control practices are focused on preventing horizontal transmission of the pathogen and on controlling CDI associated risk factors. Hospitalized patients are exposed to *C. difficile* by indirect contact with healthcare workers, the contaminated environment or by direct contact with infected patients. At every level, measures can be taken to control the transmission of the pathogen. First of all, patients suffering from CDI should be isolated in a private room or at most in a room with another patient with CDI to prevent direct patient-to-patient transmission and limit contamination of the direct environment. To limit spread of the pathogen by health care workers, they are encouraged to pay attention to good hand hygiene. Since *C. difficile* spores are resistant to the commonly used alcohol based antiseptics, they should thoroughly wash their hands with water and soap or use chlorhexidine based antiseptics. In addition, contaminated surfaces (floors, toilets, bed frames,…) should also be disinfected with chlorhexidine based products. If possible single-use disposable equipment (e.g. thermometers) can be used or medical equipment should be dedicated to a single patient. All equipment should be carefully cleaned and disinfected using the proper antiseptics after use on a patient suffering from CDI. Use of disposable gloves or protective clothing can also aid in limiting the spread of the pathogen.[99, 100, 117]

Since antibiotic consumption is one of the most important risk factors for CDI, a good antibiotic stewardship might aid in controlling the incidence of CDI. Several studies show that the restricted use of antibiotics has a beneficial effect on the incidence of CDI.[118-120] Restricted use of antibiotics helped to control an epidemic hospital outbreak of the highly fluoroquinolone resistant NAP1/BI/027 strain.[120, 121] Moreover, limited use of clindamycin, an antibiotic at high risk for developing CDI,

decreased the incidence of CDI.[119, 122] The replacement of cefotaxime by the low risk antibiotic piperacillin-tazobactam was associated with a reduction in CDI rate of more than 50%.

3 CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE IN ANIMALS

C. difficile has been isolated from several animal species including calves, cattle, deer, goats, sheep, cats, dogs, horses, piglets, pigs and poultry. Most studies focus on the prevalence of the pathogen in healthy animals and the risk of spread to the environment and humans. Many reports describe the presence of *C. difficile* in animals regardless of the presence of diarrhea. Clinical symptoms due to CDI vary widely between and within species.[123, 124]

C. difficile has been recognized as an important intestinal pathogen in horses of all ages with disease outcomes ranging from mild diarrhea to life-threatening necrotizing enterocolitis.[124-127] As in humans, hospitalization and antibiotic exposure are two important risk factors.[128] Antibiotic administration in horses has been associated with changes in the intestinal microbiota and an increase in the amount of *C. difficile*.[129] Moreover, *C. difficile* spores have been isolated from the floor, stables, medical equipment and footwear of the medical personal in a veterinary hospital.[130] Antibiotic exposure is however not always a prerequisite to develop *C. difficile* associated disease (CDAD). Other reported predisposing factors are dietary changes, stress and pre- and post-surgical feed withdrawal.[127] Fatal enterocolitis has been reported in horses without a history of hospitalization or antibiotic exposure.[125, 131] On the other hand, toxigenic and non-toxigenic *C. difficile* strains have been isolated from horses outside the hospital environment.[128, 132, 133] Rodriguez et al. postulate that horses frequently harbor toxigenic and non-toxigenic *C. difficile* strains regardless of hospitalization. The development of diarrhea appears to be more unusual.[128] Noteworthy, a synergism between *C. difficile* and *C. perfringens* has been reported in gastrointestinal disease in horses.[125]

In neonatal piglets, *C. difficile* is an important cause of diarrhea, mesocolonic edema and colitis.[134] Different authors report outbreaks of *C. difficile* infection in piglets.[135, 136] Up to two thirds of litters can be affected and mortality rates as high as 16% have been reported.[134, 137] Yaeger et al. detected *C. difficile* toxin in 29% of piglets with diarrhea. In 19% of diarrheal piglets, *C. difficile* was the sole pathogen present.[138] Experimental inoculation of piglets with *C. difficile* spores led to the development of characteristic *C. difficile* associated disease indicating that *C. difficile* is a porcine pathogen.[139, 140] However, *C. difficile* colonization is not always associated with disease. It is commonly found in the feces of piglets. Prevalence of *C. difficile* in neonatal piglets is as high as 100% within 48h after birth.[141] Remarkably, colonization significantly decreases over time.[142] In pigs at

slaughter age, *C. difficile* is often not detected.[132, 143-145] On the other hand, a Belgian study isolated *C. difficile* from 1% of pigs at slaughter age and 7% of pig carcasses in the slaughter house.[146] Several studies report the lack of association between the presence of *C. difficile* and the development of diarrhea.[141, 147, 148] In contrast to the situation in humans, antibiotic use does not affect the prevalence of *C. difficile* in pigs. No differences were detected in prevalence, antimicrobial susceptibility and toxinotypes of *C. difficile* strains isolated from pigs and environmental samples in conventional versus antibiotic free farms.[145] Interestingly, administration of a non-toxigenic *C. difficile* strain as a probiotic to piglets successfully lowered the prevalence of toxin-positive feces, mesocolonic edema and microscopic lesions as compared to control piglets.[149]

The importance of *C. difficile* as a bovine pathogen is less clear. *C. difficile* has been isolated from healthy calves as well as calves suffering from diarrhea.[150-152] Several reports describe that *C. difficile* is isolated to a higher extent in young calves with a sharp decrease when they reach slaughter age.[153-155] On the other hand, several authors still describe the presence of *C. difficile* in calves at slaughter age and on calve carcasses.[146, 150, 155] Remarkably, one study reports that the use of antimicrobials was associated with increased shedding of the pathogen in calves.[153]

In dogs, the role of *C. difficile* in gastrointestinal disease is not defined. *C. difficile* has been isolated from healthy animals as well as from animals suffering from diarrhea.[156-161] A positive association has however been reported between the detection of *C. difficile* toxin and the presence of diarrhea in dogs.[162] Clooten et al. reported a significant association of CDI with antibiotic exposure and hospitalization.[162] Initially, a significant association between the presence of *C. difficile* toxins and acute hemorrhagic diarrheal syndrome was reported.[163] Recently, this observation was countered since Busch et al. reported low detection rates of toxigenic *C. difficile* strains with no differences between healthy and diarrheal dogs. Therefore, the role of *C. difficile* toxin A and B in the pathogenesis of acute hemorrhagic diarrheal syndrome was questioned.[164] As in horses, a synergism between *C. difficile* and *C. perfringens* has been suggested for enteric disease in dogs.[160]

Less information is available about the role of *C. difficile* in enteric disease in cats. *C. difficile* has been isolated from cats lacking any sign of disease.[159, 162] On the other hand, Weese et al. detected *C. difficile* toxins in cats suffering from acute diarrhea.[165] Another report describes the isolation of toxigenic *C. difficile* from cats with diarrhea that responded to subsequent metronidazole therapy.[166] Although these results might propose a possible role of *C. difficile* in feline enteric disease, no definite conclusions can be made due to the limited information available.

Although the data available on the contribution of *C. difficile* as a pathogen in animals are accumulating, it remains difficult to get a clear picture. Rodriguez-Palacios et al. postulated that it

might be important to take into account geographical, climate and seasonal factors when studying the prevalence of *C. difficile* in the intestinal tract of animals and the environment.[132] Great concern exists on the possible zoonotic potential of the organism. Since *C. difficile* has been reported in household pets and farm animals, they might provide a reservoir for toxigenic isolates. Moreover, identical toxigenic isolates have been detected in humans and animals. Especially ribotype 078 has been commonly found in animals and is pathogenic in humans. In addition, *C. difficile* has been found in several food products such as processed meat products, edible shellfish and raw vegetables questioning its potential role in foodborne transmission. The contribution to human CDI has however not been extensively studied.[167, 168,169-171]

CLOSTRIDIUM PERFRINGENS

1 THE ORGANISM

Clostridium perfringens is a ubiquitous microorganism that can be found throughout the environment and as a member of the normal intestinal microbiota of humans and animals.[172, 173] It is however also a common and important human and animal pathogen causing a spectrum of diseases. *C. perfringens* is known as the leading cause of gas gangrene and one of the most common causes of food poisoning.[174] It has been associated with antibiotic associated diarrhea and human necrotic enteritis.[175] As an animal pathogen, *C. perfringens* is the causative agent of enterotoxemia in cattle, sheep, rabbits, pigs and horses.[174] In this chapter, we shall not review all the different diseases associated with *C. perfringens* in animals and humans, but limit to necrotic enteritis in broiler chickens. Much research has been done to understand the role of *C. perfringens* in broiler necrotic enteritis. Since the ban on the use of antibiotics as growth promoters, the incidence of necrotic enteritis in broilers has been rising. Several studies indicate that enterotoxigenic *C. perfringens* strains can be found in poultry derived products.[176] Improper handling of contaminated food has been associated with transmission of enterotoxigenic *C. perfringens* strains to humans.[177] Control of this pathogen is necessary to limit production losses, decrease mortality, improve animal welfare, decrease the risk of contamination of poultry products for human consumption and protect human health.

1.1 GENERAL CHARACTERISTICS

Clostridium perfringens is a Gram-positive, rod-shaped bacterium.[172, 178] It grows in anaerobic conditions but is less strictly anaerobic than other Clostridia since it can survive prolonged exposure to oxygen.[179] Vegetative cells are 1.3 – 19.0 µm long and 0.6 – 2.4 µm wide.[172, 178] Although no flagella are present, the bacterium gains its motility due to the presence of type IV pili.[180] Under optimal growth conditions (43-45°C), *C. perfringens* is known as the most rapidly multiplying organism with generation times less than 10 min. Growth is accompanied by excessive gas production. The bacterium is able to survive unfavorable conditions due to the formation of highly resistant spores.[178, 181] *C. perfringens* is heterotrophic since it needs the presence of 13 amino acids for optimal growth. As a consequence, it cannot grow in an environment where an amino acid supply is limited. By the production of extracellular toxins and enzymes, *C. perfringens* can make these nutrients available and import them inside the cell for its own use.[182]

1.2 GENETICS

C. perfringens belongs to the family of the Clostridiaceae and cluster I of the genus *Clostridium*.[11] The first whole genome sequence was published in 2002 from *C. perfringens* strain 13. This strain naturally occurs in the soil. It is classified as a toxinotype A strain which is commonly associated with gas gangrene in humans. The genome consists of a circular chromosome of 3 031 430 bp and a plasmid of 54 310 bp. In contrast to *C. difficile*, the *C. perfringens* genome contains only few mobile genetic elements.[182]

1.3 VIRULENCE FACTORS

C. perfringens exerts its toxic effect by the secretion of a variety of toxins and enzymes that damage the hosts tissue. Four major toxins can be distinguished: alpha-, beta-, epsilon- and iota-toxin. Only the gene encoding alpha-toxin is located on the chromosome. The other three major toxins are plasmid-borne.[183] Several excellent reviews can be consulted on the role of these toxins in disease.[184-187] *C. perfringens* strains are classified into five toxinotypes (A-E) based on their differential production of the four major toxins.[183, 188] (Table 2) This differential toxin production determines the pathogenicity of the *C. perfringens* isolate and is associated with specific human and animal disease. The majority of strains that cause necrotic enteritis in broilers, gas gangrene, food poisoning and diarrhea in humans belong to toxinotype A.[189-191]

Table 2. *Clostridium perfringens* toxinotypes.[183]

Toxinotype	Alpha-toxin	Beta-toxin	Epsilon-toxin	Iota-toxin
A	x			
B	x	x	x	
C	x	x		
D	x		x	
E	x			x

In addition to the major toxins, an enterotoxin and several minor toxins are known. Enterotoxin is a membrane acting toxin produced during sporulation. It is associated with human food poisoning.[192] Several minor toxins are produced, including theta-toxin or perfringolysin O, which is responsible for the hemolysis of red blood cells and beta2-toxin, which is associated with enteritis in piglets.[193, 194] The designation major and minor toxin is solely intended for classification of strains into toxinotypes and does not refer to the virulence of the toxins.

2 NECROTIC ENTERITIS IN POULTRY

Necrotic enteritis (NE) is the most common enteric disease in broilers. In 1961, *C. perfringens* was identified as the causative agent of NE.[195] The disease is characterized by necrosis and inflammation in the gastrointestinal tract. Clinical signs are depression, anorexia, diarrhea, dehydration and ruffled feathers. Macroscopic lesions are mainly confined to the small intestine but can extend to the caeca, liver and kidneys. The intestines are thin walled, inflamed and filled with gas.[196] A spectrum of mucosal lesions can be detected ranging from focal necrosis or ulceration to the presence of necrotic patches or a pseudomembrane.[197] NE can manifest in a clinical or subclinical manner. The acute clinical form of NE leads to an increased mortality rate in broiler flocks. Mortality rates are generally between 2 - 10% but rates as high as 50% have been reported. Subclinical disease is characterized by damage to the intestinal mucosa leading to decreased digestion and absorption, reduced growth rate and impaired feed conversion ratios. It often remains undetected and has the greatest impact on performance.[191, 198, 199] The total annual cost due to NE in broilers has been estimated to be over $2 billion.[200]

C. perfringens is part of the normal broiler gut microbiota. Counts of $0 – 10^5$ cfu/g have been found in the intestinal content of healthy broilers. On the other hand, broilers suffering from NE can have up to $10^6 – 10^8$ cfu/g in their intestinal content.[201, 202] NE arises if an environment is created that favors the overgrowth of *C. perfringens*. Two important issues that are associated with this are the nature of the feed and the presence of coccidiosis caused by *Eimeria* species. In addition, virulent *C. perfringens* strains are required to induce NE.[203] A recent review offers an excellent update on the pathogenesis of *C. perfringens* associated NE.[196]

It has long been thought that alpha-toxin was the key virulence factor in NE since toxinotype A strains are the most predominantly found in NE, oral inoculation of broilers with semi-purified alpha-toxin or alpha-toxin containing supernatant was associated with high mortality rates and immunization of broilers with alpha-toxin based vaccines significantly protected against NE.[189, 204-207] However, the role of alpha-toxin was questioned by several authors.[189] The most conclusive evidence was presented by Keyburn et al. who proved that an alpha-toxin deficient strain was still able to induce disease in an *in vivo* NE model.[197] Discovery of the novel toxin NetB provided comprehensive evidence that this is the most important toxin involved in NE.[208] For example, a NetB mutant failed to induce disease while complementation of the NetB mutant with the wild type NetB gene successfully caused NE.[208] In addition, a 100% correlation has been found between the production of NetB and the ability to induce NE in an *in vivo* model.[209, 210, 701] NetB is exclusively found in chicken isolates,

with just one exception.[211] Moreover, immunization of broilers with NetB toxoid conferred partial protection against NE.[212, 213]

A remarkable observation is that while a high degree of genetically different *C. perfringens* isolates can be found in healthy animals, single strain dominance is observed in birds suffering from NE. Pulsed field gel electrophoresis indicated that *C. perfringens* isolates from NE positive flocks are usually genetically identical within a flock.[189, 204] The exact mechanism behind this is not yet understood. However, isolates from NE positive flocks appear to be more capable of inhibiting other *C. pefringens* strains as compared to isolates from healthy birds. Key components behind this mechanism might be the production of bacteriocins. In the case of *C. perfringens* and NE, a bacteriocin called perfrin was recently discovered. Perfrin was bactericidal for other *C. perfringens* strains and associated with NetB positive strains from NE positive flocks.[214, 215]

2.1 CURRENT TREATMENT STRATEGY

2.1.1 ANTIBIOTIC THERAPY

NE has long been controlled by the use of antibiotics as in-feed growth promoters. The European Union has however banned the use of in-feed antibiotics leading to a flare in the incidence of NE in broilers. Antibiotics are now used for therapeutic reasons when clinical signs of NE are apparent. Amoxicillin, tylosin, lincomycin and bacitracin are commonly used antibiotics to control NE. *In vivo* experiments studying the effect of these antibiotics on broiler NE are however limited. Only one study describes the protective effect of amoxicillin treatment on the development of NE lesions.[216] Lincomycin, tylosin and bacitracin have been successfully used to control broiler NE but resistance against these antibiotics has already been reported.[216-223] In addition, imprudent use of these antibiotics could even contribute to the development of antibiotic resistance.[216]

THE QUEST FOR ALTERNATIVE TREATMENTS

1 THE RISING NEED FOR ANTIBIOTIC ALTERNATIVES

1.1 CLOSTRIDIUM DIFFICILE

Although still efficient, the current treatment strategies using the antibiotics metronidazole and vancomycin have some major drawbacks. Metronidazole is effective against infections with anaerobic bacteria while vancomycin is able to kill Gram-positive bacteria. As such, both antibiotics do not have a narrow spectrum of activity and also act on a large range of bacteria from the gut microbiota. For both antibiotics, a limited treatment success in severe disease and a high recurrence rate has been reported.[224] Moreover, careful use of vancomycin is recommended to avoid the risk of colonization with vancomycin resistant enterococci. The emergence of highly virulent strains also requires the need for efficient treatments to rapidly cure the disease and limit spread of the pathogen.

Excessive use, over-prescription and misuse of antibiotics have led to a dramatic increase in the incidence of antibiotic resistant microbial pathogens.[225] Consumption of antibiotics is always associated with the risk of spread of resistance to other pathogens or bacteria of the indigenous microbiota. Although most *C. difficile* strains remain susceptible to metronidazole, clinical isolates with reduced susceptibility have been reported.[90, 226-228] There is great concern that the industry is not able to develop new effective antibiotics at a sufficient rate to counteract the development of antibiotic resistance.[225] Since 1987, no novel antibiotic classes have been successfully discovered.[229] Moreover, pharmaceutical companies have curtailed their antibacterial research due to its expensive and time consuming character and devious regulations.[230]

As stated by Johnson et al., it seems contradictory that standard treatment of CDI involves the administration of antibiotics when disease outcome results from the disruptive effect of antibiotics on the colonic microbiota.[231] After all, treatment with broad spectrum antibiotics might further disrupt the already abnormal microbiota and thereby enhance the growth of any leftover *C. difficile* organisms or of a newly acquired strain once antibiotic therapy is discontinued. There is a great need to look for efficient treatments that do not further disrupt the already altered indigenous microbiota,

help to restore the complex balance of the normal gut microbiota and limit the use of additional antibiotic therapy.[232]

Research has been done to develop novel antibiotics with a narrow spectrum of activity specifically targeting *C. difficile* without significantly affecting the microbiota. The only FDA and EMA approved narrow spectrum antibiotic for the treatment of CDI is fidaxomicin.[107] As mentioned before, this antibiotic is as efficient as vancomycin but has the disadvantage of being extremely expensive.[113] Cadazolid and SMT19969 are two examples of narrow spectrum antibiotics with potent *in vitro* antibacterial activity against *C. difficile*.[233, 234] Cadazolid is an oxazolidinone antibiotic that acts as an inhibitor of protein synthesis and is able to inhibit DNA synthesis in a limited extent. Results of *in vivo* experiments demonstrate complete protection against diarrhea and death and support the relevance of further clinical studies.[235] SMT19969 is a non-absorbable antibiotic that inhibits DNA synthesis. Results of *in vivo* hamster trials concluded that SMT19969 was superior to vancomycin since a greater overall survival was noted along with a delayed time to relapse and a lower recurrence rate. Moreover, fecal samples were negative for culture spores and an improved recovery of the intestinal microbiota was detected. A phase 1 clinical trial indicated that SMT19969 was safe and well tolerated and caused a minimal disturbance of the intestinal microbiota.[236-238] Both antibiotics are very promising but require further clinical studies.

Several other alternative treatments have been proposed including immune therapy, bacteriophage therapy, administration of antimicrobial peptides such as bacteriocins and lipopeptides, and use of probiotics. Due to the scope of this PhD thesis, we will focus on microbial based products as alternatives for antibiotic therapy. For more information on other treatment strategies we would like to refer to an excellent review by Hedge et al.[239]

1.2 CLOSTRIDIUM PERFRINGENS

Antibiotics have long been used as in-feed additives to improve nutrient availability, animal health, and growth performance. Hence, NE has long been constrained at the same time. However, public concern on the use of in-feed antibiotics and the alarming rise of antibiotic-resistant “superbugs” has led to a ban on the use of antibiotics as growth promoters in Europe. Consequently, a rise has been seen in the incidence of NE in broilers with a concomitant increase in the therapeutic use of antibiotics to treat NE.[191, 240, 241] Imprudent use of these antibiotics is however associated with the risk of resistance development.[216] In addition, resistance against the commonly used antibiotics to treat NE has already been reported.[216-218, 222, 223] Research has focused on alternative strategies to improve animal health. Strategies to control NE mainly focus on dietary changes, pathogen reduction and stimulation of the immune response.[242] *C. perfringens* infections have been successfully

controlled using natural feed additives, organic acids, enzymes, lysozymes or microbial derived products. Figure 5 gives an overview of the virulence mechanisms of *C. perfringens* that can be tackled to control the pathogen.[243] Due to the scope of this thesis, we will focus on microbial based products. Several excellent reviews describe these alternative strategies and can be consulted for more detailed information.[242, 243]

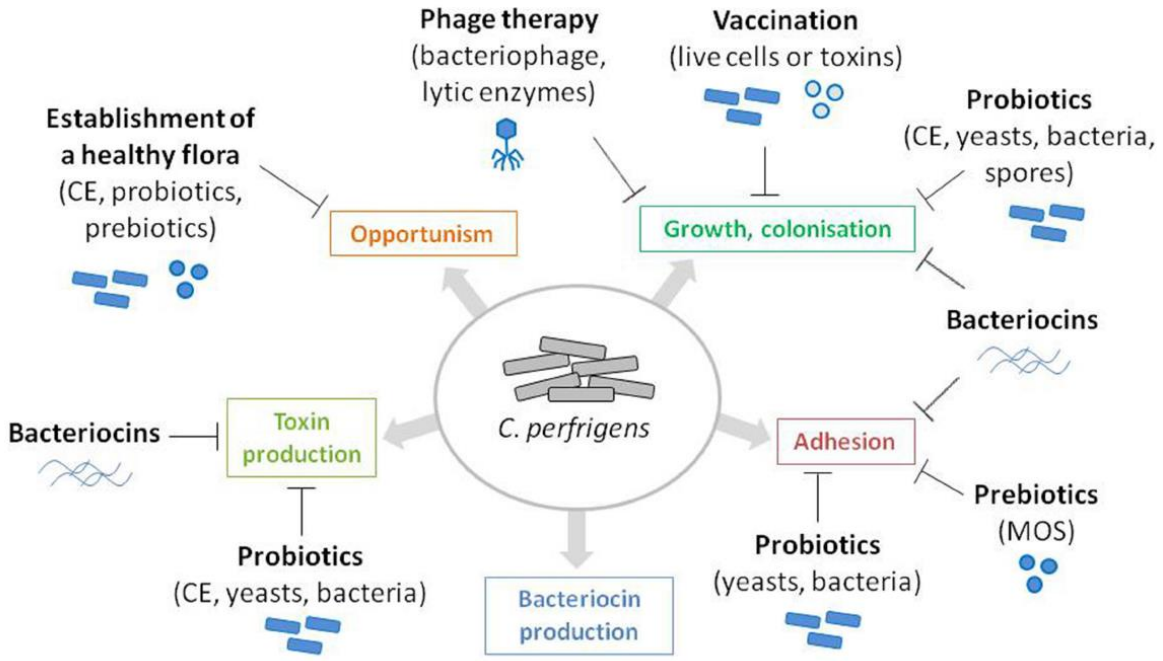

Figure 5. Potential targets for the control of NE caused by *C. perfringens* in broilers.[243]

2 MICROBIAL BASED PRODUCTS AS PROMISING THERAPEUTICS FOR INTESTINAL CLOSTRIDIAL INFECTIONS

2.1 BACTERIAL METABOLITES

The upsurge in multidrug resistant pathogens has created the need to look for alternatives to antibiotics as efficient treatment strategies. Prokaryotic microorganisms produce a vast range of secondary metabolites that are not always essential for growth and reproduction per se but aid the organisms to survive under unfavorable conditions. Singla et al. attempted to classify these bacterial metabolites based on structural relationships and proposed 17 different structural classes (Table 3). These molecules are structurally very diverse and display a broad range of activities with potential therapeutic significance including antibacterial, antiviral, antifungal, antitumor, anti-inflammatory and antibiofilm activities.[244] The bacteriocins, lantibiotics and lipopeptides will be discussed in more detail below. For more information about the therapeutic spectrum of other metabolites, we would like to refer to the recent review of Singla et al.[244]

Table 3. Classification of bacterial metabolites as proposed by Singla et al.[244] The underlined classes are discussed in more detail.

	Structural class	Bacterial metabolites examples
1	β-lactam antibiotics	Sulfazecin, isosulfazecin
2	Bacteriocin	Thuricin, coagulin
3	Cyclic lipoheptapeptide	Surfactin A-D, iturin, fengycin
4	Catecholate siderophore	Bacillibactin, petrobactin
5	Diketopiperazine	Nocazine D, E
6	Indole alkaloids	3-hydroxyacetylindole, 3-formylindole
7	Lantibiotic	Nisin, subtilin, mersacidin
8	Polyketide macrolactone	Difficidin, bacillaene, macrolactin
9	Aminopolyol	Zwittermicin
10	Isocoumarin	Amicoumacin A, B, C
11	Amino sugar	3, 3'-neotrehalosadiamine
12	Adenine nucleotide	β-exotoxin
13	Polyacetilene analog	Melanin
14	Dipeptide	Bacilysin
15	Phospholipid	Bacilysocin
16	Prenylated naphthoquinones	Fumaquinone, naptherpin, marinone
17	Synthetic analogues of cyclic lipoheptapetide	Azasurfactin, 3-epi-azasurfactin

2.1.1 BACTERIOCINS

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by one bacterium that are active against other bacteria and for which the producer has a specific mechanism of immunity. They differ from the traditional antibiotics in their biosynthetic pathway and activity spectrum. While bacteriocins are ribosomally synthesized, antibiotic synthesis is catalyzed by large multi-enzyme complexes. Most antibiotics have a broad spectrum of antibacterial activity. Bacteriocins on the other hand are mainly targeted at a narrow spectrum of bacteria often within the same species as the producing strain or closely related ones, although broad spectrum bacteriocins also exist. Another major difference is their potency against susceptible bacteria. Bacteriocins are able to kill other bacteria at nanomolar concentrations as compared to antibiotics that are needed in much higher concentrations to be effective. Moreover, bacteriocins have been demonstrated to have a low toxicity towards eukaryotic cells. Since they display a limited spectrum of activity, their use can limit collateral damage to the gut microbiota. The production of antimicrobial activity can be exploited in situ by use of these bacteria as probiotic microorganisms. Due to the peptidic nature of bacteriocins, they are amenable to bioengineering in order to ameliorate their effectiveness or to overcome possible toxicity issues. Taken together, all these characteristics suggest that bacteriocins are promising alternatives to conventional antibiotics.[245-248]

Due to the multitude of ongoing research on bacteriocins produced by Gram-positive bacteria classification schemes are constantly updated. Based on the publications of Rea et al. and Cotter et al., an overview is represented in Table 4 with the most important features for each class being displayed. Three major classes can be distinguished: the posttranslationally modified bacteriocins (class I), the unmodified bacteriocins (class II) and the bacteriolysins (class III). Both class I and class II bacteriocins are further divided in several subclasses.[247, 248] Several modes of action of bacteriocins have already been reported including inhibition of gene expression and protein synthesis. However, most bacteriocins have the membrane as a target. Some of them inhibit cell wall synthesis while others cause pore formation leading to membrane permeabilization, leakage of intracellular compounds, dissipation of the transmembrane potential and eventually cell death.[246, 247] Certain bacteriocins even encompass both mechanisms of action. For example, nisin binds lipid II and thereby prevents proper cell wall synthesis. In addition, it uses lipid II as a docking molecule to induce the formation of pores.[249]

CLASS I: POST-TRANSLATIONALLY MODIFIED BACTERIOCINS

Class I bacteriocins contain post-translational modifications. Based on the type of modification, six subclasses can be distinguished with specific features (Table 4). The most thoroughly studied are the

lantibiotics. Lantibiotics are small peptides (< 5 kDa) that contain the unusual amino acids lanthionine, β-methyllanthionine, dehydroalanine or dehydrobutyrine. Lantibiotics are known to have a potent and broad spectrum of activity, a low degree of antibiotic resistance development and a negligible amount of cytotoxicity. Nisin is the best characterized lantibiotic and is widely used as food preservative. Several classification schemes have been proposed to further subdivide the lantibiotics based on their structure, biosynthetic pathway, similarity of propeptides or absence or presence of antimicrobial activity.[248, 250, 251] A second subclass of post-translational bacteriocins are the sactibiotics. These bacteriocins contain intramolecular cross-linkage between sulphur atoms of cysteine residues and α-carbons of certain amino acids. Although some members possess a broad spectrum of activity, certain sactibiotics only have a limited spectrum of activitiy. Thuricin CD is known to selectively target *C. difficile* and is discussed in more detail below.[248, 252] A relatively new subclass are the thiopeptides. They consist of a central pyridine, dihydropyridine or piperidine ring that serves as a scaffold for at least one macrocyclic structure and tail which both contain dehydrated amino acids and azoles. Several thiopeptides are known to encompass antibacterial activity against important bacterial pathogens such as MRSA and VRE. Major drawbacks are however its large molecular size and poor solubility in water which limits their use as therapeutics.[248, 253, 254] Three other classes are also known that only contain a few members. Linaridins are bacteriocins with a linear structure that contain unusual dehydrated amino acids. Bottromycins contain a macrocyclic amine, a decarboxylated carboxy-terminal thiazole and carbon-methylated amino acids. A last small class, the glycocins, contain S-linked glycopeptides.[247]

CLASS II: UNMODIFIED BACTERIOCINS

The class II bacteriocins consist of a heterogenous group of peptides (< 10 kDa) composed of standard amino acids. Four subclasses are distinguished. Members of class IIa, called the pediocin-like bacteriocins, have a narrow spectrum of activity and are all able to inhibit the growth of *Listeria monocytogenes*. Antibacterial activity has been reported against important human pathogens such as *S. aureus* and VRE. Since they only have a limited spectrum of activity, the deleterious effect on the commensal microbiota is minimized which offers a major advantage. All class IIa bacteriocins share a common amino-terminal region (YGNGV), called the pediocin box, which is thought to facilitate nonspecific binding with target cell surfaces.[247, 255] Subclass IIb are the two-peptide bacteriocins that are composed of two separate peptide chains that need to interact to obtain optimal activity. Great variations exist in amino acid sequence and structure of these bacteriocins but a common GxxxG motif is conserved in all peptides. They possess a limited spectrum of activity.[256] Circular bacteriocins are classified in subclass IIc. During their synthesis, the peptide backbone undergoes head-to-tail cyclization. Their circular nature provides enhanced stability. They are heat stable and relatively

resistant to proteolytic digestion. Circular bacteriocins encompass a broad spectrum of activity. All three subclasses function by targeting the cell membrane inducing pore formation and eventually cell death.[257] Subclass IId contains the linear, unmodified, non-pediocin-like bacteriocins. These bacteriocins do not show sequence similarity to the other class II bacteriocins. As such, they show great variations in structure and modes of action.[258]

CLASS III: BACTERIOLYSINS

A final class are the bacteriolysins which are the least well characterized. Bacteriolysins are large, heat unstable bacteriocins. They are made up of different domains, each responsible for receptor binding, translocation and antibacterial activity. The small number of bacteriolysins known target the bacterial cell membrane. Bacteriolysins with narrow and broad spectrum of activity have been reported.[259]

2.1.1.1 BACTERIOCINS FOR THE CONTROL OF CLOSTRIDIUM DIFFICILE

The antibacterial activity of certain bacteriocins against *C. difficile* has already been reported.[260] However, the potential use of bacteriocins as alternative therapeutics for CDI must meet a number of criteria. First of all, it needs to be as least as effective as the currently used antibiotics. Most ideally, it would encompass a narrow spectrum of activity with high specificity for *C. difficile* resulting in restricted collateral damage to the gut microbiota. In addition, the possibility of resistance development in *C. difficile* or other species should be minimal. The bacteriocin itself must be able to survive transit through the gastrointestinal tract. It should be delivered in a stable and functional way at the target site. Finally, the bacteriocin needs to be non-toxic for the host.[261] Several studies have highlighted the potent *in vitro* activity of certain class I bacteriocins against *C. difficile*. These bacteriocins belonged to the subclasses of the lantibiotics, sactibiotics and thiopeptides. No bacteriocins with anti-*C. difficile* activity have yet been reported in the other three subclasses.

CLASS I: POST-TRANSLATIONALLY MODIFIED BACTERIOCINS

Lantibiotics. Different lantibiotics are reported to be at least as effective as metronidazole and vancomycin in killing *C. difficile*. These lantibiotics include nisin and lacticin 3147 produced by *Lactococcus lactis*, actagardine A produced by *Actinoplanes garbadinensis* ATCC31049 and mutacin 1140 produced by *Streptococcus mutans* JH1140.[260, 262-265] Lacticin 3147 was even reported to efficiently kill *C. difficile* in an *in vitro* gut model.[263] Moreover, engineering of nisin and mutacin 1140 has led to the discovery of mutants with elevated antibacterial activity against *C. difficile*.[265] NVB302 is a semi-synthetic derivative of the lantibiotic deoxyactagardine B produced by *Actinoplanes ligurae* which proved to be non-inferior to vancomycin in an *in vitro* human gut model of CDI.[266] Results of a recent phase I clinical trial indicated that high concentrations of NVB302 were recovered in fecal

samples and that it was safe and well-tolerated in healthy test subjects.[267] Unfortunately all of these lantibiotics show a broad spectrum of activity and will have a considerable impact on the resident gut microbiota.

Sactibiotics. A very promising bacteriocin with a narrow spectrum of activity is thuricin CD produced by *B. thuringiensis* DPC 6431. Thuricin CD is a posttranslationally modified bacteriocin that consists of two distinct peptides, Trn-α and Trn-β, that act synergistically to kill *C. difficile*.[268] Its use in an *in vitro* distal colon model indicated that it was equally effective as the standard antibiotics at killing *C. difficile* but had no significant impact on the composition of the microbiota.[269] One of the major problems concerning bioavailability of bacteriocins is their sensitivity to the harsh environmental conditions in the digestive tract.[270-273] Oral administration of unprotected thuricin CD to pigs revealed that no functional thuricin CD could be detected in the porcine intestinal tract. While Trn-α appeared to be resistant to gastric enzymes, Trn-β was degraded by gastric enzymes *in vitro* and *in vivo* resulting in no functional intestinal thuricin CD. In an attempt to deliver functional thuricin CD to the gut, spores of the producing organism *B. thuringiensis* DPC 6431 were fed to mice. Unfortunately, 99% of the spores were excreted and no thuricin CD was detected in the intestinal tract indicating that ingestion of *B. thuringiensis* spores is not a suitable vehicle for delivery of thuricin CD in the gut. However, rectal administration of thuricin CD to mice significantly decreased *C. difficile* shedding. As such, rectal administration of thuricin successfully overcomes stability issues and might be a mode of delivery of functional thuricin CD to the colon.[271]

Thiopeptides. LFF571 is a semi-synthetic derivative of the natural occurring thiopeptide bacteriocin GE2270 A. It displays antimicrobial activity against a range of Gram-positive bacteria by targeting protein synthesis.[274] LFF571 has an excellent *in vitro* activity against *C. difficile*.[275] Moreover, results of a hamster trial indicated that LFF571 was more efficient than vancomycin in a lower dose, with fewer recurrences.[276] Additional *in vivo* studies in hamsters and rats proved that LFF571 was poorly absorbed and high intestinal concentrations were detected.[274] Results of a phase I clinical trial indicated that LFF571 was well tolerated by the healthy test subjects with no serious side effects being reported. Moreover, low serum concentrations and high fecal concentrations were measured.[277] Recently, a phase II clinical trial was conducted evaluating the safety and efficacy of LFF571 in adults with primary CDI or a first recurrent episode. A clinical cure and recurrence rate of 90.6% and 15.4% were reported for LFF571-treated patients respectively. Both rates were better than those reported for vancomycin-treated patients for which a clinical cure rate of 78.3% and recurrence rate of 25.3% was described. These results indicate that LFF571 is non-inferior to vancomycin for the treatment of CDI.[278]

CLASS II: UNMODIFIED BACTERIOCINS

Less research has been done to study the antibacterial effect of class II bacteriocins on *C. difficile*. Only two bacteriocins have been reported to show *in vitro* activity against *C. difficile*, namely CECT7121 produced by *Enterococcus faecalis* CECT7121 and acidocin LCHV produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine. Since these bacteriocins have no modifications, one of the major problems encountered might be their sensitivity to proteolytic digestion in the digestive tract as compared to the posttranslationally modified bacteriocins. To overcome this problem, in situ production of the bacteriocins could be achieved by the use of probiotics. *E. faecalis* CECT7121 is a promising candidate for use as a probiotic with therapeutic significance. Several reports have indicated that this strain is able to induce an adequate immune response in the host.[279-281] More research is however necessary to evaluate the production of functional bacteriocin in the gut and its probiotic efficacy in the prevention or treatment of CDI. *L. acidophilus* is a commonly used probiotic and part of the Bio-K+® probiotic mixture with proven effectiveness against CDI.[282]

R-TYPE BACTERIOCINS

R-type bacteriocins are phage-tail like particles that are produced by some *C. difficile* strains. They are classified as bacteriocins because they comprise antibacterial activity against other *C. difficile* strains. They share genetic relatedness and structural similarities with temperate phages, hence it is believed that they share a common ancestor. Due to their high specificity, they offer a promising alternative approach to selectively kill *C. difficile* without a detrimental effect on the resident gut microbiota or the fear of developing antibiotic resistance. R-type bacteriocins kill target bacteria by attaching to a bacterial receptor followed by perforation of the cell membrane causing rapid membrane depolarization and instant cell death. Recognition of target bacteria is mediated by the receptor binding protein (RBP) from the bacteriocin which determines the killing spectrum. AvidBiotics created genetically modified R-type bacteriocins by changing the RBP and called them Avidocin™ proteins. Results of an *in vivo* mouse trial indicated that the tested Avidocin™ survived passage through the mouse gastrointestinal tract, did not alter the gut microbiota and prevented colonization of *C. difficile* NAP1/BI/027 spores which makes it worthwhile to further investigate its potential prophylactic and therapeutic use.[283-285]

Noteworthy, synergistic effects of bacteriocins and conventional antibiotics have been reported. Authors hypothesize that initial bacteriocin-induced cell wall damage make the target bacteria more accessible for antibiotic uptake increasing their antibacterial effect.[286]

Table 4. Classification of bacteriocins produced by Gram positive bacteria based on the classification by Rea et al. and Cotter et al.[247, 248] The underlined bacteriocins show potent *in vitro* activity against *C. difficile*. Bacteriocins highlighted in bold have reported activity against *C. perfringens*. *Acidocin LCHV and CECT7121 are reported to belong to the class II bacteriocins but are not yet assigned to a subclass.

Class	Subclass	Distinctive features	Examples
I	Lantibiotics	Unusual amino acids: lanthionine, β-methyellanthionine, dehydroalanine, dehydrobutyrine	**Nisin**, actagardine, lacticin 3147, mutacin 1140, NVB302, **ruminococcin A/C**
	Sactibiotics	Intramolecular cross-linkage between sulphurs of cysteine residues and α-carbons of certain amino acids	Thuricin CD
	Thiopeptides	Central pyridine, dihydropiridine or piperidine ring and heterocycles	GE2270A, LFF571
	Linaridins	Unusual dehydro amino acids	Cypemicin
	Bottromycins	Macrocyclic amidine, decarboxylated carboxy-terminal thiazole and carbon-methylated amino acids	Bottromycin A2
	Glycocins	S-linked glycopeptides	Sublancin 168
II	IIa: pediocin-like	Conserved N-terminal region (pediocin box), anti-*Listeria* activity, narrow spectrum of activity	**Pediocin A**, **divercin**
	IIb: two-peptide	Two peptides that need to interact to form an active complex, common GxxxG motif in all peptides	Plantaricin S, lactococcin
	IIc: circular	Covalent linkage between N- and C-terminal of the peptide resulting in a circular backbone	Gramicidin S
	IId: linear, non-pediocin-like	Heterogeneous group of bacteriocins that do not belong to any of the other classes	Acidocin LCHV, CECT7121*
III	Bacteriolysins	Large, heat-labile bacteriocins with different domains for translocation, receptor binding and lethality	Helveticin J, Zoocin A

2.1.1.2 BACTERIOCINS FOR THE CONTROL OF CLOSTRIDIUM PERFRINGENS

Bacteriocins represent a promising alternative to conventional antibiotics. Little is known about the use of bacteriocins as feed additives for poultry although more and more research is being done nowadays.[287] Intestinal colonization of *Campylobacter* sp. in poultry was successfully controlled by dietary addition of certain class II bacteriocins.[287-289] Few *in vivo* trials have been conducted to test the efficacy of bacteriocin administration on NE in broilers.

CLASS I: POST-TRANSLATIONALLY MODIFIED BACTERIOCINS

Lantibiotics. Several bacteriocins have already been identified with potent *in vitro* inhibitory activity against *C. perfringens*. The well characterized nisin produced by *Lactococcus lactis* was shown to inhibit the outgrowth of spores and vegetative cells.[290] Inhibitory activity of two lantibiotics, ruminococcin A produced by *Ruminococcus gnavus* E1 and a lantibiotic produced by *Bifidobacterium longum* DJO10A, against several pathogenic clostridia, including *C. perfringens* and *C. difficile*, has been reported.[291, 292] Inoculation of rats with *R. gnavus* E1 revealed that ruminococcin A was only poorly expressed in the gastrointestinal tract. However, researchers identified another lantibiotic, ruminococcin C, which was active against *C. perfringens* and expressed *in vivo*. A recent report describes the beneficial effect of sublancin, a class I bacteriocin produced by *B. subtilis* strain 168, on NE in broilers.[293]

CLASS II: UNMODIFIED BACTERIOCINS

In-feed supplementation of pediocin A, a class IIa bacteriocin produced by *Pediococcus pentosaceus* FBB61, improved the growth performance of broilers challenged with *C. perfringens*.[294] Addition of lyophilized divercin produced by *Carnobacterium divergens*, to the feed was associated with improved broiler performance and maintenance of the histomorphology of the gastrointestinal tract.[295]

BACTERIOCIN LIKE INHIBITORY SUBSTANCES

Several bacteriocin-producing bacteria have been isolated from the gastrointestinal tract of healthy animals. The inhibitory activity towards *C. perfringens* and the proteinaceous nature of these compounds has been confirmed *in vitro*.[296-298] Bacterial metabolites with antimicrobial activities similar to those of bacteriocins that have not yet been purified, identified and characterized are temporarily classified as bacteriocin-like inhibitory substances (BLIS). Recently, *Brevibacillus borstelensis* AG1 was associated with the production of a thermostable BLIS of 12 kDa with potent activity against *C. perfringens*.[299]

2.1.2 NONRIBOSOMALLY SYNTHESIZED LIPOPEPTIDES

Lipopeptides are low molecular weight amphiphilic molecules composed of a lipid tail linked to a short linear or cyclic peptide and produced by a wide variety of bacteria, yeasts and fungi. Their synthesis is catalyzed by nonribosomal peptides that contain 3 distinct domains: an adenylation, condensation and thioesterase domain. Each domain is involved in the stepwise incorporation of an amino acid in the peptide backbone. First, amino acids and peptides are activated by adenylation and covalently linked to the peptidyl carrier protein. Next, a peptide bond is formed between two amino acids. The peptide is finally released after thioester hydrolysis which is mostly accompanied with cyclization. Several enzymes catalyze the attachment of the fatty acid chain to the N-terminal part of the peptide. A high structural diversity among the lipopeptides is observed due to variations in length, configuration, number and composition of lipids and amino acids. Most lipopeptides are directed against the membrane of target cells. After initial binding, oligomerization induces the formation of pores causing membrane damage and eventually cell death. Lipopeptides possess an extensive range of properties including emulsifying, foaming, solubilizing and biosurfactant capacities. Moreover, they display antibacterial, antiviral, antifungal, hemolytic, antitumor and insecticidal activities which make them promising therapeutic alternatives.[300-302] Some lipopeptides are already registered as commercial antibiotics. Daptomycin is the first FDA approved lipopeptide antibiotic used for the treatment of systemic and life threatening infections caused by Gram-positive bacteria.[303]

Lipopeptides from *Bacillus* species are of the most intensively studied. Three different classes of cyclic lipopeptides can be distinguished: surfactins, fengycins and iturins. Iturins are composed of a peptide backbone of 7 α-amino acids linked to a β-amino fatty acid (C14-C17). All iturins share the common sequence β-amino fatty acid – Asx – Tyr – Asx with variable amino acids at the other positions. Iturin, bacillomycin, bacillopeptin and mycosubtilin belong to the iturin family. The peptide moiety in fengycins is made up of 10 α-amino acids and is linked to a β-hydroxy fatty acid (C14-C18). Lipopeptides of the fengycin family are the only ones that contain unusual amino acids such as ornithine and allo-threonine. Variations in the peptide backbone allow us to distinguish fengycin and plipastatin. Both iturins and fengycins are known for their strong antifungal activities. Surfactins consist of 7 α-amino acids linked to a β-hydroxy fatty acid with a variable chain length of 12 to 16 carbon atoms. Surfactins are very powerful biosurfactants and have demonstrated antibacterial capacities. Among the surfactin family, surfactin, lichenysin, esperin and pumilacidin can be distinguished based on diversity of the peptide sequence.[300]

Surfactin is the most extensively studied and the major representative of all lipopeptides. Several *Bacillus* species are able to produce surfactin, including *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* and *B. licheniformis*.[304-307] It is a negatively charged molecule due to the presence of amino acids glutamate and aspartate on positions 1 and 5 in the heptapeptide. The other positions are occupied by hydrophobic amino acids (Figure 6). Surfactin variants with differences in the composition of the amino acid sequence and length of the fatty acid chain have already been isolated. At positions 2, 4 and 7, a valine, leucine or isoleucine can occur.[304, 308, 309] A surfactin variant with an alanine at position 4 has also been reported.[310] These changes are associated with altered culture conditions and can affect the activity of surfactin. For example, replacement of valine at position 4 with leucine or isoleucine renders surfactin variants with higher affinity for hydrophobic solvents and doubles their surfactant activity.[304] Moreover, shorter fatty acid chains are associated with better foaming properties while longer fatty acid chains increase the hemolytic activity.[311, 312] Usually a mixture of surfactin isoforms is produced by the bacterium with differences in peptide sequence and fatty acid chain length.[304, 313-315]

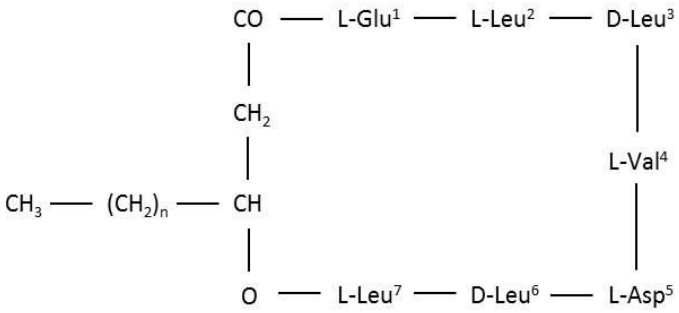

Figure 6. Primary structure of surfactin (n = 9 – 11)

Surfactin has a compact structure due to its cyclization. The peptide backbone folds back together and adopts the 3D conformation of a stable β-sheet. On one side of the molecule, amino acids 2 and 6 face each other in close vicinity of the acidic amino acids Glu-1 and Asp-5 which constitute a minor polar domain. On the opposite side, a major hydrophobic domain is formed in which residue 4 faces the connection of the fatty acid chain. The side chains of residue 3 and 7 contribute to a lesser extent to the hydrophobicity of this major domain (Figure 7). Below the critical micelle concentration, the fatty acid chain adopts an extended conformation freely in solution. Above this concentration, supramolecular structures like micelles are formed due to hydrophobic interactions between the fatty acid chains.[316, 317] The two acidic groups form a well suited claw which can bind divalent cations. Ca^{2+} is known to stabilize the surfactin conformation, decrease the critical micellar concentration and thus facilitate micelle formation.[316, 318, 319]

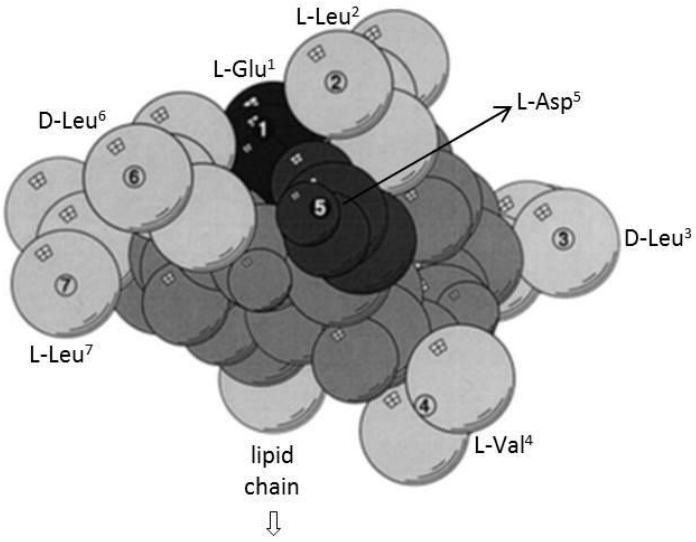

Figure 7. 3D structure of surfactin peptide sequence. The amino acids found in standard surfactin are represented on their appropriate position. Hydrophobic amino acids in positions 2, 3, 4, 6 and 7 and the attachment site of the fatty acid chain are represented in pale grey. Acidic amino acids 1 and 5 are in black and dark grey respectively. Peptide backbone atoms are in grey.

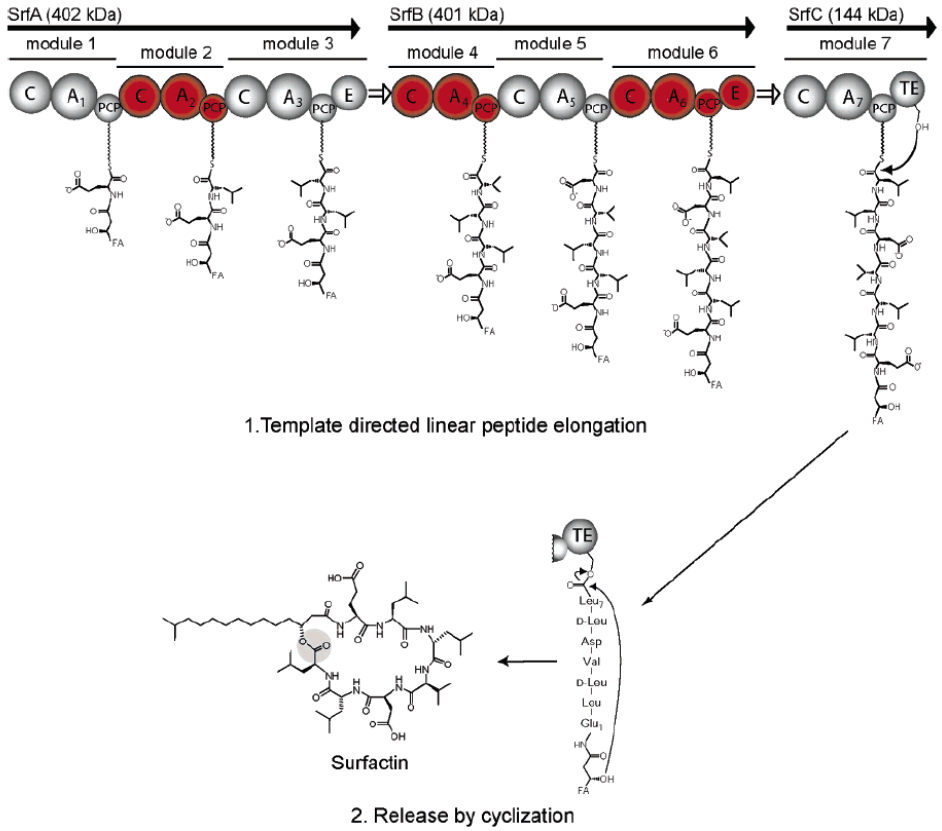

Figure 8. Surfactin biosynthesis. The surfactin synthetase complex consists of seven modules (grey and red) responsible for the specific incorporation of seven amino acids. These modules are further subdivided in 24 catalytic domains encompassing 1 of the following functions: adenlyation (A), protein carrier peptide (PCP), condensation (C), epimerization (E) and thioesterase (TE) activity. In a final step, peptide release is catalyzed by the TE domain and accompanied by cyclization.[320]

The surfactin synthetase complex contains four enzymatic subunits with a modular organization. Seven modules can be distinguished that are each responsible for incorporation of an amino acid which are further organized in 24 catalytic domains responsible for substrate recognition, activation, binding, modification, elongation and release of the lipopeptide (Figure 8). Surfactin synthesis is initiated by activation of the relevant amino acids in a two-step reaction. After initial binding, the adenylation domain A catalyzes the formation of an aminoacyl adenylate intermediate. This molecule is converted to a thioester by transfer to the peptidyl carrier protein (PCP) also known as the thiolation domain T. PCP provides a way of transport of the substrates and elongation intermediates to the different catalytic domains. Elongation of the peptide occurs by the formation of peptide bonds between the amino acid building blocks bound to the PCPs and is catalyzed by the condensation domain C. Release of the final product is catalyzed by the C-terminal thioesterase domain TE and is accompanied by macrocyclization. The surfactin synthetase complex also contains additional editing domains. Module 3 and module 6 encompass an epimerization domain responsible for modification of L-amino acids to D-amino acids. As a consequence, the heptapeptide in surfactin has the chiral sequence LLDLLDL. *In vitro* studies indicate that modules 2, 4 and 7 are able to recognize and accept several aliphatic amino acids rendering surfactin variants with differences in peptide sequence as discussed earlier.[320, 321]

The genes required for surfactin biosynthesis are organized in a 25kb operon srfA which is also involved in sporulation. Four open reading frames can be distinguished srfA-A, srfA-B, srfA-C and srfA-D, which corresponds to the four enzymatic subunits. SrfA-A, srfA-B and srfA-C are involved in adenylation, condensation and elongation of the growing peptide sequence. At the end of *srfA-C*, a thioesterase domain is encoded responsible for release and cyclisation of the peptide. The presence of these three subunits is sufficient for surfactin synthesis. However, the presence of a fourth subunit, SrfA-D, encoding a thioesterase/acyltransferase enzyme, is associated with enhanced surfactin production. SrfA-D stimulates the formation of initiation products and is involved in transfer of the fatty acid chain to SrfA-A inducing formation of β-hydroxyacyl-glutamate.[316, 320-322]

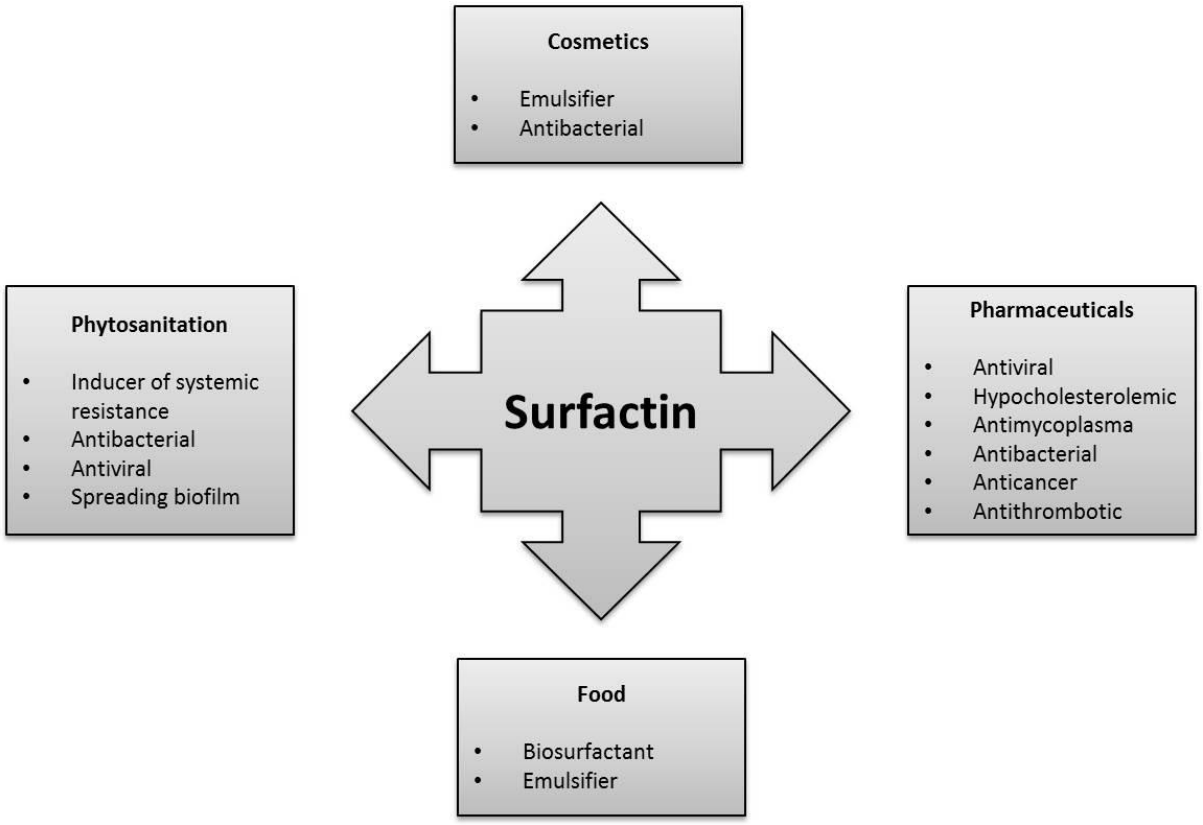

Figure 9. Scope of surfactin usage.[302]

Surfactin is a versatile molecule which acts as a powerful biosurfactant with exceptional emulsifying and foaming properties. It displays a wide array of interesting biological activities including antitumor, antibacterial, antiviral and hemolytic activities.[323-328] An overview of all its potential applications is presented in Figure 9.[302] Its mode of action is a direct consequence of the interaction with the target membrane and the alteration of the membrane properties. As an amphiphilic molecule, it can tightly associate with lipid layers and induce membrane perturbation and pore formation. Using artificial membranes, it was demonstrated that the degree of membrane damage is highly dependent on the surfactin concentration. Dimerization of surfactin molecules in the lipid bilayers appears to be an essential step for membrane destabilization and leakage. At low concentrations, surfactin inserts into the outer leaflet of the membrane inducing limited perturbation. It is miscible with the phospholipids and forms mixed micelles. With increasing concentrations, temporary membrane permeabilisation is achieved but membranes are able to recover. Further rise in surfactin concentration induces the development of surfactin-rich domains in the membrane eventually leading to the formation of pores. The presence of high surfactin concentrations leads to the complete disruption of the membrane with the formation of mixed micelles. Surfactins membrane interaction is enhanced by the presence of cations which conceal the

negative charge of the Glu-1 and Asp-5 amino acids making a deeper insertion of the lipopeptide into the membrane possible.[321, 329, 330]

2.1.2.1 LIPOPEPTIDES FOR THE CONTROL OF CLOSTRIDIUM DIFFICILE

CB-183,315 or surotomycin is a cyclic lipopeptide structurally related to daptomycin with significant activity against Gram-positive bacteria and limited activity against Gram-negative bacteria. It is the only lipopeptide known with potent inhibitory activity against *C. difficile*. *In vitro* studies showed that surotomycin has MIC values ≤ 1 µg/ml against *C. difficile* and is more potent than vancomycin and metronidazole.[331, 332] Results of a phase II clinical trial indicated that CB-183,315 was safe and well tolerated. Moreover, a high clinical cure rate and a statistically significant reduction in recurrence rate was reported for CB-183,315 as compared to vancomycin.[333] A phase III clinical trial has just been completed but no results have yet been reported.

2.1.2.2 LIPOPEPTIDES FOR THE CONTROL OF CLOSTRIDIUM PERFRINGENS

As for *C. difficile*, surotomycin is the only lipopeptide with reported antibacterial activity against *C. perfringens*. Research, however, focusses on its use against CDAD and no information is available yet for a possible use against *C. perfringens* associated diseases.[334]

2.1.3 LIMITATIONS OF BACTERIOCINS AND LIPOPEPTIDES AS THERAPEUTICS

Bacteriocins and lipopeptides are very promising alternatives to antibiotics, but do have some major drawbacks. First of all, purification of these compounds is not straightforward and yields are generally low which makes their production costs high.[297, 298] It has therefore been postulated that it might be more efficient to supply probiotic bacteria that produce the bacteriocins in situ.[287] Although these antimicrobial peptides are amenable to bioengineering, it cannot be guaranteed that the engineered molecules have the same activities as the natural peptides. Many antimicrobial peptides are reported to be non-toxic for the host but some of them might be indeed toxic for eukaryotic cells due to their hemolytic activity. The activity spectrum of the peptides can be narrow but broad spectrum activity has also been reported which has an adverse effect on the composition of the normal microbiota. This feature is however advantageous when bacteriocins are used as e.g. food preservatives.[335] Moreover, there is still the possibility that bacteria develop resistance against these antimicrobial peptides. To date, bacteriocins have not been extensively used as therapeutics. Hence, little is known about resistance development. Although nisin has long been used as food preservative, no resistance of food spoilage bacteria has yet been reported in the food industry. Our knowledge on bacteriocin resistance development relies solely on *in vitro* experiments.[336] The effect of antimicrobial peptides on the body is largely unknown since *in vivo* studies about

pharmacodynamics, pharmacokinetics, and stability of these molecules are rare. Stability issues, toxicity, immune responses and other issues still need to be studied thoroughly.[337] Finally, the possibility exists that the compounds are not able to survive the harsh conditions in the gastrointestinal tract such as the acidity of the stomach or proteolytic digestion.[337]

2.2 PROBIOTICS

The intestinal microbiota has an important role in maintaining the hosts health and wellbeing. It fulfills several important functions including the digestion of carbohydrates that otherwise remain unavailable for host cells, mucosal cell development, vitamin production, intestinal transit regulation, immune system stimulation and colonization resistance. Colonization resistance is the first line of defense against harmful invasion by microbial pathogens. Suppression of microbial pathogen growth by colonization resistance includes the physical obstruction of attachment sites, competition for essential nutrients, production of toxic metabolic end products, production of antimicrobial compounds and immune system stimulation.[225, 338, 339] Disturbances of the complex homeostasis of the gut microbiota leads to a vast range of diseases from the obvious inflammatory bowel diseases such as Crohn's disease to the activation of chronic human immunodeficiency virus infection, cancer and even allergies.[340-343]

2.2.1 PROBIOTICS FOR THE CONTROL OF CLOSTRIDIUM DIFFICILE

One of the most common causes of imbalance of the gut microbiota is antibiotic consumption. Up to 25% of patients receiving antibiotics develop antibiotic associated diarrhea (AAD) with *C. difficile* as the most important cause.[344, 345]

Probiotics are defined as live microorganisms that confer a health benefit to the host when consumed in appropriate amounts.[225] The principle of using harmless bacteria for the protection against pathogens has been known for many years. Already in 1907, Metchnikoff postulated that human health could be improved by manipulation of the gut microbiota due to the consumption of healthy bacteria from yoghurt.[346] One of the most widely recognized uses for probiotics refers to the gastrointestinal tract e.g. in the control of travelers' diarrhea, inflammatory bowel disease and irritable bowel syndrome.[347] Several studies indicate the beneficial effect of probiotics in reducing the risk of AAD. The most conclusive results have been found for the single strain probiotics *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* and indicate that both strains are able to significantly reduce the risk for AAD.[348-354]

Since *C. difficile* is the most common cause of AAD, probiotics might offer an attractive alternative to standard antibiotic therapy. Probiotics can protect the host against invading pathogens in different

ways: they can support the restoration of the normal gut microbiota by repopulation of the gut with non-pathogenic flora, improve the gut mucosal barrier function, positively affect the hosts immune system and provide direct protection against harmful infections by the production of antimicrobial compounds that act against these invading pathogens.[355] A major advantage of probiotics is their relatively low production cost and the unlikeliness of increasing the incidence of antibiotic resistance.[225]

Many recent reviews and meta-analyses postulate the positive effect of probiotic administration on the primary prevention of CDAD. A review by Johnston et al. (20 trials) showed a 66% reduction in the incidence of CDAD and a meta-analysis by McFarland et al. (6 trials) indicated a 41% reduction in CDAD due to probiotic consumption.[356, 357] Avadhani and Miley (8 trials) even reported a CDAD reduction rate of 71%.[358] A systematic review and meta-analysis by Johnson et al. (11 trials) also suggests that primary prevention of CDAD with probiotics might be achievable.[359] A recent Cochrane analysis indicates the safe and effective use of probiotics in the prevention of CDAD.[360] There is, however, still no consensus reached due to the diversity of probiotic strains used, small sample sizes, low overall CDI rates and methodological flaws in study design of the clinical trials.[361] The observed results are, however, promising but require larger, well defined studies to be able to draw definite conclusions. A recent Delphi study involving the expert opinion of 8 specialists in the field does however recommend the use of specific probiotics to prevent *C. difficile* overgrowth.[362] The microorganisms that are most commonly studied are the yeast *Saccharomyces boulardii* and bacteria of the genus *Lactobacillus*.

YEASTS

Saccharomyces boulardii is a non-pathogenic yeast which is one of the most commonly used probiotics. Several studies have been performed evaluating the effect of *S. boulardii* administration on CDI. Supplementary administration of *S. boulardii* reduced the risk of CDI in children receiving antibiotic therapy but not in adults.[348, 363, 364] Although *S. boulardii* is not effective in preventing a primary episode of CDI, several studies indicate that combined use of standard vancomycin therapy and *S. boulardii* is efficient in preventing recurrent CDI.[61, 365, 366] Although the exact mechanism behind the protection against CDI is yet unknown, several possibilities have been suggested. *S. boulardii* produces a 54 kDa serine protease that not only degrades *C. difficile* toxins A and B but also inactivates the toxin A receptor on intestinal epithelial cells.[367] Moreover, it stimulates the hosts immune system and enhances the total intestinal IgA response as well as the specific anti-toxin A IgA response.[368] Evidence exists that *S. boulardii* also exerts its anti-inflammatory activity by modulation of the hosts MAP kinase signaling pathways.[369] Tasteyre et al. showed that *S. boulardii* is able to

inhibit *in vitro* cell adherence of *C. difficile* indicating a competition for attachment sites between both microorganisms.[370]

LACTIC ACID BACTERIA

Members of the genus *Lactobacillus* have a long record of safety and have already been used for many years in fermented foods such as yogurt, soy products and kefir.[371, 372] Several studies have investigated the efficacy of *Lactobacillus* species in the primary prevention of CDI. A probiotic drink containing *L. casei* SHIROTA (Yakult) was able to decrease the rate of CDI. Six percent of the patients in the untreated control group developed CDI as compared to none of the patients in the treatment group.[373] Hickson et al. proved that a mixture of *L. casei*, *L. bulgaricus*, and *Streptococcus thermophilus* reduced the incidence of CDI from 17% in the control group to 0% in the probiotic treatment group.[374] A four strain probiotic mixture of *L. acidophilus* NCFM, *L. paracasei* Lpc-37, *Bifidobacterium lactis* Bi-07 and *B. lactis* Bl-04 lowered the risk of CDAD in a dose-dependent manner.[375] Plummer et al. showed that administration of *L. acidophilus* and *B. bifidum* significantly reduced the fecal excretion of *C. difficile* toxins as compared to untreated patients indicating a possible mode of action for the probiotic, i.e. toxin neutralization rather than prevention of colonization.[376] *L. plantarum* 299v was the only *Lactobacillus* based probiotic studied for the secondary prevention of CDI. Although the trial was stopped early due to a low number of study subjects, it gave evidence for a reduction in recurrence rate since administration of the probiotic in conjunction with standard metronidazole therapy rendered a recurrence rate of 36% as compared to 67% in the control group receiving only metronidazole.[377] *L. plantarum* 299v was also shown to reduce the colonization level of *C. difficile* in critically ill patients.[378]

The Federal Department of Public Health in Canada has recently approved the use of Bio-K+® (Bio-K Plus International Inc., Laval, Quebec, Canada) for the prevention of antibiotic associated diarrhea due to *C. difficile*. Bio-K+® is a commercially available probiotic containing *L. acidophilus* CL1285®, *L. casei* LBC80R® and *L. rhamnosus* CLR2®. Initial studies by Beausoleil et al. and Sampalis et al. both indicated that the daily administration of Bio-K+® was safe and well tolerated. Moreover, they both concluded that the treatment was effective in the prevention of AAD in hospitalized patients. Unfortunately, no definite conclusions on the effect of CDAD could be made due to the small sample sizes and low overall CDI incidence.[379, 380] Gao et al. demonstrated that Bio-K+® was effective in reducing CDAD in hospitalized patients on antibiotics in a dose-dependent way. Administration of the probiotic formula once or twice daily significantly reduced the CDI incidence to 9.4% and 1.2% respectively as compared to 23.8% for the placebo group.[381] Recently, Maziade et al. reported the results of an 10-year study evaluating the impact of Bio-K+® on CDAD in addition to existing preventive measures in a Canadian hospital. In 2003, this hospital suffered from a *C. difficile*

NAP1/027/BI outbreak which they were unable to control by standard preventive measures. It was decided to administer the probiotic Bio-K+® formula to all adult in-patients on antibiotics within 12 hours of antibiotic prescription. In the first 2 years of the study, they proved that Bio-K+® resulted in a reduction of 73% for all CDI cases, 76.4% for severe CDI cases and 39% for recurrent CDI. Moreover, during the following 8 years of follow up, primary CDI incidences decreased from 18.0 cases per 10 000 patient days to 2.3 cases per 10 000 patient days and remained at such low level. Moreover, this average CDI rate was consistently lower than that observed in similar other hospitals in Quebec.[282, 382]

Several studies fail to prove a positive effect of probiotic administration on the prevention of CDAD. Different reasons can explain this heterogeneity in effectiveness including the quantity of probiotic administered, the patient population studied and the distribution of risk factors among the patient population.[282] It is important to keep in mind that the effect of the probiotic bacteria under investigation are strain specific. This means that the observed results from one study only relate to that strain and cannot be extrapolated to other strains. For example, *L. rhamnosus* GG has a beneficial effect on CDAD but other *L. rhamnosus* strains might not. The reason for this observation is that individual strains have certain specific characteristics such as gastric acid and bile resistance, ability to colonize the mucosa and the potential to produce specific antimicrobial compounds.[282, 351, 383, 384]

NONTOXIGENIC CLOSTRIDIUM DIFFICILE

Nontoxigenic *C. difficile* isolates lack the toxin A and B genes. As a consequence, they do not produce active toxins and are unable to induce disease.[385] Already in 1983, researchers discovered that colonization of hamsters with nontoxigenic strains of *C. difficile* before administration of a toxigenic strain suppressed the cecal colonization of this toxigenic strain and improved survival rates.[386] Treatment of two patients with recurrent disease with oral nontoxigenic *C. difficile* proved to be successful in resolving relapsing CDI.[387] Previous studies indicate that asymptomatic colonization with *C. difficile* correlates with a decreased risk of CDAD.[388] Moreover, acquisition of a toxigenic *C. difficile* strain mounted an adequate serum IgG antibody response against toxin A to prevent CDAD.[389] Based on these findings, Sambol et al. postulated that colonization with a nontoxigenic *C. difficile* strain is harmless for the host and will possibly provide protection against colonization with toxigenic *C. difficile* strains that induce CDAD. Results of their hamster trial proved that prior colonization with nontoxigenic CD strains is highly effective in preventing CDAD after subsequent challenge with toxigenic CD strains.[390] Merrigan et al. proved that the nontoxigenic strain *C. difficile* VP20621 successfully protected hamsters against challenge with toxigenic strains.[391] A recent study evaluated the safety of a VP20621 spore suspension in healthy adults and proved that VP20621 was well

tolerated and able to colonize the gastrointestinal tract.[392] Moreover, results of a recent phase II clinical trial showed that VP20621 significantly reduced CDI recurrence.[393] Taken together, these findings support the possible use of nontoxigenic *C. difficile* strains as probiotics to prevent primary or recurrent CDI.

BACILLUS BASED PROBIOTICS

Bacillus species have already been used as probiotics for human as well as veterinary use for a long time.[394] One of the major assets of *Bacillus* species is their ability to easily produce spores. These spores are very stable which is beneficial for the products shelf life and enables them to survive the harsh conditions of the gastrointestinal tract.[395, 396] Several studies show that the number of excreted fecal spores exceeds the number of orally dosed spores which supports the hypothesis that *Bacillus* spores are able to germinate, grow, proliferate and re-sporulate in the intestinal tract.[397-400] More conclusive evidence is found using a reverse-transcriptase PCR assay measuring the expression of vegetative or sporulation specific genes in the mouse gut indicating significant levels of germination and sporulation.[397, 399]

Orally ingested *Bacillus* probiotics can exert their beneficial probiotic effect through a combination of different mechanisms. Competitive exclusion of bacterial pathogens by *Bacillus* spores has been specifically demonstrated as a mode of action in aquaculture for the fish pathogen *Aeromonas hydrophila* and in poultry for *Salmonella* Enteritidis, *Clostridium perfringens* and *Escherichia coli*.[401, 402] Moreover, several studies indicate that orally administered bacteria are able to efficiently induce an immune response in the host.[403, 404] Duc et al. evidenced that *B. cereus*, *B. clausii* and *B. pumilus* probiotics generated systemic IgG antispore responses and elicited cytokine responses in the gut associated lymphoid tissue.[405] As described above, *Bacillus* species are known as excellent producers of antimicrobial compounds which can also contribute to their probiotic effectiveness. For example, the product Enterogermina® (Sanofi Winthrop, Milan, Italy) has been marketed in Italy for more than 30 years. The probiotic contains spores of four antibiotic resistant *Bacillus clausii* strains and its use is recommended to cure and prevent intestinal microbiota disorders during antibiotic treatment. The probiotic provides protection by stimulation of IgA secretion and the production of AMPs during sporulation. Since antibiotics are ineffective against spores, it is suitable to use in conjunction with antibiotic therapy.[406-410]

Different *Bacillus* probiotics have been successfully used to reduce the rate of AAD. *B. clausii* has been reported to reduce the rate of diarrhea due to anti-*Helicobacter pylori* therapy.[411] Spielholz observed lower AAD and CDAD incidences after administration of a symbiotic tablet containing *S. boulardii* and *B. coagulans*.[412] A recent report showed that administration of a single strain

probiotic containing *B. subtilis* 3 or a two strain probiotic containing *B. subtilis* 3 and *B. licheniformis* 31 during antibiotic therapy decreased the incidence of AAD. Moreover, both probiotics were well tolerated by the patients without side effects.[413] A Chinese study successfully used *B. licheniformis* to reduce the rate of AAD in the elderly.[414]

Although few studies have focused on the anti-*Clostridium difficile* activity of *Bacillus* strains, results are promising. Urdaci et al. showed that *B. claussii* strains are able to produce antimicrobial substances that inhibit *C. difficile in vitro*.[415] According to a recently published patent, the growth and activity of *Clostridium* pathogens including *C. difficile* is successfully inhibited by a novel *B. licheniformis* strain.[416] Several *in vivo* studies have been conducted to investigate the potential use of *Bacillus* strains as probiotics to prevent CDAD. Fitzpatrick et al. showed that administration of *B. coagulans* GBI-30 (BC30) prolonged survival and significantly improved stool consistency of *C. difficile* infected mice.[417] Moreover, an additional study indicated that BC30 limited the recurrence after vancomycin withdrawal and significantly attenuated histological and biochemical markers of infectious colitis in mice.[418] Colenut and Cutting evaluated the use of *B. subtilis* PXN21 spores as potential treatment against CDI using a murine model. Not only did they find an improvement in the symptoms of CDI, results of their *in vitro* work suggest a stimulation of the innate immunity with an upregulation of the TLR2 receptor and an induction of the pro-inflammatory cytokines IL-6 and TNF-α. Moreover, they provide indirect evidence that the germination of live spores to vegetative cells is essential in the suppression of CDI.[419]

2.2.2 PROBIOTICS FOR THE CONTROL OF CLOSTRIDIUM PERFRINGENS

In recent years, the use of probiotics as alternatives to antibiotic growth promoters has gained more and more attention[242]. Much research has been done concerning the use of probiotics to control NE in broilers. Several microbial feed additives are already commercially available.[243]

YEASTS

Although yeast cells are known to have good probiotic properties such as immunostimulatory and antimicrobial activities, research on the use of yeasts to control NE is limited. Yeast extracts have been successfully used to improve broiler performance and intestinal health.[420] NuPro® and Actigen™, produced by Alltech Inc., and SafMannan®, produced by Phileo Lesaffre Animal Care, are three commercially available yeast based probiotics with reported beneficial effects on the detrimental effects of NE.[221, 421, 422]

LACTIC ACID BACTERIA

Several reports describe the antibacterial activity of lactic acid bacteria against *C. perfringens*. For example, a *L. reuteri* and *L. amylovorus* strain isolated from the porcine gastrointestinal tract exhibited *in vitro* antagonistic effects against *C. perfringens*.[423] A commercially available probiotic containing *L. plantarum* also inhibited the growth of *C. perfringens in vitro*.[424] Gérard et al. administered a commercial *Lactobacillus* probiotic in the drinking water to young broiler chickens and observed a decrease in the amount of *C. perfringens* in the ceca. Moreover, the probiotic did not affect the composition of the commensal microbiota.[425] A recent study tested the efficacy of FloraMax® B-11, a probiotic mixture of several lactic acid bacteria and inactivated *S. cerevisiae*, against NE in broilers. Probiotic administration had a beneficial effect on body weight and a remarkable reduction in mortality and intestinal lesion scores were observed when compared with the untreated control group. Moreover, this probiotic controlled and reduced NE associated mortality in a field outbreak.[242, 243, 426]

BACILLUS BASED PROBIOTICS

Bacillus species are widely used probiotics and known to be excellent producers of antimicrobial compounds.[394] Bacteriocins produced by *B. cereus* 8A and *B. subtilis* with anti-*C. perfringens* activity have already been reported.[296, 427] Several *Bacillus* species (*B. licheniformis*, *B. pumilus*, *B. subtilis*) isolated from the chicken gastrointestinal tract have demonstrated *in vitro* inhibitory activity against *C. perfringens*.[297, 395] NE has been successfully controlled by the administration of *Bacillus* strains to broilers. *B. subtilis* PB6 has demonstrated *in vitro* anti-*C. perfringens* activity and was used as feed additive to control NE in broilers. Results indicated that supplementation of *B. subtilis* PB6 improved broiler gut health and significantly reduced NE associated intestinal damage.[428] In addition, beneficial effects on broiler performance and the control of NE associated disease have also been reported for in-feed supplementation of *B. subtilis* QST 713 spores and *B. licheniformis*.[429, 430]

BACILLUS AMYLOLIQUEFACIENS

1 THE ORGANISM

Bacillus amyloliquefaciens is a Gram-positive, rod-shaped, spore-forming micro-organism commonly found in the soil. The species *B. amyloliquefaciens* belongs to the genus *Bacillus*. It is further classified in the family Bacillaceae, the order Bacillales and finally the phylum Firmicutes. The bacterium gains its motility due to the presence of peritrichous flagella.[431] It is a member of the *Bacillus* subtilis group that contains 6 closely related species: *B. subtilis*, *B. licheniformis*, *B. vallismortis*, *B. velezensis*, *B. mojavensis* and *B. amyloliquefaciens*. Since their 16S rRNA sequences show more than 99% similarity, distinction at species level relies on the sequencing of other house hold genes such as *gyrA* or *gyrB*.[432] *B. amyloliquefaciens* is used at industrial level as an important source of α-amylase, protease and BamHI restriction enzyme.

2 A PROMISING PROBIOTIC

The EFSA has placed *B. amyloliquefaciens* on the Qualified Presumption of Safety (QPS) list based on the absence of toxigenic activity and acquired antibiotic resistance genes.[433] *B. amyloliquefaciens* possesses several interesting properties which make it a suitable probiotic. First of all, *B. amyloliquefaciens* is able to form spores which benefits its survival during passage in the gastrointestinal tract. Several researchers have demonstrated that the bacterium is tolerant to low pH and bile salts. In addition, it has DNA protective and antioxidant activities due to its ability to scavenge hydroxyl and DPPH free radicals. An immune stimulatory effect of probiotic *B. amyloliquefaciens* administration has also been reported.[434-436]

A very valuable property of *B. amyloliquefaciens* is its ability to produce a wide array of ribosomally and non-ribosomally synthesized compounds with profound antibacterial and antifungal activity. Complete genome analysis of *B. amyloliquefaciens* FZB42 indicated that up to 8.5% of its genome is dedicated to the production of secondary metabolites with antimicrobial activity.[437] For example, the bacteriocin mersacidin is able to inhibit Gram-positive pathogens such as *Staphylococcus aureus* and *Enterococcus faecium* but no activity against Gram-negative bacteria or fungi has been reported.[438] Amylocyclicin and plantazolicin are two bacteriocins with antibacterial activity against closely related

Bacillus species.[439, 440] The two polyketides difficidin and bacilysin are two promising agents for the biocontrol of phytopathogens. Both molecules can successfully inhibit the growth of *Erwinia amylovora*, the causative against of fire blight disease in e.g. apple and pear trees, and *Xanthomonas oryzae* pv. *oryzae*, responsible for bacterial blight in rice plants.[441, 442] In addition, bacilysin encompasses anticyanobacterial activity which can be exploited for the control of harmful algal blooms.[443] The lipopeptides fengycin and bacillomycin D have been successfully used for the control of brown rot in stone fruits caused by *Monilinia fructicola*.[444] Fengycin and iturin also possess antifungal activity against the phytopathogens *Fusarium oxysporum*, *Cladosporium cucumerinum* and *Botrytis cinerea*.[445] Surfactin, another lipopeptide produced by *B. amyloliquefaciens*, has no significant impact on fungal growth but comprises growth inhibiting activity against important bacterial pathogens including *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* sp.[446-448]

Due to these valuable probiotic properties, *B. amyloliquefaciens* has been extensively studied to investigate its potential beneficial effect on the host. Oral administration of *B. amyloliquefaciens* was considered to be safe in mammals since no acute mortality or visible disease signs were observed in mice.[449] In addition, *B. amyloliquefaciens* appears to have a beneficial effect on the pathogenesis and progression of certain intestinal disorders. For example, orally administered *B. amyloliquefaciens* significantly reduced bacterial translocation in weaned mice.[450] Weaned piglets benefited from dietary supplementation with probiotic *B. amyloliquefaciens* since an improvement in growth performance and decrease in the incidence of diarrhea was observed.[451] In healthy dogs, supplementation of a probiotic mixture containing *B. amyloliquefaciens* and *Enterococcus faecium* was associated with a reduction in the number of pathogenic Clostridia.[452] The bacterium has also been proposed as a novel probiotic for a better management of inflammatory bowel disease since it successfully reduced intestinal inflammation in mice.[453] *In vitro* antibacterial activity of *B. amyloliquefaciens* has been demonstrated against the important fish pathogens *Edwardsiella tarda*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *V. harveyi*. Subsequent *in vivo* tests were conducted to study the potency of probiotic *B. amyloliquefaciens* administration in preserving fish health after pathogen challenge. Interestingly, the probiotic significantly increased survival rates of catla challenged with *E. tarda* and eels challenged with *A. hydrophila*.[449, 454]

EFSA recognizes the use of *B. amyloliquefaciens* in animal production either directly as a feed additive or indirectly as a production source of enzymes as feed additives.[433] Several reports indicated that probiotic supplementation of *B. amyloliquefaciens* to broilers had a beneficial effect on growth performance, maintenance of the normal gut microbiota, nutrient digestibility, ammonia production and immune response.[455-457] Ecobiol® is a commercially available probiotic containing *B. amyloliquefaciens* used as a feed-additive to stimulate fattening of broilers. The beneficial effect of

Ecobiol® on broilers was confirmed using large *in vivo* trials. Results indicated that the probiotic improves nutrient digestibility by the secretion of enzymes that break down the non-starch polysaccharides and proteins in the feed. In addition, *B. amyloliquefaciens* produces lactic acid during fermentation of carbohydrates, which is associated with a rise in the amount of intestinal lactobacilli. On the other hand, the amount of *E. coli* in the caeca is significantly reduced. A large meta-analysis concluded that the probiotic improves feed conversion ratios and reduces mortality. Besides in broilers, a positive effect of in-feed Ecobiol® supplementation has also been described in laying hens where it improves albumen and egg shell quality among others.[458] As mentioned before, *B. amyloliquefaciens* is also an important production source of digestive enzymes. For example, the enzyme preparations Kemzyme® PLUS and Avizyme® 1505, both used as a feed additive in poultry for fattening, contain α-amylase produced by *B. amyloliquefaciens*. This enzyme is able to break down large polysaccharides such as starch thereby improving nutrient availability.[433, 459]

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SCIENTIFIC AIMS

Clostridium difficile and *Clostridium perfringens* are two important human and animal pathogens able to cause severe intestinal inflammation. *C. difficile* is an opportunistic pathogen that causes disease when the intestinal microbiota is disturbed, mainly due to the oral intake of antibiotics. Current treatment strategies rely on the administration of broad spectrum antibiotics but have major disadvantages including treatment failures with high recurrence rates, risk for antibiotic resistance development and, most importantly, they do not aid in the recovery of the intestinal microbiota. *C. perfringens* is the causative agent of necrotic enteritis in broiler chickens. The use of antibiotics as growth promoters has long constrained this disease. However, public concern on the use of in-feed antibiotics and the alarming rise of antibiotic-resistant pathogens has led to a ban on the use of antibiotics as growth promoters in Europe. Consequently, a rise has been seen in the incidence of necrotic enteritis in broilers with a concomitant increase in the therapeutic use of antibiotics.

Although the discovery of broad spectrum antibiotics has definitely been a major breakthrough for human and veterinary medicine, we have evolved into an era in which its imprudent use has led to the emergence of antibiotic resistant bacterial pathogens. There is great fear that the industry is not able to develop new effective antibiotics at a sufficient rate to counteract the development of antibiotic resistance. Nowadays, researchers have largely shifted their attention from the discovery of novel broad spectrum antibiotics to finding alternatives in an attempt to battle the development of antibiotic resistance, a valuable property that can be exploited in two ways. On one hand, the antimicrobial compounds in se can be used as antibiotic replacers and their potential therapeutic use is definitely worth investigating. On the other hand, there is the possibility that these bacteria could be used as probiotics. Probiotics have become more and more popular in recent years. Probiotic bacteria that produce antimicrobial compounds targeting specific pathogens are particularly interesting since they tackle the causative disease agent and restrict collateral damage to the intestinal microbiota. In addition, they can support the restoration of the normal gut microbiota.

During laboratory practice, we isolated an environmental *B. amyloliquefaciens* strain that was able to inhibit the growth of *C. difficile* and *C. perfringens in vitro*. Several studies already indicated that *B. amyloliquefaciens* possesses many suitable probiotic properties. In addition, the European Food Safety Authority has put *B. amyloliquefaciens* on the Qualified Presumption of Safety list. Ongoing research concerning probiotics mainly focusses on probiotic strains that are already commercially available. We specifically selected this *B. amyloliquefaciens* strain based on its potential to inhibit the growth of *C. difficile* and *C. perfringens*. Therefore, the general aim of this thesis was to investigate whether *B. amyloliquefaciens* could be used as an alternative for conventional antibiotics in the control of *Clostridium difficile* and *Clostridium perfringens* associated intestinal diseases.

The first aim of this thesis was to identify the compound responsible for the antibacterial activity of *B. amyloliquefaciens* against *C. difficile*. Using acid precipitation, solid phase extraction and reversed-phase HPLC, the compound was first purified. Identification was achieved using HPLC-ESI-Orbitrap mass spectrometry. In addition, we characterized the anti-*C. difficile* activity of *B. amyloliquefaciens* and had a closer look at pH, temperature and protease stability, the antibacterial spectrum, hemolytic activity and cytotoxicity against intestinal epithelial cells.

Since *B. amyloliquefaciens* shows great promise as a probiotic micro-organism, the second aim of this thesis was to investigate if *B. amyloliquefaciens* administration could prevent *C. difficile* associated disease. We first analyzed the antibacterial activity of *B. amyloliquefaciens* against a collection of 24 different *C. difficile* ribotypes. Afterwards, a well described mouse model for *C. difficile* associated disease was used to investigate if prophylactic administration of *B. amyloliquefaciens* could prevent *C. difficile* associated disease in these animals.

Several *Bacillus* species are used as an in-feed supplement for broilers to improve broiler performance and intestinal health. In-feed supplementation of *B. amyloliquefaciens* has been reported to improve the performance of broiler chickens. The third aim of this study was to analyze if *B. amyloliquefaciens* could protect broilers from developing necrotic enteritis. Since *C. perfringens* is the causative agent for broiler necrotic enteritis, we started with analyzing the *in vitro* activity of *B. amyloliquefaciens* against NetB negative and NetB positive *C. perfringens* strains. In a second part, an *in vivo* broiler model was used to study whether in-feed supplementation of vegetative *B. amyloliquefaciens* cells could prevent *C. perfringens* associated necrotic enteritis.

EXPERIMENTAL STUDIES

CHAPTER 1
CHARACTERIZATION OF THE ANTI-CLOSTRIDIUM DIFFICILE ACTIVITY OF BACILLUS AMYLOLIQUEFACIENS SG1

ABSTRACT

In the past decade, *Clostridium difficile* has emerged as an important human pathogen with steady increases in incidence and severity worldwide. Current treatment strategies with broad spectrum antibiotics have some major drawbacks such as treatment failures with high recurrence rates and the destructive effect on the intestinal microbiota. In addition, antibiotic consumption is always associated with the fear of antibiotic resistance development. This has created the urgent need to find alternative treatment options.

We isolated a *Bacillus amyloliquefaciens* strain that is able to inhibit the growth of *C. difficile*. In this study, we characterized the anti-*C. difficile* activity of cell free supernatant obtained from a *B. amyloliquefaciens* SG1 overnight culture. Determination of the antibacterial spectrum indicated only a narrow spectrum of activity. The antibacterial activity was stable over a wide pH and temperature range. No loss of activity was observed after treatment of the supernatant with catalase, amylase, pepsin, protease P, protease K, chymotrypsin and pronase with the exception of only a minor loss of activity due to trypsin treatment. Overall, the antibacterial activity was relatively resistant to proteolytic digestion. The antibacterial activity was impaired by exposure to pancreatin and lipase indicating the presence of a lipid moiety. Lysozyme treatment affected the antibacterial activity weakly. The supernatant showed no hemolytic activity and was not cytotoxic towards IPEC-J2 cells. Next, the antimicrobial compound was purified using acid precipitation, solid phase extraction and reversed phase-HPLC. Mass spectral analysis of the antibacterial fraction revealed the presence of a major peak at 1036.65 Da. MSMS analysis indicated that this peak corresponded to the lipopeptide surfactin.

Our results indicate that *B. amyloliquefaciens* SG1 produces at least one compound, the lipopeptide surfactin, with potent antibacterial activity towards *C. difficile*, making the strain or the lipopeptide a potential alternative treatment option for *C. difficile* associated disease.

INTRODUCTION

The discovery of broad spectrum antibiotics has definitely been a major breakthrough for human and veterinary medicine. However, their inappropriate and extensive use has led to the emergence of multidrug resistant bacterial pathogens. This has become a global public health problem since the number of multidrug resistant infections is constantly rising threatening effective prevention and treatment. There is great concern that the industry is not able to develop new effective antibiotics at a sufficient rate to counteract the development of antibiotic resistance.[1]

In the past decade, *C. difficile* has emerged as an important hospital associated pathogen with steady increases in incidence and severity worldwide.[2, 3] It has evolved into the most important cause of health care associated diarrhea and is mainly related to prior exposure to antibiotics.[4] Antibiotics administered to manage any kind of infection induce a disturbance in the indigenous intestinal microbiota giving *C. difficile* the chance to colonize the intestinal tract and produce its toxins responsible for the disease.[5] Current treatment strategies rely on the administration of the broad spectrum antibiotics metronidazole and vancomyin but have major disadvantages. Both antibiotics are associated with a limited treatment success since high recurrence rates are being reported.[6] In addition, *C. difficile* strains with reduced susceptibility for both antibiotics have already been isolated.[7, 8] Vancomycin use is associated with the risk of colonization with vancomycin resistant enterococci, hence, cautious practice is recommended.[9] Antibiotic use is always associated with the danger of spread of antibiotic resistance to other pathogens or strains of the resident gut microbiota.[10] Since metronidazole and vancomycin have a broad spectrum of activity, they cause a considerable amount of collateral damage to other strains of the gut microbiota.[11] Altogether, this has created an urgent need to develop new treatment strategies that efficiently target *C. difficile* but only have a minimal impact on the resident gut microbiota.

Nowadays, researchers have largely shifted their attention from the discovery of novel broad spectrum antibiotics to finding alternative treatments in an attempt to limit the development of antibiotic resistance. Bacteria produce many secondary metabolites other than the classical broad spectrum antibiotics. These metabolites often possess potent antibacterial activity. Bacteriocins and lipopeptides are two classes of secondary metabolites with promising characteristics for use as antibiotic alternative. Several bacteriocins with antibacterial activity against *C. difficile* have already been reported. Nisin and lacticin 3147, both produced by *Lactococcus lactis* strains, exhibit excellent *in vitro* activity against *C. difficile* and are promising candidates for the treatment of CDAD.[12, 13] Thuricin CD is a bacteriocin produced by *Bacillus thuringiensis* with a narrow spectrum of activity against *C. difficile*. This antimicrobial peptide offers the possibility of a targeted therapy with a

minimal impact on the resident gut microbiota.[14] NVB302 is a semi-synthetic derivative of deoxyactagardine B with elevated *in vitro* antibacterial activity against *C. difficile*. A recent phase I clinical trial indicated that its use in healthy subjects is safe and well-tolerated.[15, 16] LFF571 is derived from the bacteriocin GE2270 A and results of a phase II clinical trial demonstrated better clinical cure and recurrence rates as vancomycin therapy.[17-19] CB-183,315 is a novel lipopeptide with a more potent *in vitro* activity against *C. difficile* than metronidazole and vancomycin.[20, 21] In addition, a phase II clinical trial concluded that its use is safe and well-tolerated and results showed higher clinical cure and recurrence rates than vancomycin.[22] Taken together, the use of bacterial metabolites as alternative for antibiotic therapy shows great promise for the treatment of CDAD.

During laboratory practice, a bacterial strain able to inhibit *C. perfringens* grew on agar plates as a contaminant. This strain was isolated and identified as *B. amyloliquefaciens* based on 16S and gyrase B gene sequencing and named *B. amyloliquefaciens* SG1. Additional testing revealed an even higher level of *in vitro* inhibitory capacity against *C. difficile*. Due to this high amount of antibacterial activity, the focus of the current study was to characterize the anti-*C. difficile* activity of *B. amyloliquefaciens* supernatant and to identify the components involved in its antibacterial activity.

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS

B. amyloliquefaciens SG1 was grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) and incubated for 24 h at 30°C with agitation for the preparation of cell free supernatant (CFSN). For purification and identification of the antimicrobial compound, *B. amyloliquefaciens* SG1 was grown for 48 h at 30°C in Medium Optimized for Lipopeptide Production (MOLP) as described previously.[23]

Clostridium difficile VPI 10463 was used as indicator strain to screen for residual antibacterial activity of CFSN by means of an agar well diffusion assay. The strain was inoculated in reinforced clostridial medium (RCM) (Oxoid) and incubated for 24 h at 37°C in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) workstation (Ruskinn Technology, Bridgend, UK).

Other bacterial indicator strains used to determine the antibacterial spectrum of CFSN are listed in Table 1 with their appropriate medium. All Gram-negative strains, *Clostridium perfringens* strains, *Staphylococcus* sp. and *Streptococcus* sp. were grown in brain heart infusion broth (BHI) (Merck, Darmstadt, Germany). *Lactobacillus* sp. were grown in MRS broth (MRS) (Oxoid). All strains were incubated overnight at 37°C.

CHARACTERIZATION OF ANTIBACTERIAL ACTIVITY

CELL FREE SUPERNATANT

For preparation of CFSN, *B. amyloliquefaciens* SG1 was grown as described above. Bacterial cells were removed by centrifugation at 5000 x g for 15 min at 4°C. The supernatant was filter-sterilized and used for characterization of the antibacterial activity. The activity of CFSN was tested against *C. difficile* VPI 10463 using an agar well diffusion assay. A 24 h culture of *C. difficile* VPI 10463 was diluted 1/50 in autoclaved, cooled reinforced clostridial agar (Oxoid) and poured in sterile petri dishes to obtain a confluent layer. Using the back of sterilized tips, wells were made in the agar and filled with 20 – 40 µl of the CFSN. The plates were incubated overnight at 37°C in an anaerobic (84%N_2, 8% CO_2 and 8% H_2) workstation (Ruskinn Technology, Bridgend, UK) and checked for the presence of growth inhibition zones around the wells. The size of the inhibition zones was measured. All experiments were performed in triplicate. Results are means of three independent replicates ± standard error on the mean.

INHIBITION SPECTRUM OF B. AMYLOLIQUEFACIENS SG1

The antimicrobial activity of the CFSN was tested against a selection of Gram-negative and Gram-positive bacteria as represented in Table 1. All strains were obtained from our own collection. Overnight cultures were diluted 1/10 in sterile phosphate buffered saline. One hundred microliter was swabbed on the appropriate agar plate. Wells were made in the agar and filled with 40 µl CFSN. After overnight incubation, plates were evaluated for the presence of growth inhibition zones around the wells. The sensitivity was determined by measuring the size of the growth inhibition zone. Results are expressed as follows, -: no growth inhibition; +: ≤ 5 mm; ++: 5 – 10 mm; +++: ≥ 10 mm.

EFFECT OF PH AND HEAT TREATMENT

The antimicrobial activity of CFSN was tested for sensitivity to pH and heat treatment. Heat sensitivity was analyzed by incubation of 500 µl aliquots of CFSN at 30 – 120°C with 10°C intervals for 10, 30 or 60 min using a heat block. Samples were immediately transferred to ice after incubation. The influence of the pH was determined by adjusting the pH of 20 ml aliquots of CFSN from 2 to 12 with 6 N HCl or 3 M NaOH. Samples were incubated at 4°C for 1 h and centrifuged at 5000 x g for 15 min at 4°C. The supernatant was collected and neutralized to pH 7. Any precipitated material was dissolved in 20 ml sterile GIBCO® Hank's Balanced Salt Solution (Life Technologies, Gent, Belgium). Both fractions were tested for residual antibacterial activity against *C. difficile* VPI 10463 as described above. The activity of an untreated sample was determined as a control and was considered 100%. Activity of all treated samples was expressed as the percentage of residual activity as compared to the control sample.

EFFECT OF PROTEOLYTIC ENZYMES

The sensitivity of the antimicrobial metabolites in the CFSN to enzymatic digestion was determined by incubation of aliquots of CFSN with 1 mg/ml of pepsin, proteinase P, proteinase K, trypsin, chymotrypsin, pronase, catalase, amylase, lipase, lysozyme or pancreatin. All enzymes were dissolved in HBSS and filter-sterilized prior to use. All samples were incubated at 37°C for 1 h. An untreated sample was included as a control. To exclude any influence of the enzymes themselves on the growth of *C. difficile*, the tested enzymes were diluted in sterile HBSS to achieve identical concentration as compared to the treated supernatant and tested for antibacterial activity. Activity of all treated samples was compared with the activity of an untreated sample and expressed as % residual activity.

HEMOLYTIC ACTIVITY

The hemolytic activity of CFSN was analyzed using commercial blood agar plates. Three drops of 20 µl were spotted on a columbia agar plate supplemented with 5% sheep blood (Oxoid). The plate was left to dry and incubated overnight at 37°C in an aerobic atmosphere.

KINETICS OF ANTIBACTERIAL ACTIVITY PRODUCTION

To monitor the production of antimicrobial metabolites along the bacterial growth, a *B. amyloliquefaciens* SG1 overnight culture was diluted 1/2000 in TSB and incubated at 30°C with agitation. A 3 ml sample was collected at regular time intervals. To follow the bacterial growth, the OD was measured at 600 nm. The sample was centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was filter-sterilized and screened for antibacterial activity against *C. difficile* VPI 10463.

CYTOTOXIC EFFECT OF CFSN ON INTESTINAL EPITHELIAL CELLS

To assess the cytotoxic effect of CFSN on intestinal epithelial cells, a neutral red uptake assay was performed as described previously.[24] Porcine intestinal epithelial cells (IPEC-J2) were grown in Dulbecco's Modified Eagle Medium (DMEM), nutrient mixture F12, supplemented with 5% fetal calf serum, 1% insulin-transferrin-selenium (ITS), 1% penicillin-streptomycin and 1% kanamycin (Life Technologies, Gent, Belgium). IPEC-J2 cells were seeded in a collagen coated 96-well plate at a concentration of ± 5 x 10^4 cells/well and incubated for 24 h at 37°C in the presence of 5% CO_2. Cell medium was removed and cells were washed with pre-warmed HBSS containing Ca^{2+} and Mg^{2+}. One hundred microliters of CFSN was added to the cells and plates were incubated for 3 hours at 37°C. CFSN was removed and neutral red medium (DMEM, F12, ITS, 1 ml neutral red solution (Sigma, Diegem, Belgium)) was added. After 3 hours of incubation, cells were washed with HBSS containing Ca^{2+} and Mg^{2+} and treated with neutral red desorb solution (50% absolute ethanol, 49% distilled water, 1% glacial acetic acid) for 15 min at room temperature to extract neutral red from the cells.

Absorbance was measured at 550 nm. TSB was included as a negative control, 100% SDS was used as a positive control. CFSN was tested in triplicate and plates were measured three times.

PURIFICATION AND IDENTIFICATION OF ANTIBACTERIAL COMPOUD

PURIFICATION

The supernatant of a *B. amyloliquefaciens* SG1 culture grown in MOLP was used for purification of the antimicrobial compound. The supernatant was first concentrated by acid precipitation. The pH of the supernatant was adapted to pH 2 using a 6 M HCl solution. After overnight incubation at 4°C, the white precipitate was collected by centrifugation at 5000 x g for 15 min. The precipitate was dissolved in HBSS. The pH of the solution was neutralized using 3 M NaOH and the solution was filter sterilized. The sample was further subjected to solid phase extraction. The sample was applied on a Bond Elut C18 (Agilent Technologies, Diegem, Belgium) column. The column was washed with 40 ml distilled water followed by 40 ml of a 50% MeOH solution. The antimicrobial compound was eluted with 40 ml 100% MeOH. Fractions of 20 ml were collected every time. All collected fractions were tested for antibacterial activity against *C. difficile* VPI 10463 by means of an agar well diffusion assay. The fraction with the highest antibacterial activity was selected for further purification using RP-HPLC. This fraction was divided in 2 ml aliquots which were vacuum dried for enrichment reasons and dissolved in 80 µl 0.1% TFA/50% ACN. The samples were centrifuged at 16000 x g for 8 min at 4°C. The supernatant was subjected to RP-HPLC using a PTC-C18 column (PTC Spheri-5 220x2.1 mm Higgins Analytical) at a flow rate of 100 µl/min using gradient elution and detection at 220 and 280 nm. Mobile phase A consisted of 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. The elution conditions were 50 – 80% B in 54 min, 80 – 100% B in 10 min followed by 100% B for 5 min. All collected fractions were analyzed for antibacterial activity against *C. difficile* VPI 10463 using an agar well diffusion assay including both buffers A and B to exclude any effect of the buffers themselves. Uncultured MOLP medium was subjected to the same protocol and used as a blanc to be able to exclude background peaks.

IDENTIFICATION

The fractions with the highest antibacterial activity, corresponding to the same peak in the chromatogram, were pooled and vacuum dried for enrichment reasons. Afterwards, high-resolution mass spectra of this sample were generated by HPLC-ESI-Orbitrap mass spectrometry (1260 series HPLC system; Agilent Technologies, Waldbronn, Germany) coupled to an HR-ESI-Orbitrap mass spectrometer (Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was carried out on a Grom-Sil 120 ODS-5 ST column (100 x 2 mm; 5 µm, Grace Davison, Deerfield, IL, USA) by using a mobile phase consisting of H_2O (A) and ACN (B) each containing 0.1 % formic acid

with a gradient from 5 – 99 % B over 10 minutes followed by a 2 min hold at 100 % B using positive ionization mode. Fragmentation of molecules was carried out using collision-induced dissociation (CID). In this study CID measurements performed at 40 % normalized collision energy have been represented. To exclude any background, the same procedure was applied to the pooled fractions from the RP-HPLC of uncultured MOLP medium.

RESULTS

CHARACTERIZATION OF THE ANTIBACTERIAL ACTIVITY

CFSN of *B. amyloliquefaciens* SG1 was tested against a range of Gram-positive and Gram-negative bacteria as represented in Table 1. *B. amyloliquefaciens* SG1 displayed a limited spectrum of activity. No activity was detected against the Gram-negative bacteria *Escherichia coli*, *Salmonella* sp., *Pasteurella* sp. and *Pseudomonas aeruginosa*. Only the growth of *Yersinia pseudotuberculosis* was inhibited to a minor extent. Moreover, the growth of several Gram-positive bacteria belonging to *Clostridium* cluster I, IV, XIVa and XVI, 7 out of 8 tested *Lactobacillus* strains and all tested *Staphylococcus* sp. was not affected by the supernatant. Only *Clostridium perfringens*, *Enterococcus cecorum* and the two tested *Streptococcus* species were inhibited by the supernatant.

Table 1. Antibacterial spectrum of CFSN from *B. amyloliquefaciens* SG1 against a selection of indicator micro-organisms. * - : no inhibition; +: ≤ 5 mm; ++: 5 – 10 mm; +++: ≥10 mm

	Strains	Medium	Activity*
Gram-negative	*Escherichia coli* (n=5)	BHI	-
	Pasteurella sp. (n=1)	BHI	-
	Pseudomonas aeruginosa (n=2)	BHI	-
	Salmonella Enteritidis (n=1)	BHI	-
	Salmonella Typhimurium (n=1)	BHI	-
	Yersinia pseudotuberculosis (n=2)	BHI	+
Gram-positive	*Clostridium* cluster I (n=2)	M2GSC	-
	Clostridium cluster IV (n=3)	M2GSC	-
	Clostridium cluster XIVa (n=1)	M2GSC	-
	Clostridium cluster XVI (n=4)	M2GSC	-
	Clostridium difficile VPI 10463 (n=1)	RCA	+++
	Clostridium perfringens (n=1)	BHI	++
	Enterococcus cecorum (n=1)	BHI	+
	Lactobacillus acidophilus (n=1)	MRS	-
	Lactobacillus brevis (n=1)	MRS	-
	Lactobacillus buchneri (n=1)	MRS	-
	Lactobacillus paracasei subsp. paracasei (n=1)	MRS	-
	Lactobacillus plantarum (n=1)	MRS	+
	Lactobacillus sakei subsp. carnosus sp. (n=1)	MRS	-
	Lactobacillus salivarius sp. (n=1)	MRS	-
	DNAse negative *Staphylococcus* sp. (n=1)	BHI	-
	Methicillin Resistant *Staphylococcus aureus* (n=1)	BHI	-
	Staphylococcus intermedius (n=5)	BHI	-
	Streptococcus bovis (n=1)	BHI	+
	Streptococcus equi subsp. *zooepidemicus* (n=3)	BHI	++

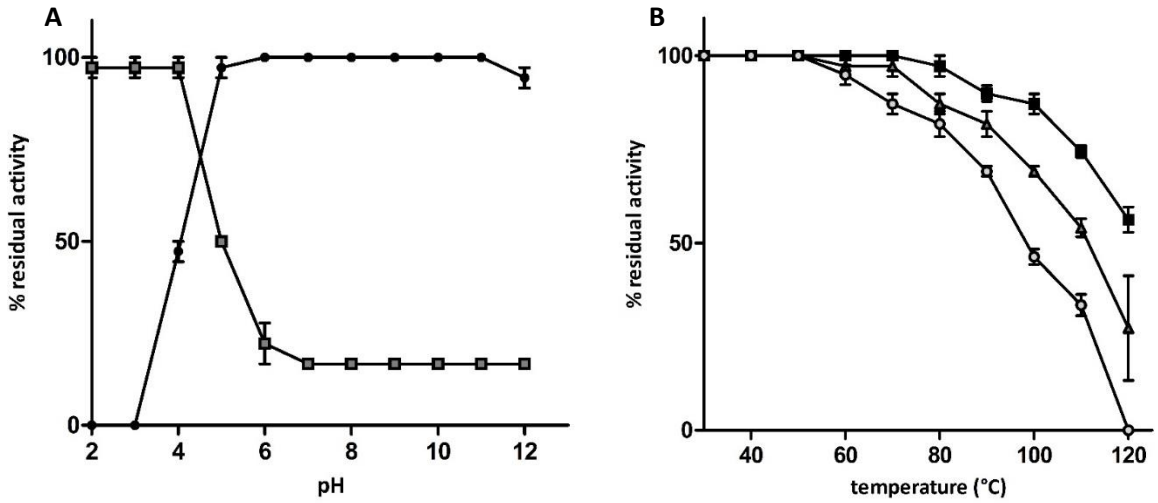

Figure 1. Effect of pH and temperature on the relative anti-*C. difficile* activity of CFSN from a *B. amyloliquefaciens* SG1 culture. (A) Effect of pH on CFSN (●) or dissolved precipitate (■) (B) Effect of exposure to various temperatures for 10 min (■), 30 min (▲) or 60 min (○) The remaining antibacterial activity was compared with an untreated sample and expressed as % residual activity. Results are means of three independent replicates ± standard error on the mean.

The effect of exposure to different pH values and temperatures on the antibacterial activity of CFSN is represented in Figure 1. The antibacterial activity was relatively resistant to changes in pH (Figure 1A). Antimicrobial metabolites secreted by *B. amyloliquefaciens* SG1 remained stable at pH values between 6 and 11 with only minor loss of activity at pH 5 and pH 12. At low pH however, the antibacterial activity was abolished but could be completely recovered after suspending the collected precipitate in the original volume. Antibacterial activity was unaffected by heat treatment of CFSN at 30-70°C for 10 min and 30-50°C for 30 min and 60 min. Loss of activity was observed after incubation at 80°C for 10 min, 60°C for 30 min and 60 min. Heating of the CFSN for 60 min at 120°C resulted in a complete loss of antibacterial activity (Figure 1B).

The effect of various enzymes on the antibacterial activity of CFSN against *C. difficile* is represented in

Table 2. The antibacterial activity was unaffected by exposure catalase, amylase, pepsin, protease P, protease K, chymotrypsin and pronase. Only a minimal loss of activity was observed due to trypsin and lysozyme treatment. The antibacterial activity was diminished due to the addition of pancreatin and lipase indicating the presence of a lipid moiety.

Table 2. Effect of various enzymes on the anti-*C. difficile* activity of CFSN from a *B. amyloliquefaciens* SG1 culture. *Antimicrobial activity of untreated CFSN equalizes 100%. Results are means of three individuals replicates ± standard error on the mean.

Test product	Residual activity (%)*
1% lysozyme	94.5 ± 3.2
1% pancreatine	83.3 ± 0.5
1% amylase	100
1 mg/ml pepsin	100
1 mg/ml protease P	100
1 mg/ml protease K	100
1 mg/ml trypsin	98.2 ± 1.,8
1 mg/ml chymotrypsin	100
1 mg/ml pronase	100
1 mg/ml catalase	100
1 mg/ml lipase	90.8 ± 2.,5

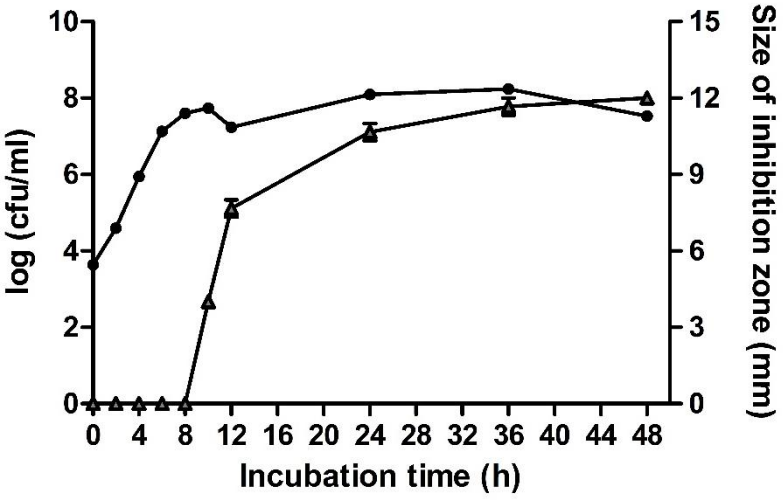

Figure 2. Growth and antibacterial metabolite production by *B. amyloliquefaciens* SG1 in TSB at 30°C. Log cfu/ml (●) and diameter of growth inhibition zone (▲).

CFSN showed no hemolytic activity. The cytotoxicity of CFSN was determined using IPEC-J2 cells. No cell death was observed with 100% of supernatant as compared to the negative control consisting of cell medium. The secretion of antimicrobial metabolites in the supernatant by *B. amyloliquefaciens* SG1 coincides with the growth of the bacterium. The amount of antimicrobial activity rises during the late exponential growth phase of the bacterial culture and reaches its maximal production during the stationary growth phase. No loss of activity is noted during the stationary growth phase (Figure 2).

PURIFICATION AND IDENTIFICATION OF ANTIBACTERIAL COMPOUND

The antimicrobial compound was first concentrated using acid precipitation. All antibacterial activity was retained after dissolving the collected precipitate. The sample was subjected to solid phase extraction and all eluted samples were tested for antibacterial activity against *C. difficile*. The highest amount of activity was obtained after elution with 100% methanol. This fraction was concentrated and subsequently subjected to RP-HPLC for further purification. All eluted samples were tested for antibacterial activity against *C. difficile*. A high amount of antibacterial activity was obtained in the samples corresponding to a large peak with a retention time of 54 – 56 minutes. The fractions corresponding to this peak were pooled, vacuum dried and further analyzed with HPLC-ESI-Orbitrap mass spectrometry (MS). The obtained mass spectrum is represented in Figure 3 A. The peaks on the left part of the spectrum were also found in the blank sample and are, as such, not responsible for the antibacterial activity. The peak with the highest concentration has a molecular mass of 1036.65. MSMS analysis indicated a spectrum characteristic for the lipopeptide surfactin (Figure 3 B). This observation was confirmed by comparison of the mass spectra with those obtained from commercially available surfactin (Figure 4). The 1036.65 and 1058.63 peak correspond respectively to surfactin with a lipid chain containing 14 carbon atoms and its sodium adduct.

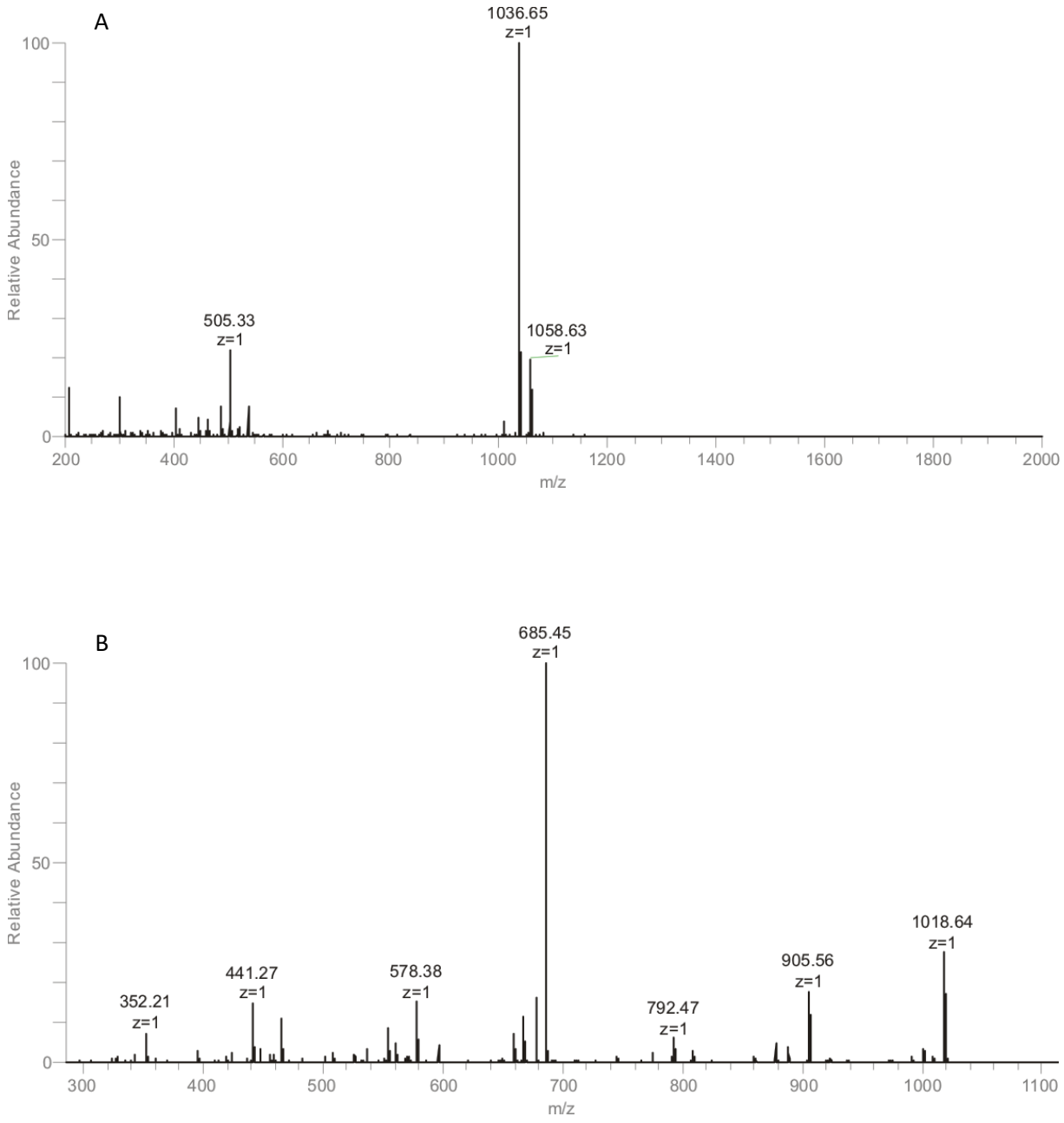

Figure 3. MS (A) and MSMS (B) analysis of the purified antibacterial compound produced by *B. amyloliquefaciens* SG1 active against *C. difficile*. MSMS analysis was conducted on the 1036.65 peak.

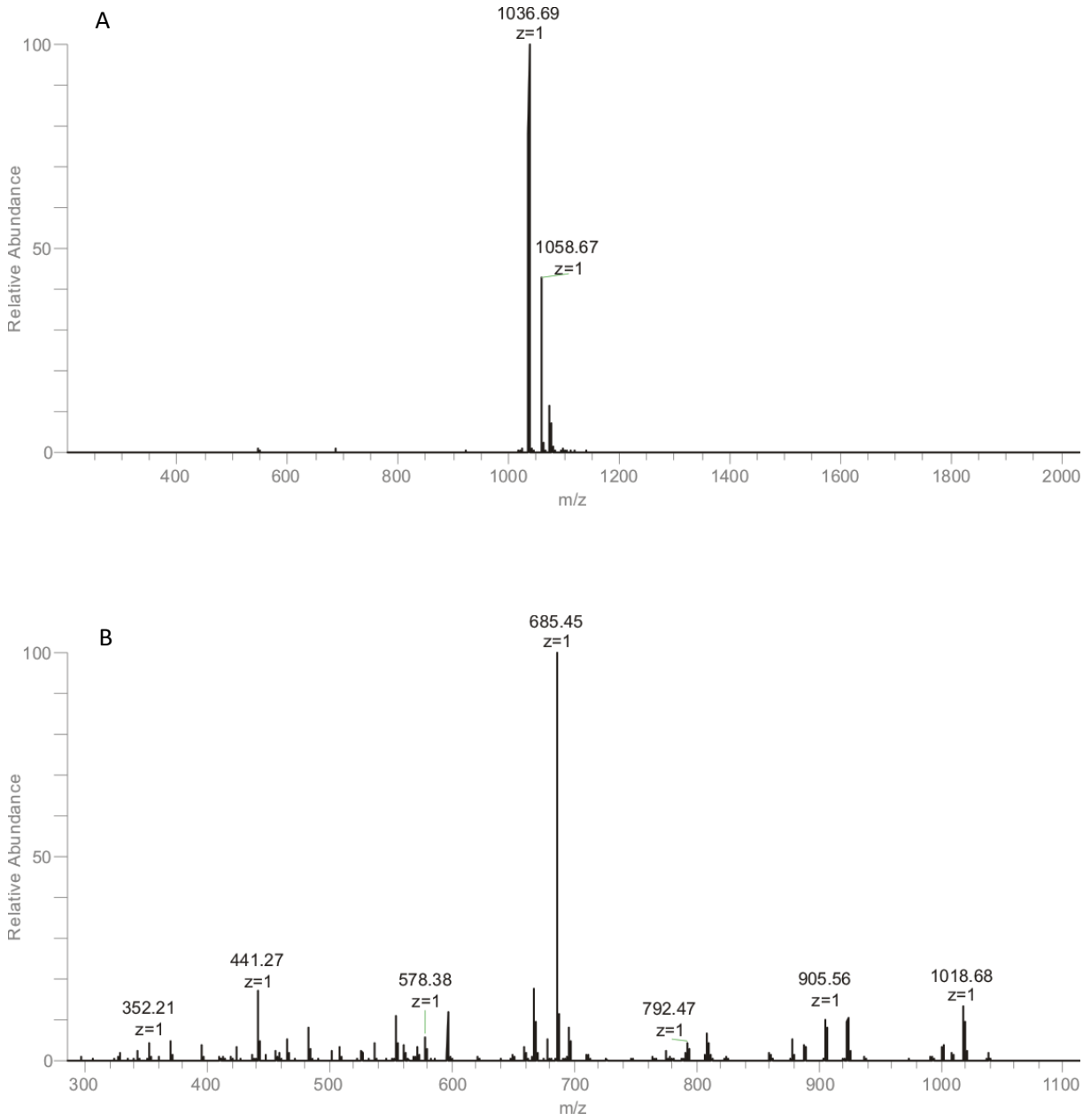

Figure 4. MS (A) and MSMS (B) analysis of commercially available surfactin. MSMS anlysis was conducted on the 1036.69 peak.

The purification and identification procedure was repeated to confirm the result. Surfactin was again identified in the sample although different homologues were present. The most abundant peaks present had a molecular mass of 1022.67 and 1044.65. These peaks correspond to surfactin with a lipid chain containing 13 carbon atoms and its sodium adduct. In addition, an unknown peak at 503.31 was detected. Unfortunately, the identity of this compound could not be detected after MSMS analysis because its concentration was too low.

To analyze if surfactin is indeed responsible for the antibacterial activity against *C. difficile*, commercially available surfactin (Sigma) was purchased. Surfactin was dissolved in methanol and tested for antibacterial activity against *C. difficile* using an agar well diffusion assay. Methanol was included as a control. Overnight incubation revealed that surfactin was able to inhibit the growth of *C. difficile* (Figure 5).

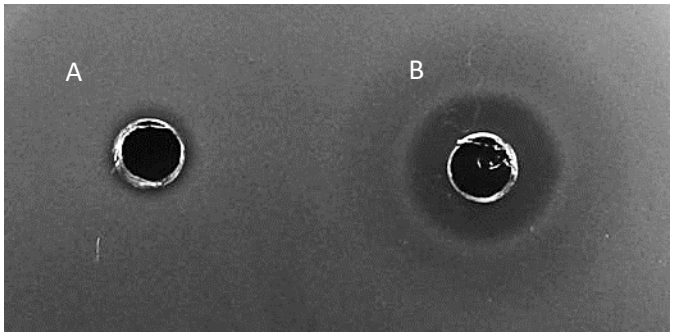

Figure 5. Antibacterial activity of (A) control (methanol) and (B) commercially available surfactin against *C. difficile*.

DISCUSSION

In the past decade, the incidence and severity of *C. difficile*-associated disease has increased worldwide.[25] Current treatment strategies with the broad spectrum antibiotics metronidazole and vancomycin are regularly failing.[6] In addition, the fear of antibiotic resistance development has created the urgent need to find alternatives to broad spectrum antibiotic therapy.[26] Lipopeptides are secondary bacterial metabolites that often encompass significant antibacterial activity and show great promise as therapeutics.[27] In this study, we demonstrated that the lipopeptide surfactin is responsible for the antibacterial activity *of B. amyloliquefaciens* SG1 against *C. difficile*.

Surfactin is a cyclic lipopeptide made up of a peptide chain containing seven amino acids that undergoes cyclization, and a hydrophobic fatty acid chain. The antibacterial effect of surfactin against important human pathogens such as *Escherichia coli*, *Salmonella* Enteritidis and *Listeria monocytogenes* has been documented in several studies.[28-30] However, we clearly demonstrated that *B. amyloliquefaciens* SG1 supernatant does not encompass antibacterial activity against the *E. coli* and *Salmonella* strains tested in our study. Interestingly, Sabaté and Audisio reported that the antibacterial capacity of surfactin is strain dependent. Surfactins purified from different *B. subtilis* strains showed a large variation in antibacterial efficacy against *Listeria monocytogenes*. Surfactin purified from *B. subtilis* subsp. *subtilis* C4 inhibited the pathogen with a concentration of 0.125 mg/ml while the lowest inhibitory effect was observed for *B. subtilis* subsp. *subtilis* M1, which only inhibited the pathogen at 1 mg/ml of surfactin.[28] Crude supernatant inhibited the growth of *C. difficile* VPI 10463 without the necessity for enrichment. The surfactin produced by our strain encompasses a high antibacterial effect against *C. difficile* but not against other bacteria. When used for the treatment of *C. difficile* associated disease, this limited spectrum of activity may spare the indigenous intestinal microbiota from collateral damage.

Lipopeptides have already been suggested as promising alternatives for antibiotic therapy.[27] Nevertheless, lipopeptide production has long been seen as evidence for toxicity. This observation is however based on results of *in vitro* experiments and *in vivo* toxicity has not been confirmed. Results of our *in vitro* tests clearly indicate that *B. amyloliquefaciens* SG1 supernatant does not present cytotoxic or hemolytic activity. Since crude supernatant significantly inhibits *C. difficile* without the necessity of enrichment, the concentration of surfactin present is sufficiently low to avoid toxicity. Despite the fact that literature concerning the use of lipopeptides *in vivo* is not elaborate, their beneficial effects have been described in the few available reports. Good safety profiles were reported for the oral administration of a crude lipopeptide mixture from *Bacillus mojavensis* A12 to mice and the intraperitoneal injection of lipopeptide biosurfactant from *B. subtilis* SPB1 to mice. Both

were reported as promising for therapeutic application.[31, 32] Intraperitoneal administration of surfactin C, produced by *B. subtilis*, significantly improved survival and reduced the number of *E. coli* in a mouse model of septic shock.[33] In addition, many health benefits have been attributed due to lipopeptide producing *Bacillus* species. Several *Bacillus* species are recognized as probiotics for human and veterinary use. For example, Enterogermina® is a *Bacillus clausii* containing probiotic used for the prevention of infantile diarrhea.[34] *Bacillus* fermented foods have a long history of safe use. The Japanese product Natto is made of soy beans fermented by *B. subtilis var. natto*. Consumption of Natto has been associated with health benefits. The presence of substanial amounts of the lipopeptide surfactin has been demonstrated indicating a low toxicity towards humans.[35] Moreover, *in vivo* tests with *B. subtilis var. natto* indicated no signs of toxicity or virulence.[36] Oral administration of *B. subtilis* PB6 significantly improved intestinal pathology in a rat model of inflammatory bowel disease. This protection was attributed to the production of surfactins. In addition, a clinical study indicated that the strain was well tolerated in healthy volunteers.[37] Finally, recent evidence suggests that lipopeptide producing *Bacillus* species can be part of the indigenous intestinal microbiota from humans.[38] Taken together, these results indicate that surfactin has a low toxicity towards humans and shows great promise as possible alternative for conventional antibiotic therapy.

In humans, *C. difficile* infection is localized in the colon. To achieve a stable delivery at the site of target, orally administered antimicrobials need to be able to survive the harsh conditions of the gastrointestinal tract such as the acidity of the stomach and proteolytic digestion. Researchers previously reported that changes in pH or addition of proteases did not affect the antibacterial activity of surfactin.[28] We demonstrated that *B. amyloliquefaciens* SG1 supernatant retained its antibacterial activity against *C. difficile* over a quite large pH range. However, at very acidic pH values, all antibacterial activity disappeared but could be completely recovered by a subsequent increase in the pH. It is generally known that lipopeptides precipitate at low pH. Acid precipitation is commonly used to enrich lipopeptides as an initial step during the purification process.[39] In the acidic environment of the stomach, surfactin will probably precipitate but during further passage in the intestinal tract, it could regain its activity since it encounters a gradual rise in pH. The antibacterial activity was not affected by several digestive enzymes including amylase, pepsin and chymotrypsin. Loss of activity was noted due to trypsin, pancreatine and lipase treatment suggesting proteolytic digestion during gastrointestinal transit. However, all of these problems could be easily overcome by use of the *B. amyloliquefaciens* SG1 strain itself to obtain in situ production of surfactin at the site of target. *B. amyloliquefaciens* is on the Qualified Presumption of Safety list of the European Food Safety Authority based on the absence of toxigenic activity and acquired antibiotic resistance

genes.[40] Several studies indicate that *B. amyloliquefaciens* administration has a beneficial effect on the pathogenesis and progression of certain intestinal disorders. For example, orally administered *B. amyloliquefaciens* significantly reduced bacterial translocation in weaned mice.[41] Moreover, it has been proposed as a novel probiotic for a better management of inflammatory bowel disease since it successfully reduced intestinal inflammation in mice.

As most lipopeptides, surfactin exerts its antibacterial effect by targeting the bacterial cell membrane where it induces the formation of pores leading to membrane depolarization and eventually cell death.[42] Since surfactin is not yet used as a therapeutic, little information is available concerning the possible resistance development by bacteria. Resistance to surfactin has been reported for the soil bacterium *Streptomyces* sp. Mg1 due to the secretion of a hydrolytic enzyme that breaks down surfactin.[43] It is however postulated that development of resistance against lipopeptides is not straightforward since it involves the adaptation of a long evolved conserved structure. CB-183,315 is a novel cyclic lipopeptide analogue of daptomycin with potent activity against Gram-positive bacteria including *C. difficile*. Researchers reported the unlikelihood of resistance development based on its bactericidal mode of action, spontaneous resistance incidence studies and serial passages. Interestingly, enhanced activity has been reported towards *C. difficile* strains resistant to fluoroquinolones, clindamycin and metronidazole. CB-183,315 shows a limited activity against Gram-negative bacteria suggesting little collateral damage to and a rapid restoration of the normal gut microbiota. Its effectiveness in preventing initial and recurrent CDAD has been demonstrated using the classical hamster model. In addition, results of clinical trials indicate its use is safe and well tolerated. A phase 3 clinical trial is currently performed.[20, 44, 45]

Mass spectral analysis of the purified fraction showed that surfactin was responsible for the antibacterial activity against *C. difficile*. Confirmation of this results with a newly prepared sample however, also revealed the presence of a second peak with a molecular mass of 503. Due to its low concentration, we were unable to reveal its identity using MSMS analysis. Comparison of the mass spectra obtained from the first and second sample indicated a difference in the main surfactin homologues present. The first sample contained mainly C14-surfactin while the C13 surfactin homologue was most abundant in the second sample. It has been reported previously that the production of surfactant homologues can be influenced by the differences in the composition of the growth medium and incubation conditions.[46-48] To obtain additional evidence that surfactin is responsible for the antibacterial activity against *C. difficile*, commercially available surfactin was purchased and tested. A clear growth inhibition zone was detected demonstrating its antibacterial effect towards *C. difficile*. However, it still remains possible that *B. amyloliquefaciens* SG1 also produces other compounds with antibacterial activity against *C. difficile*.

Overall, these results indicate that surfactin produced by *B. amyloliquefaciens* SG1 encompasses antibacterial activity against *C. difficile* and could be a promising alternative for broad spectrum antibiotics in the treatment of CDI.

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CHAPTER 2

BACILLUS AMYLOLIQUEFACIENS SG1 AS PROPHYLACTIC TREATMENT FOR CLOSTRIDIUM DIFFICILE ASSOCIATED DISEASE IN A MOUSE MODEL

Adapted from Geeraerts, S., Ducatelle, R., Haesebrouck, F. & Van Immerseel, F. (2015) Journal of Gastroenterology and Hepatology 30(8) 1275-1280.

ABSTRACT

Probiotics might offer an attractive alternative for standard antibiotic therapy to treat *Clostridium difficile* infections. We specifically selected a *Bacillus amyloliquefaciens* strain for its high *in vitro* antibacterial activity against *C. difficile* and tested its efficacy to prevent CDI in a mouse model. *B. amyloliquefaciens* SG1 supernatant was tested against a large collection *C. difficile* strains using an agar well diffusion test. *B. amyloliquefaciens* SG1 was orally administered to C57BL/6 mice in which CDI was induced using *C. difficile* VPI 10463 and its effect was compared with control mice receiving no treatment and mice receiving *Saccharomyces boulardii*. Mice were followed up daily for signs of disease including weight loss. At necropsy, the colon was collected and subjected to histopathological analysis. *C. difficile* toxin A/B levels and colon weight/length and colon/body weight ratios were calculated. *B. amyloliquefaciens* SG1 supernatant was able to inhibit the growth of all *C. difficile* strains. Results of the *in vivo* trial indicated a significant weight loss for untreated and *S. boulardii* treated mice as compared to *B. amyloliquefaciens* SG1 treated mice. *C. difficile* toxin A and B levels were significantly higher for untreated and *S. boulardii* treated mice than *B amyloliquefaciens* SG1 treated mice. A significantly lower degree of colon damage was detected for *B. amyloliquefaciens* SG1 treated mice as compared to untreated and *S. boulardii* treated mice, based on histopathological analysis, colon weight/length and colon/body weight ratios. In conclusion, administration of *B. amyloliquefaciens* SG1 was successful in preventing CDI in a mouse model.

INTRODUCTION

The intestinal microbiota is important to preserve gut health since it forms an effective barrier against pathogens.[1] Disturbance of this complex ecosystem due to antibiotic consumption makes the host susceptible to gastrointestinal infections. Up to 25% of patients receiving antibiotics develop antibiotic associated diarrhea (AAD).[2] In humans, *Clostridium difficile* is responsible for 15 – 25% of all AAD cases and is the main cause of pseudomembranous colitis.[3]

Alteration of the normal intestinal microbiota is the most important trigger for CDI. Antibiotics administered to control any kind of infection can alter the resident gut microbiota, allowing ingested and subsequently germinated *C. difficile* spores to colonize the gut. Standard treatment involves the discontinuation of the inducing antibiotic, if possible, and administration of metronidazole or vancomycin, if necessary.[2] As stated by Johnson et al.[4], it seems contradictory that standard treatment of CDI involves the administration of antibiotics when disease outcome results from the disruptive effect of antibiotics on the colonic microbiota.

There is an urgent need to develop efficient methods for prevention and treatment of CDI that do not further disrupt the altered indigenous microbiota, help to restore the complex balance of the normal gut microbiota and limit the need for additional antibiotic therapy.[5] Probiotics might offer an attractive alternative to standard antibiotic therapy. They can protect the host against invading pathogens by repopulation of the gut with non-pathogenic microbiota, activation of the hosts immune system and production of antimicrobial compounds that specifically act against pathogenic microorganisms. A major advantage of probiotics is their relatively low production cost and the unlikeliness of increasing the incidence of antibiotic resistance.[1] Different probiotics have already been studied to prevent AAD and *C. difficile* associated diarrhea. The most conclusive results have been found for *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii*, both associated with a significant reduction in AAD.[6-11] Moreover, administration of *S. boulardii* combined with standard antibiotic therapy is efficient and safe in patients with recurrent CDI.[12]

Only commercially available probiotics have already been used for the treatment or prevention of CDI. To the best of our knowledge, probiotic microorganisms have never before been selected for their specific antibacterial activity against *C. difficile*. In this study, we evaluated the *in vitro* antimicrobial activity of *B. amyloliquefaciens* SG1 culture supernatant against a collection of *C. difficile* strains and its efficacy to prevent CDI in a mouse model.

MATERIALS & METHODS

GROWTH CONDITIONS AND INOCULUM PREPARATIONS

The antimicrobial activity of *B. amyloliquefaciens* SG1 was tested against a collection of 24 *C. difficile* ribotypes as listed in Table 1. *B. amyloliquefaciens* SG1 was grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) for 24h at 30°C. *C. difficile* strains were inoculated in reinforced clostridial medium (RCM, Oxoid) and incubated for 24h at 37°C in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) workstation (Ruskinn Technology, Bridgend, UK).

Table 1. *C. difficile* ribotypes used in the *in vitro* work. VPI 10463 corresponds to the reference strain.

BR001	BR017	BR053	BR087
BR002	BR020	BR056	BR095
BR003	BR023	BR070	BR106
BR012	BR027	BR075	BR126
BR014	BR029	BR078	BR131
BR015	BR046	BR081	VPI 10463

For the *in vivo* trial, *C. difficile* VPI 10463 was inoculated in broth containing proteose peptone (40 g/l), Na_2HPO_4 (5 g/l), KH_2PO_4(1 g/l), $MgSO_4.7H_2O$ (0.1 g/l), NaCl (2 g/l) and fructose (6 g/l) and incubated anaerobically for 36h at 37°C. *B. amyloliquefaciens* SG1 was inoculated in TSB and grown for 24h at 30°C. *Saccharomyces boulardii* was isolated from Enterol® (Biocodex, Gentilly Cedex, France), inoculated in sabouraud liquid medium (Oxoid) and incubated for 24h at 37°C. Cells were collected by centrifugation at 5000 x g for 15 min and suspended in GIBCO® Hank's Balanced Salt Solution (HBSS) (Life Technologies, Bleiswijk, The Netherlands). *C. difficile* VPI 10463 was suspended in HBSS with 0.1% cysteine-HCl (Merck, Darmstadt, Germany).

AGAR WELL DIFFUSION ASSAY

Cell free supernatant was prepared by filter sterilizing the supernatant of *B. amyloliquefaciens* SG1 grown in TSB. *C. difficile* cultures were diluted 1/50 in reinforced clostridial agar (Oxoid) and poured in sterile petri dishes. Wells were made in the agar and filled with 40 µl supernatant. Plates were incubated anaerobically overnight at 37°C and evaluated for growth inhibition zones.

IN VIVO TRIAL

MICE

Nine week old female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed in groups of 4 or 5 animals per cage of 820 cm^2. Cages, water, feed and bedding were autoclaved prior to contact with the animals. Feed and water were accessible ad libitum. Upon arrival, mice were acclimated for two weeks in the research facilities.

CLOSTRIDIUM DIFFICILE INDUCED COLITIS

CDI was induced based on the protocol described by Chen et al.[13] An antibiotic cocktail [kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml) and vancomycin (0.045 mg/ml)] was administered in the drinking water for 3 days (days -6 to -3) followed by an i.p. clindamycin injection (20 mg/kg) two days later (day -1). After 24 hours, animals were infected with *C. difficile* VPI 10463 (± 1 x 10^6 cfu) by oral gavage and monitored for signs of disease. Moribund and all surviving mice at day 5 post infection were euthanized. All experimental procedures involving animals were approved by the ethical committee of the Faculty of Veterinary Medicine of Ghent University.

EXPERIMENTAL TREATMENT

Mice were randomly allocated to 1 of 3 different groups with 14 animals per group and treated with *B. amyloliquefaciens* (± 6 x 10^8 cfu), *S. boulardii* (± 3 x 10^8 cfu) or remained untreated. All treatments were administered by oral gavage. The first treatment dose was given 1 hour after clindamycin injection and repeated 3 times daily with a 4 hour interval during 6 days. HBSS was administered to the control group receiving no treatment. Figure 1 gives an overview of the study design used in this experiment.

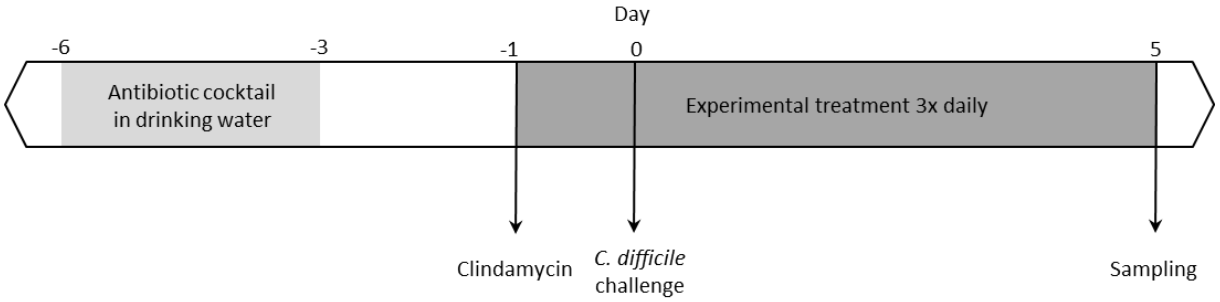

Figure 1. Experimental set up. CDI was induced in C57BL/6 mice after disruption of the gut microbiota using an antibiotic cocktail [kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml) and vancomycin (0.045 mg/ml)] by the oral route and clindamycin (20 mg/kg) by injection. Experimental treatments consisted of orally administered *B. amyloliquefaciens* SG1 (± 6 x 10^8 cfu) or *S. boulardii* (± 3 x 10^8 cfu). HBSS was administered to the untreated control group. All treatments were started 1 day prior to *C. difficile* challenge and repeated 3x daily until 5 days post infection.

NECROPSY

Surviving animals were sacrificed by cervical dislocation 5 days post infection. The colon of each mouse was harvested and the content collected. Colon weight/length (mg/cm) and colon/body weight (mg/g) ratios were calculated for each mouse individually.

HISTOPATHOLOGICAL ANALYSIS

For each mouse, small segments of colon, at the transition of the proximal and distal part, were collected and fixed in a 4% phosphate buffered formaldehyde solution. Paraffin embedded samples were sliced in 5 µm sections and stained with hematoxylin-eosin for light microscopic examination. Histological damage of colon segments was evaluated based on a scoring system reported by Chen et al.[13] The severity of the following parameters was taking into account: neutrophil margination and tissue infiltration, hemorrhagic congestion and edema of the mucosa and epithelial cell damage. For each parameter, a score of 0 to 3 was given corresponding to an increasing severity of damage (no, mild, moderate, severe). The sum of the individual parameter scores was calculated as the total histological score for each sample.

QUANTIFICATION OF C. DIFFICILE TOXINS IN THE COLON CONTENT

The presence of *C. difficile* toxins was determined using the commercial C. DIFF TOXA/B II ELISA kit (Techlab, Blacksburg, Virginia, USA) according to a modified protocol as described by the suppliers. Colonic content samples were stored at -20°C prior to analysis. Samples were thawed and diluted 1:9 in assay diluent. A two-fold dilution series of the positive control supplied in the kit was made with assay diluent to generate a standard curve. As a negative control, only assay diluent was used. Fifty microliters of conjugate containing labeled antibodies against toxins A and B, and 100 µl of the

samples or controls were transferred to the assay wells and incubated at 37°C for 50 min. Toxin-antibody complexes were retained by binding to immobilized antibodies on the assay wells. The wells were rinsed four times with the provided wash solution to remove any unbound material. To estimate the amount of toxin present in the samples, a color reaction was induced by adding 100 µl of the substrate solution. The plate was incubated for 10 minutes at room temperature. Fifty microliters of 0.6 N sulfuric acid was added to stop the color reaction and the optical density of each well was measured at 450 nm using a Multiskan RC/MS/EX (Artisan technology group, Champaign, Illinois, USA) ELISA reader. The total toxin titer was determined by applying a Hill function to the concentration-response data (GraphPad Prism 5, GraphPad Software, San Diego, CA, USA) and expressed as arbitrary units (A.U.).

STATISTICAL ANALYSIS

Results are expressed as mean values ± standard error on the mean and analyzed for statistical significance using a non-parametric one-way ANOVA with the Kruskall-Wallis test and Dunns' post-test with the GraphPad Prism 5.0 software. Survival rates between treatment groups were analyzed with the log rank (Mantel-Cox) test. In all analyses, p-values less than 0.05 were considered as statistically significant.

RESULTS

B. AMYLOLIQUEFACIENS SG1 SUPERNATANT INHIBITS THE GROWTH OF C. DIFFICILE

Antimicrobial activity of *B. amyloliquefaciens* SG1 culture supernatant was tested against different *C. difficile* ribotypes. After incubation, zones of growth inhibition were observed against all *C. difficile* ribotypes. Figure 2 represents the growth inhibition of *C. difficile* VPI 10463 by *B. amyloliquefaciens* SG1 supernatant. Little variation in size of the growth inhibition zones was observed against all *C. difficile* ribotypes.

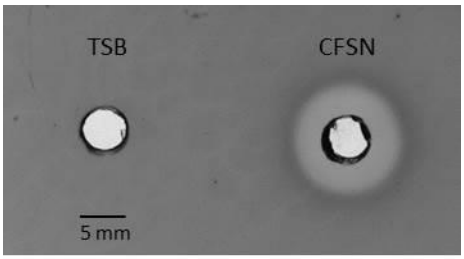

Figure 2. Growth inhibition of *C. difficile* VPI 10463 by cell free supernatant of a *B. amyloliquefaciens* SG1 overnight culture grown in TSB. TSB was included as a control to exclude any influence of the medium itself.

B. AMYLOLIQUEFACIENS IMPROVES CLINICAL SIGNS AND SURVIVAL AFTER CDI

To assess the potential prophylactic use of *B. amyloliquefaciens* SG1 against CDI, C57BL/6 mice were treated with *B. amyloliquefaciens* SG1 prior to CDI. Untreated and *S. boulardii* treated mice showed signs of severe diarrhea and dehydration 48h post infection. *B. amyloliquefaciens* SG1 treated mice had no or very little diarrhea. At necropsy, formed stool could be detected in the colon of *B. amyloliquefaciens* SG1 treated mice while no or watery stool was observed in the colon of control or *S. boulardii* treated mice. In the control and *S. boulardii* treated group, respectively 50% and 57% of mice became moribund while none of the *B. amyloliquefaciens* SG1 treated mice reached clinical end points requiring euthanasia. Figure 3 shows Kaplan-Meier survival plots for all groups.

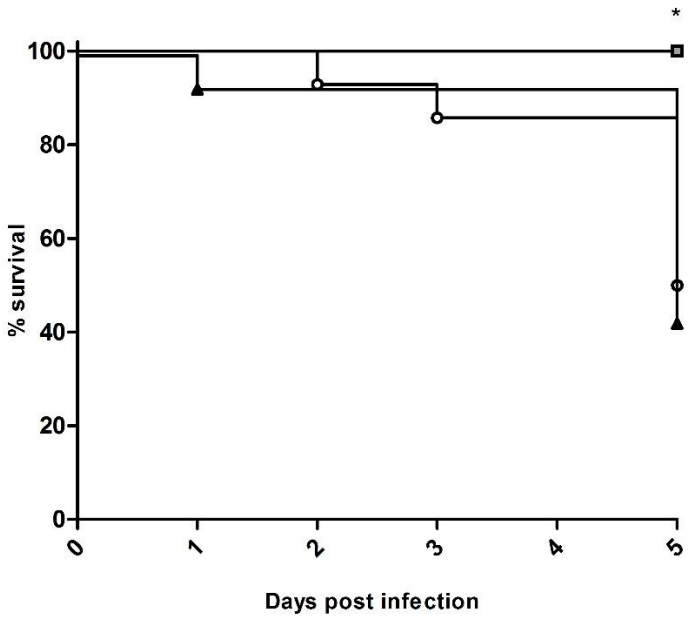

Figure 3. Kaplan-Meier survival plot for mice treated with *B. amyloliquefaciens* SG1 or *S. boulardii* and untreated control mice. During the experiment, 50% of the untreated control mice and 57% of the *S. boulardii* treated mice died or became moribund and required euthanasia. All mice treated with *B. amyloliquefaciens* SG1 survived throughout the study period with statistical significant differences as compared to both other groups (* $p < 0.5$). Control; *B. amyloliquefaciens*; *S. boulardii*.

B. AMYLOLIQUEFACIENS TREATMENT PROTECTS MICE FROM SEVERE WEIGHT LOSS

Weights were recorded daily and relative weight losses were calculated starting one day prior to infection (Figure 4). All mice showed a progressive weight loss 48h after CDI. *B. amyloliquefaciens* SG1 treated mice showed a smaller decrease in weight in comparison with control and *S. boulardii* treated mice, with significant differences from day 2 until the end of the experiment. A complete overview of all weight losses is represented in table 2. On day 5 post infection, *B. amyloliquefaciens* SG1 treated mice showed an average weight loss of 8.32% which was significant less than 19.41% for untreated and 17.14% for *S. boulardii* treated mice.

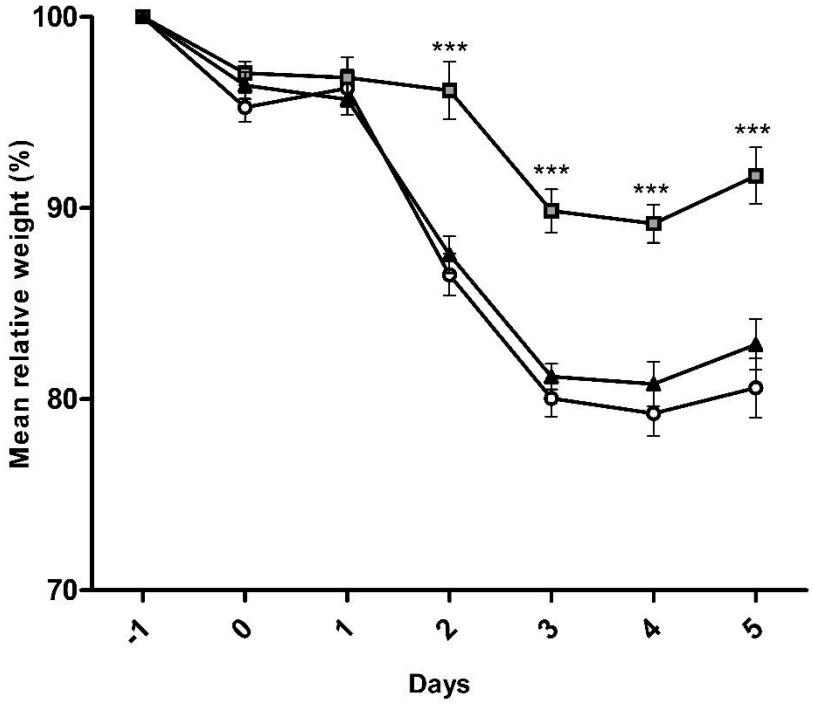

Figure 4. Mean relative weight graph of all infected mice (mean ± standard error on the mean). Treatment with *B. amyloliquefaciens* SG1 and *S. boulardii* started 1 day prior to infection (day -1) and lasted for 6 consecutive days. Relative weight was based on the weight at day -1. An average weight loss of 19.41% and 17.14% was detected for the untreated control and *S. boulardii* treated mice compared to only 8.32% for the *B. amyloliquefaciens* SG1 treated mice on day 5 post infection. Statistical significant differences between the *B. amyloliquefaciens* SG1 treated mice and untreated control mice are represented (*** $p < 0.001$).

Control; *B. amyloliquefaciens*; *S. boulardii*.

Table 2. Observed average relative weight losses for untreated control, *B. amyloliquefaciens* SG1 and *S. boulardii* treated mice from the day of infection until the end of the experiment. All mice showed progressive weight loss but *B. amyloliquefaciens* SG1 treatment protected mice from severe weight loss as compared to untreated control and *S. boulardii* treated mice with statistical significant differences from day 2 until day 5 post infection. Different superscripts indicate significant differences between the groups ($p < 0.05$).

Treatment group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Control	95.27 ± 0.76	96.28 ± 0.97	86.52 ± 1.10^{a}	80.03 ± 0.96^{a}	79.26 ± 1.19^{a}	80.59 ± 1.55^{a}
***B. amyloliquefaciens* SG1**	97.06 ± 0.61	96.82 ± 1.08	96.15 ± 1.51^{ab}	89.85 ± 1.13^{ab}	89.18 ± 0.99^{ab}	91.69 ± 1.48^{ab}
S. boulardii	96.42 ± 0.71	95.68 ± 0.81	87.56 ± 0.96^{b}	81.18 ± 0.67^{b}	80.79 ± 1.17^{b}	82.86 ± 1.34^{b}

B. AMYLOLIQUEFACIENS SG1 TREATMENT REDUCES COLONIC C. DIFFICILE TOXIN

To demonstrate that the observed pathology was caused by *C. difficile*, toxin A and B levels were determined in colonic content samples after necropsy using a *C. difficile* toxin A/B II ELISA kit. High mean toxin levels were detected in the colon contents of control and *S. boulardii* treated mice, resp. 63.59 ± 10.97 A.U. and 77.63 ± 7.27 A.U., whereas mean toxin levels remained significantly lower in *B. amyloliquefaciens* SG1 treated mice with an average of 4.99 ± 1.74 A.U. (Figure 5).

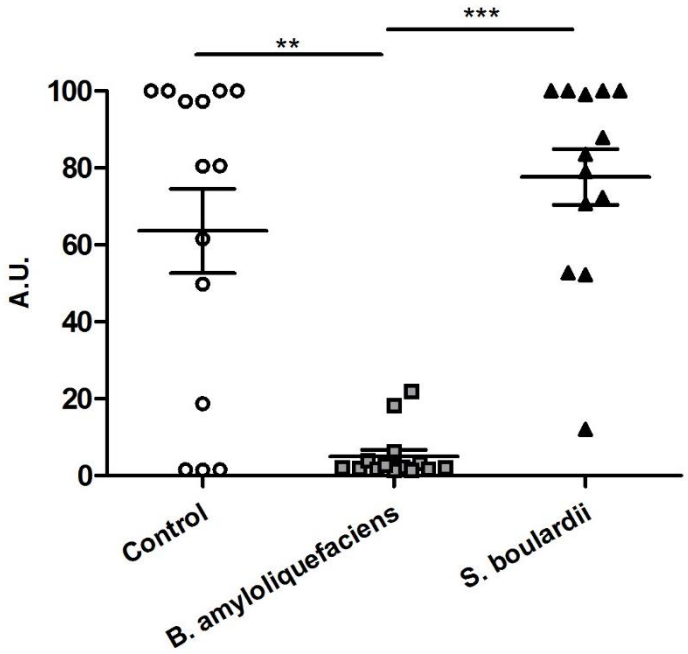

Figure 5. *C. difficile* toxin A/B levels were measured in colon samples of all mice using a *C. difficile* toxin A/B II ELISA kit and expressed as activity units (A.U.) (mean ± standard error on the mean). Toxin levels in colon samples from untreated control mice and *S. boulardii* treated mice reached 63.59 A.U. and 77.63 A.U. respectively whereas toxin levels remained low or undetectable in the group treated with *B. amyloliquefaciens* SG1 with an average of 4.99 A.U. (** $p < 0.01$; *** $p < 0.001$).

B. AMYLOLIQUEFACIENS SG1 TREATMENT IMPROVES COLONIC HISTOPATHOLOGY

Examination of colon samples from control mice clearly indicated the specific histopathology associated with CDI in mice including the presence of submucosal edema, neutrophil infiltration and epithelial cell damage (Figure 6).[13] Figure 7 shows images of three mice per treatment group. An average score of 4.21 ± 1.89 was calculated for control mice compared to 2.14 ± 1.17 for *B. amyloliquefaciens* SG1 treated mice. For *S. boulardii* treated mice, the total score was 3.29 ± 1.38. Significances were only found between control and *B. amyloliquefaciens* SG1 treated mice. Intestinal inflammation was also assessed by calculating colon weight/length and colon/body weight ratios for each mouse individually and were respectively 42.77 ± 2.87 mg/cm and 14.71 ± 0.90 mg/g for the

control group, 30.05 ± 1.26 mg/cm and 10.98 ± 0.35 mg/g for the *B. amyloliquefaciens* SG1 group and 40.21 mg/cm ± 1.79 and 15.38 ± 0.64 mg/g for the *S. boulardii* group (Figure 8). Both ratios were significantly lower for *B. amyloliquefaciens* SG1 treated mice as compared to control and *S. boulardii* treated mice.

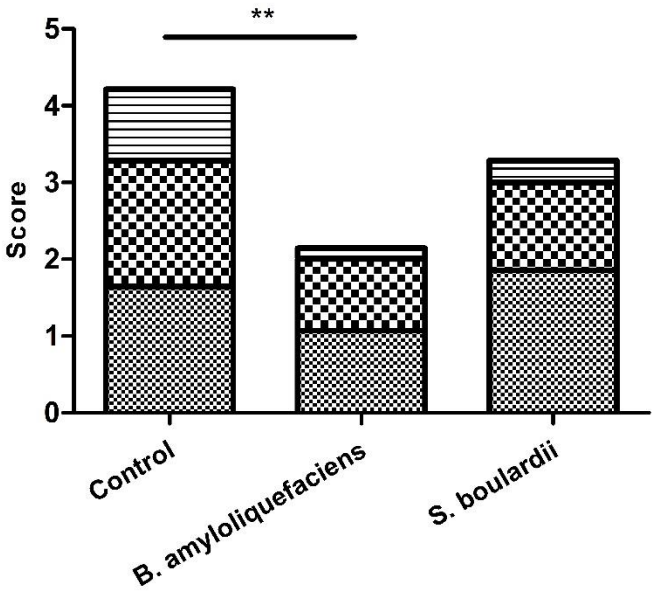

Figure 6. Histopathological scores of colon samples. Intestinal damage was graded based on the degree of epithelial cell damage, the amount of congestion and submucosal edema and the infiltration of neutrophils. The total score was calculated by summing the scores of all three individual parameters and is as follows: control, 4.21 ± 0.50; *B. amyloliquefaciens* SG1 group, 2.14 ± 0.31; *S. boulardii* group, 3.29 ± 0.37. A statistically significant difference was observed between the control group and the *B. amyloliquefaciens* SG1 group (** $p < 0.01$). epithelial cell damage; congestion/edema; neutrophil infiltration.

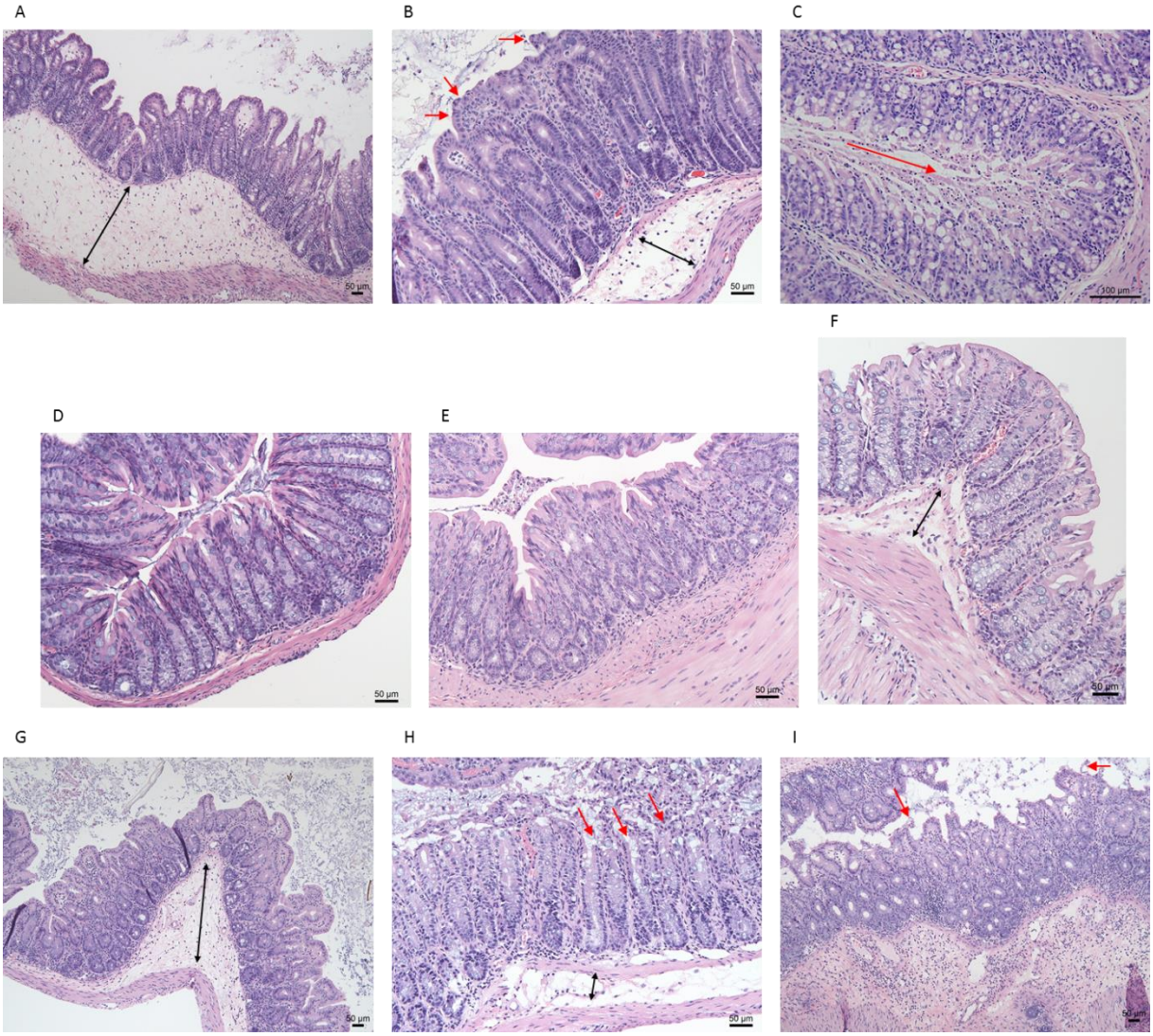

Figure 7. Light microscopic image of colon sections of three mice per treatment group. Red arrows indicate the presence of epithelial cell damage. Black arrows point out areas with edema of the submucosa. A – C: untreated control mice; A – histopathological score = 4 (severe edema in the lamina propria and submucosa, presence of inflammatory cells); B – histopathological score = 5 (great influx of inflammatory cells, low amount of edema of the submucosa, minor epithelial cell damage); C – histopathological score = 6 (severe epithelial cell damage). D – F: *B. amyloliquefaciens* SG1 treated mice; D – histopathological score = 1 (small amount of inflammatory cells, no edema of the submucosa, no epithelial cell damage); E – histopathological score = 1 (small amount of inflammatory cells in the lamina propria and submucosa, no edema of the submucosa, no epithelial cell damage); F – histopathological score = 2 (small amount of inflammatory cells, some edema of the submucosa, no remarkable epithelial cell damage). G – I: *S. boulardii* treated mice; G – histopathological score = 3 (influx of inflammatory cells, edema of the mucosa, no remarkable damage of epithelial cells); H – histopathological score = 3 (moderate damage to the epithelium, presence of inflammatory cells in the lamina propria); I – histopathological score = 4 (severe inflammation in the lamina propria and the submucosa, some epithelial cell damage)

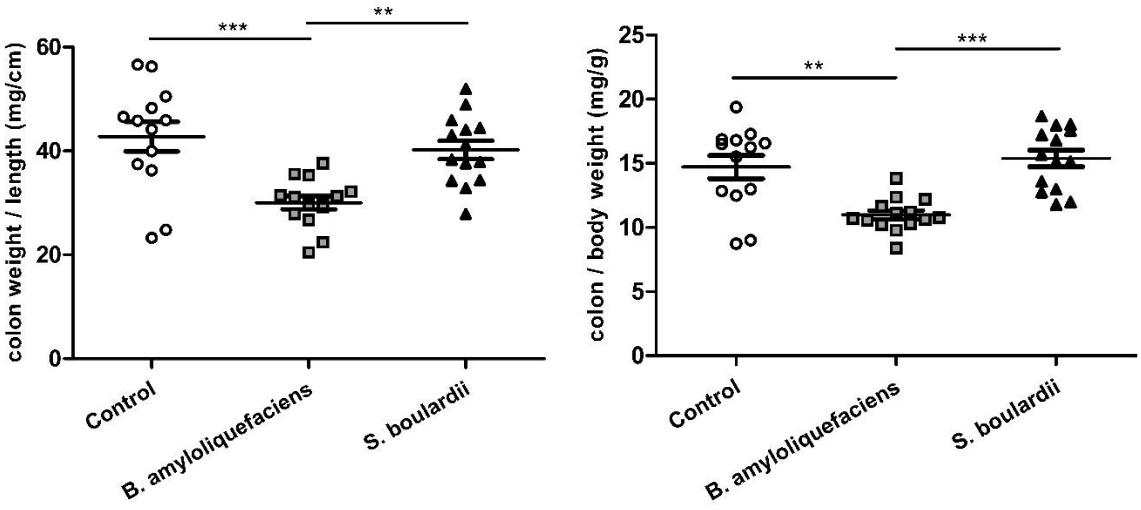

Figure 8. (A) Colon weight/length (mg/cm) and (B) colon/body weight (mg/g) ratios. Both ratios were calculated as parameters of intestinal inflammation. The average colon weight/length ratios and colon/body weight ratios were respectively 42.77 ± 2.87 mg/cm and 14.71 ± 0.90 mg/g for the control group, 30.05 ± 1.26 mg/cm and 10.98 ± 0.35 mg/g for the *B. amyloliquefaciens* SG1 group and 40.21 mg/cm ± 1.79 and 15.38 ± 0.64 mg/g for the *S. boulardii* group (mean ± SEM). For both ratios, the *B. amyloliquefaciens* SG1 treated mice showed statistically significant differences when compared to the untreated and *S. boulardii* treated mice.

DISCUSSION

Nowadays, the search for alternative treatments against CDI is receiving more and more attention. Probiotics have already been successfully used to prevent AAD.[6, 7, 14, 15] A recent open prospective study states that administration of a probiotic in addition to the existing standard preventive measures for CDI leads to a reduction in CDI rates.[16] Moreover, different meta-analyses indicate that the use of probiotics reduces the risk of AAD and CDI.[17-20] This study provides data of an *in vivo* model in which a possible probiotic strain is tested for its potential to control CDI. To our knowledge, this is the first report of a possible probiotic treatment in which the probiotic strain is specifically selected for its antibacterial activity against *C. difficile*. Screening of a collection of clinical *C. difficile* isolates revealed high susceptibility of all isolates against *B. amyloliquefaciens* SG1 supernatant. Although *B. amyloliquefaciens* SG1 is not generally recognized as safe, it is considered by the European Food Safety Authority to be suitable for the qualified presumption of safety approach to safety assessment. [21, 22]

S. boulardii is a probiotic yeast successfully used in clinical trials to decrease the incidence of AAD and to prevent recurrent CDI.[7, 8, 12, 23] Results of animal studies indicate that S*. boulardii* improves survival rates of hamsters after clindamycin induced mortality and that continuous treatment with *S. boulardii* in the drinking water protected gnotobiotic and axenic mice from *C. difficile* induced mortality.[24-26] Therefore, *S. boulardii* was included in our study as a reference treatment. In our experiments, treatment with *S. boulardii* did not confer any protection against CDI. As observed previously, the administered concentration of the probiotic organism is important for protection against CDI.[17, 25] In our study, similar doses of approximately 10^8 cfu *B. amyloliquefaciens* SG1 and *S. boulardii* were administered to mice to compare the effect of both treatments. However, previous studies used higher doses of *S. boulardii* which might explain the lack of protection in our trial.[24, 25, 27] Another important difference is the *in vivo* model used. Previous studies focused on axenic and gnotobiotic mice or hamsters while our study is based on the recently published mouse model for CDI that has already been successfully used to study this disease.[13, 24, 25]

CDI was successfully induced in the animals since all control mice suffered from severe diarrhea and dehydration after *C. difficile* challenge. However, as compared to the original publication, the concentration clindamycin needed to be doubled and a tenfold higher *C. difficile* inoculum had to be used to induce CDI. In spite of these changes, the average histopathological score for the control mice was only 4.2 as compared to 7.0 as reported by Chen et al.[13] A number of reasons could explain this discrepancy including the mouse supplier, the used mouse strain and the animal care facilities.

B. amyloliquefaciens SG1 administration was started 24 hours prior to CDI to give the bacteria the chance to colonize the intestines and produce their antimicrobial substances. Results of our *in vivo* trial show that oral administration of 10^8 cfu *B. amyloliquefaciens* SG1 three times daily was successful in preventing CDI. *C. difficile* toxin A/B titers were determined in colonic content samples to confirm that *C. difficile* was the cause of the observed disease. High toxin levels were found in control and *S. boulardii* treated mice indicating that CDI was successfully established. Use of the ELISA kit only estimates the amount of *C. difficile* toxins but does not quantify the numbers of *C. difficile* present in the colon.[28] Low levels of *C. difficile* toxins were found in the colon content of *B. amyloliquefaciens* SG1 treated mice. Unfortunately, the amount of colon content was insufficient to quantify the toxin titers as well as the actual number of *C. difficile* so it cannot be concluded if *B. amyloliquefaciens* SG1 only neutralizes the toxins or can indeed prevent the colonization of *C. difficile*.

Hell et al. postulated that only a multistrain probiotic which resembles the indigenous microbiota could be effective for CDI.[29] Results of our *in vivo* trial however clearly indicate that use of the single strain *B. amyloliquefaciens* SG1 is sufficient in the protection against CDI and eliminates the need to search for complex probiotic formulations. Since *B. amyloliquefaciens* SG1 was specifically selected for its high *in vitro* antibacterial activity against *C. difficile*, this might be the major explanation for the observed protection *in vivo*. Moreover, *Bacillus* species are known to produce different antimicrobial peptides including antibiotics, bacteriocins and lipopeptides. Genome analysis of *B. amyloliquefaciens* FZB42 estimates that as much as 8.5% of the genome is devoted to antimicrobial peptide production.[30] Results of our *in vitro* tests demonstrate that *B. amyloliquefaciens* SG1 produces antimicrobial compounds that act against *C. difficile*. Further research is necessary to determine the nature of these antimicrobial compounds and the effect on the gut microbiota composition.

Preventive treatment with a probiotic during antibiotic therapy implies that the probiotic strain is able to withstand the administered antibiotic and colonize the gut. Several bacterial strains have been successfully used in clinical trials during antibiotic therapy to protect against AAD and CDI indicating the possibility of bacteria to colonize the gut and resist antibiotics. Administration of a probiotic drink containing *Lactobacillus casei* SHIROTA during antibiotic therapy successfully reduced the incidences of AAD and CDI as compared to the control receiving a placebo.[31, 32] Moreover, combinations of *Lactobacillus* and *Bifidobacterium* or *Streptococcus* were able to lower the incidence of CDI.[33, 34] The potential of *B. amyloliquefaciens* SG1 to resist antibiotics and protect against AAD and CDI requires further research.

In conclusion, in the present study, we document a protective effect of *B. amyloliquefaciens* SG1 against CDI in an animal model. Administration of *B. amyloliquefaciens* SG1 as a probiotic to people at risk for developing CDI might help to control morbidity and mortality due to this disease and reduce the associated healthcare costs. Further research is necessary to evaluate the safety and efficacy of the strain in healthy volunteers and patients, and to study the mechanisms responsible for the protection.

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CHAPTER 3

VEGETATIVE BACILLUS AMYLOLIQUEFACIENS SG1 STRAINS DO NOT CONFER PROTECTION AGAINST BROILER NECROTIC ENTERITIS DESPITE HIGH ANTIBACTERIAL ACTIVITY OF ITS SUPERNATANT AGAINST CLOSTRIDIUM PERFRINGENS IN VITRO

Adapted from Geeraerts, S., Delezie, E., Ducatelle, R., Haesebrouck, F., Devreese, B. & Van Immerseel, F. (2016) British Poultry Science 57 (3) 324-329.

ABSTRACT

The ban on the use of antibiotics as feed-additives has led to a flare in the incidence *of Clostridium perfringens* induced necrotic enteritis in broilers. In this study, the effect of *Bacillus amyloliquefaciens* SG1 on *Clostridium perfringens* was tested *in vitro* and *in vivo*. Using an agar well diffusion assay, the inhibitory activity of *B. amyloliquefaciens* SG1 supernatant was analyzed against a large collection of NetB positive and NetB negative *C. perfringens* strains. Although strong growth inhibiting activity was detected against all *C. perfringens* isolates, it was significantly higher against virulent NetB positive *C. perfringens* strains as compared to avirulent NetB negative isolates. Subsequently, the efficacy of in-feed administration of lyophilized vegetative cells of *B. amyloliquefaciens* SG1 to prevent necrotic enteritis was tested *in vivo* using an established experimental infection model in broilers. Ross 308 broilers received either *B. amyloliquefaciens* SG1 supplemented feed or unsupplemented feed throughout the experiment. No significant differences could be detected between the untreated positive control group and the *B. amyloliquefaciens* SG1 treated group in body weight, the number of chickens that developed necrotic lesions and in pathological lesion scores. Our results demonstrate that despite its substantial inhibitory activity *in vitro*, lyophilized vegetative *B. amyloliquefaciens* SG1 cells had no beneficial effect against necrotic enteritis in the *in vivo* model used here.

INTRODUCTION

Necrotic enteritis (NE) is an important disease in broilers and is responsible for substantial economic losses, reduced animal welfare and increased mortality. In the past, addition of growth promoting antibiotics to the feed provided effective NE control.[1, 2] However, concerns about increased antibiotic resistance development and spread has eventually led to a ban on the use of growth-promoting antibiotics in the European Union.[3] This ban has caused NE to become a re-emerging problem in broilers.[4-6] The use of probiotics as feed additives offers an attractive alternative for controlling the disease.[7]

Probiotics are live microorganisms that confer a health benefit to the host when administered in appropriate amounts. They can aid in maintaining the complex ecosystem of the gut microbiota by competitive exclusion, stimulation of the host's immune system, competition for adhesion sites and the production of antimicrobial compounds that act against invading pathogens.[8] Several probiotics have already been tested as feed additives for intestinal pathogen inhibition. A *Lactobacillus*-based probiotic was shown to reduce the severity of NE in an experimental challenge model as well as in a field outbreak of NE.[9] *Bacillus* species are widely used as probiotics in human and veterinary medicine.[10] Both *B. licheniformis* and *B. subtilis* were able to reduce NE lesion score and mortality.[11-13] These findings indicate that the use of *Bacillus* species as probiotics can be an attractive alternative for growth promoting antibiotics in the feed to protect against NE. *B. amyloliquefaciens* has interesting functional probiotic properties including high tolerance to low pH and bile salts and the production of extracellular antimicrobial compounds.[14] Jerzsele et al. indicated that administration of a spore suspension of *B. amyloliquefaciens* was ineffective in preventing NE in broilers.[15] However, previous research at our department demonstrated that vegetative cells of a *B. amyloliquefaciens* SG1 strain isolated from the environment were effective in preventing *C. difficile* associated disease in a mouse model.[16] Therefore, in the current study, we first evaluated the *in vitro* antimicrobial potential of *B. amyloliquefaciens* SG1 culture supernatant against a collection of *C. perfringens* strains isolated from healthy chickens as well as isolates from chickens suffering from NE. Thereafter, we tested whether in-feed supplementation of lyophilized vegetative *B. amyloliquefaciens* SG1 cells was effective in preventing NE caused by *C. perfringens* in an experimental infection model in broilers.

MATERIALS AND METHODS

AGAR WELL DIFFUSION ASSAY

B. amyloliquefaciens SG1 was grown overnight in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 30°C with agitation. Bacterial cells were removed by centrifugation at 5000 x g for 15 min at 4°C and the supernatant was filter-sterilized. The antibacterial effect of the supernatant was tested against a collection of 73 *C. perfringens* strains. Fifty-nine strains were isolated from broiler chickens in Belgium: 25 avirulent NetB negative strains and 34 virulent NetB positive strains.[17] An additional 14 NetB positive strains isolated from broilers with necrotic enteritis in Denmark were also included in the test and were kindly provided by Dr. L. Bjerrum.[18] All *C. perfringens* strains were grown overnight in brain heart infusion broth (BHI) (Bio-Rad, Temse, Belgium) at 37°C in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) workstation (Ruskinn Technology, Bridgend, UK). *C. perfringens* overnight cultures were diluted 1/10 in sterile GIBCO® Hank's Balanced Salt Solution (HBSS) (Life Technologies, Bleiswijk, The Netherlands) and swabbed on BHI agar plates. Using the back of sterilized tips, wells were made in the agar and filled with 40 µl of the sterilized *B. amyloliquefaciens* SG1 supernatant. Plates were left to dry and incubated anaerobically overnight at 37°C. The size of the observed growth inhibition zones was measured. Experiments were repeated three times.

IN VIVO NECROTIC ENTERITIS MODEL

BACTERIAL STRAINS AND GROWTH CONDITIONS.

B. amyloliquefaciens SG1 was grown in TSB for 24h at 30°C with agitation. Cells were collected by centrifugation at 5000 x g for 15 min at 4°C and lyophilized in 75% GIBCO® horse serum (Life Technologies, Bleiswijk, The Netherlands) supplemented with 7.5% sucrose (Acros Organics, Geel, Belgium) and 0.625% nutrient broth (Oxoid, Aalst, Belgium). The number of vegetative cells and spores in the lyophilized product was determined. A small amount of the lyophilized product was suspended in HBSS and divided in two parts. To estimate the amount of spores, one sample was placed in a water bath at 80°C for 15 min to kill all vegetative cells.[19] The other sample was left untreated and used to determine the amount of spores and vegetative cells. A tenfold dilution series of both samples was plated on Columbia agar plates with 5% sheep blood and incubated at 30°C. (Oxoid, Aalst, Belgium) After overnight incubation, the number of CFU spores and vegetative cells per gram lyophilized product was determined by counting the number of colonies on the agar plates. *C. perfringens* strain 56 was used for experimental infection of chickens. The strain was originally isolated from the intestinal tract of a broiler with severe necrotic gut lesions and has already been used successfully to induce NE in broilers on different occasions.[20-22] It is a NetB positive toxinotype A strain that produces moderate amounts of alpha toxin *in vitro*.[17] For use in experimental infection

studies, this strain was grown in anaerobic BHI. Incubation was performed at 37°C in an anaerobic (84% N_2, 8% CO_2 and 8% H_2) workstation. Broilers were inoculated 3 times daily with 1 ml of this culture. For the first inoculation, overnight cultures of the strains were used. The second and third inoculations were performed with bacteria grown for 4 hours.

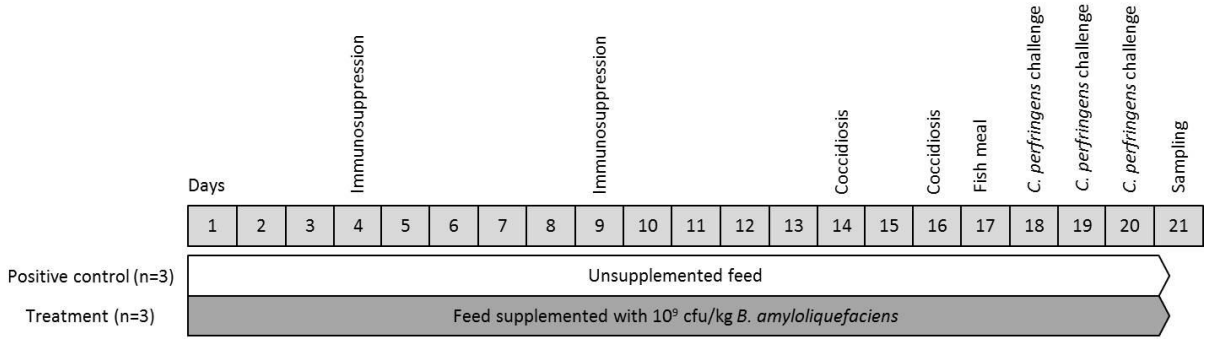

Figure 1. Experimental set up. Experimental treatment constisted of in-feed supplementation of 10^9 cfu/kg lyophilized vegetative *B. amyloliquefaciens* SG1 cells. Birds received the treatment throughout the whole trial. Positive control groups received unsupplemented feed. Immunosuppression on day 4 and day 9 was induced by oral vaccination of all animals with Poulvac Bursa Plus. On days 14 and 16, animals were predisposed for coccidiosis by oral administration of a 10-fold dose of Hipracox and Paracox-8 respectively. On day 17 the feed was changed to feed containing fish meal as a protein source. On days 18, 19 and 20, broilers were challenged with 5 x 10^8 cfu *C. perfringens* strain 56 to induce NE. All animals were euthanized on day 21.

EXPERIMENTAL DESIGN.

The *in vivo* model used was based on the method of Gholamiandehkordi et al. (2007) with some modifications. Figure 1 gives an overview of the experimental set up. One-day-old, unvaccinated Ross 308 broiler chickens were obtained from a commercial hatchery and divided in 6 groups of 27 animals. Each group was housed in a cage of 1.44 m^2. All groups were randomly distributed throughout the stable. Water and feed were available ad libitum. The animals were fed a wheat/rye-based (43%/7.5%) diet with soybean meal as protein source. The exact feed composition has been described elsewhere.[20] From day 17 until the end of the experiment the diet was changed to feed with 30% fish meal as protein source. Lyophilized cells of *B. amyloliquefaciens* SG1 were mixed with the feed at a concentration of 10^9 cfu/kg feed. To achieve statistical relevance, the treatment was supplied to three groups of animals. These broilers received the supplemented feed during the entire experiment. Birds in the three untreated, positive control groups received feed without *B. amyloliquefaciens* SG1. A light/darkness schedule of 18 h/6 h was applied. To induce mild immunosuppression, broilers were vaccinated orally on day 4 and 9 with the commercial Poulvac

Bursa Plus vaccine, containing attenuated infectious bursal disease virus (Zoetis, Zaventem, Belgium). On days 14 and 16, all animals received a tenfold dose of Hipracox (Hipra, Melle, Belgium) and Paracox-8 (MSD Animal Health, Brussels, Belgium) respectively. Both vaccines contain viable, sporulated *Eimeria* oocysts of different lines of coccidia and are administered as predisposing factors for NE. Birds were inoculated orally 3 times daily with approximately 5.10^8 cfu *C. perfringens* strain 56 on days 18, 19 and 20. On day 21, animals were euthanized with an overdose of sodium pentobarbital administered intravenously (Sigma-Aldrich, St. Louis, MO). Body weights were recorded and the intestinal tract of each bird was collected for macroscopic evaluation. All experimental procedures involving animals were approved by the ethical committee of the Faculty of Veterinary Medicine of Ghent University (EC 2015/08).

MACROSCOPIC EVALUATION.

NE was evaluated by scoring lesions in the small intestine (duodenum, jejunum, ileum) as described previously by Keyburn et al. Birds with a lesion score of 2 or more were considered as NE positive.[23]

STATISTICAL ANALYSIS.

Statistical analysis was performed using the GraphPad Prism 5.0 software. All results are expressed as mean values ± standard error on the mean (SEM). For the *in vitro* experiment, a Mann Whitney test was used to analyze significant differences in the size of the inhibition zone between NetB positive and NetB negative *C. perfringens* strains. In the *in vivo* experiment, 1 group was considered as 1 experimental unit since birds in one cage are not independent from each other. The experiment was done in triplicate yielding three independent replicates per treatment. Body weights were analyzed using an independent samples t-test. A Mann Whitney test was used to determine significance in the lesion score and number of positive chickens between the untreated control groups and the *B. amyloliquefaciens* treated groups. Differences were considered as statistical significant if $p < 0.05$.

RESULTS

AGAR WELL DIFFUSION ASSAY

An agar well diffusion assay was used to analyze the growth inhibiting ability of *B. amyloliquefaciens* SG1 against *C. perfringens*. All *C. perfringens* isolates were inhibited by *B. amyloliquefaciens* SG1 supernatant. As represented in Figure 2, virulent NetB positive isolates were inhibited to a much greater extent with inhibition zones of up to 30 mm. Seventy five percent of the NetB positive strains have an inhibition zone that is larger than the mean inhibition zone of all NetB negative strains. The average size of the inhibition zone was 14.69 ± 0.48 mm for NetB negative and 18.24 ± 0.77 mm for NetB positive *C. perfringens* strains. Statistical analysis indicated that the observed difference between both groups was significant ($p < 0.001$).

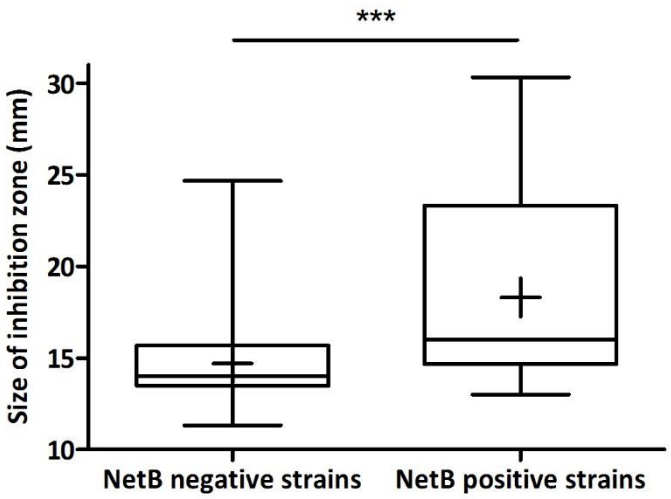

Figure 2. Box plot showing the measured inhibition zones for NetB negative and NetB positive *C. perfringens* strains. The plus represents the mean value and the whiskers are the median, the min/max value and 1st/3rd quartiles. Statistical significances are indicated (*** $p < 0.001$).

IN VIVO NECROTIC ENTERITIS MODEL

Heat treatment of a small sample of suspended *B. amyloliquefaciens* SG1 lyophilized powder revealed the presence of 2.14×10^5 spores per gram powder. A non-heated sample, consisting of spores and vegetative cells, contained 4.42×10^8 colony forming units per gram powder. This clearly shows that the lyophilized powder mainly consisted of vegetative cells and contained only 0.05% spores.

Two animals from the *B. amyloliquefaciens* SG1 treated groups and one animal from the non-treated, positive control groups died before challenge with *C. perfringens* and were excluded from the experiment. In the positive control groups, two broilers died on the first day and one broiler on the final day of challenge. Although at necropsy, extensive necrosis of the gut was visible in all three animals, they were not used for macroscopic evaluation of the lesions since no accurate scoring was possible due to rapid autolysis of the gut tissue.

Results obtained from the *in vivo* experiment are summarized in the Table. Disease was successfully established in the animals since 78.3% of all broilers in the positive control groups developed lesions characteristic for NE. Necrotic damage was limited to the duodenum and jejunum except in 2 animals that also developed necrosis in the ileum. In the *B. amyloliquefaciens* SG1 treated groups, 80.0% of the broilers scored positive for NE. Necrotic lesions were exclusively limited to the duodenum and jejunum. No significant decrease was detected in the number of NE positive chickens between the untreated control groups and *Bacillus* treated groups. When the average lesion scores and body weights of the *Bacillus* treated groups were compared with those of the untreated control groups, no significant differences were observed.

Table. Percentage of chickens with necrotic enteritis, average lesion score and body weight for all non-treated, positive control groups and *Bacillus amyloliquefaciens* SG1 treated groups. The percentage of NE positive birds, average lesion score and body weight are expressed as mean values ± standard error on the mean (SEM). No statistical differences could be detected for any of the parameters between the groups.

	Untreated groups	*B. amyloliquefaciens* SG1 treated groups
% chickens with NE	78.2 ± 6.222	79.9 ± 2.979
Average lesion score	3.00 ± 0.243	3.34 ± 0.252
Body weight (g)	750.7 ± 15.52	713.4 ± 16.69

DISCUSSION

In this study, we examined whether supplementation of lyophilized vegetative cells of *B. amyloliquefaciens* SG1 in the feed could control NE in broilers. Several studies indicate the beneficial use of probiotics in the control of this disease in broilers.[9, 11, 12, 24] One study is available where the effect of *B. amyloliquefaciens* SG1 on NE in broilers was investigated. Jerzsele et al. supplemented *B .amyloliquefaciens* spores at a concentration of 10^9 cfu/kg feed but failed to obtain a statistically significant protection against NE.[15] In our experiment, we chose to administer vegetative cells of *B. amyloliquefaciens* SG1 since previous research at our department showed that oral administration of vegetative cells of this *Bacillus* strain was able to successfully confer protection against *Clostridium difficile* associated disease in a mouse model.[16]

Most animals in the untreated control groups developed a mild, moderate or severe form of NE. Only few animals developed no obvious lesions of NE in these groups. This clearly shows a successful establishment of the disease in the animal model. It should be noted that the average lesion scores and the percentage of positive birds was higher than previously reported using the same model, indicating that the current infection resulted in a more severe form of necrotic enteritis.[20-22]

Despite the clear *in vitro* inhibitory activity of the supernatant of *B. amyloliquefaciens* SG1 against all *C. perfringens* strains, administration of vegetative *B. amyloliquefaciens* SG1 cells did not reveal a protective effect against NE in the experimental broiler model. Statistical analysis of the data indeed revealed no significant differences in weight loss or average lesion score between animals in the untreated control groups and the *B. amyloliquefaciens* SG1 treated groups. This remarkable difference between our *in vitro* and *in vivo* results could have a number of reasons.

One reason might be related to the different growth characteristics of *B. amyloliquefaciens* SG1 and *C. perfringens*. *C. perfringens* is a fast growing microorganism under anaerobic conditions while *B. amyloliquefaciens* SG1 grows best in an aerobic atmosphere. However, *B. amyloliquefaciens* SG1 is able to grow in anaerobic conditions but its growth rate is much slower. It is possible that *B. amyloliquefaciens* SG1 grows too slowly in the anaerobic gut environment to provide protection against NE. Taking into account this possibility, *B. amyloliquefaciens* SG1 was supplied in the feed throughout the whole experiment to support high colonization levels. In this way, *B. amyloliquefaciens* SG1 gets the opportunity to colonize the intestinal tract and be part of the intestinal microbiota before *C. perfringens* challenge started. For the same reason, *B. amyloliquefaciens* SG1 supernatant, and not live bacteria, was used for the *in vitro* experiments. Since the *in vivo* experiment uses the strain itself, results of the *in vitro* experiment cannot be fully extrapolated but do however give an important pointer. Moreover, it highlights the importance of

the need for animal experiments to draw definite conclusions. Due to the severity of the established infection, it might be possible that beneficial effects of *B. amyloliquefaciens* SG1 uptake were not sufficient to counterbalance the infection.

To observe a beneficial effect on intestinal health, the probiotic needs to be able to survive gastrointestinal transit and colonize the intestinal tract. A first hurdle are the detrimental conditions the probiotic encounters during passage in the gastrointestinal tract such as the acidity of the stomach and the exposure to bile salts and pancreatic juices. The possibility exists that *B. amyloliquefaciens* SG1 cannot withstand these detrimental conditions and is not able to reach the intestinal tract. It is however possible to overcome this problem by providing additional protection during transit, e.g. by encapsulation.[25-28]

A second issue depends on the capacity of the strain to colonize the gut and to adhere to the intestinal epithelium. Adherence and colonization can be influenced by the composition of the resident gut microbiota. The adhesion capacity of a probiotic strain is strain specific and depends on cell surface properties such as hydrophobicity and extracellular protein profiles.[29] An important selection criterion for probiotics in poultry is that the microbial strain preferably needs to be isolated from the microbiota of the host species in order to facilitate intestinal colonization.[25, 26, 28, 30] Since *B. amyloliquefaciens* SG1 was isolated from the environment, it is possibly not able to colonize the broiler gut.

However, previous research demonstrated successful protection against *C. difficile* associated disease in a mouse model indicating that *B. amyloliquefaciens* SG1 can indeed reach and colonize the intestinal tract in mice.[16] On the other hand, *C. difficile* infections in mice are localized in the colon while *C. perfringens* in broilers affects the small intestine. It is possible that *B. amyloliquefaciens* SG1 is unable to attach or multiply in the intestine of broilers due to environmental differences as compared to the murine colon. In addition, in the mouse trial, *B. amyloliquefaciens* SG1 was administered as live cells to the animals while lyophilized cells were used for practical reasons in the broiler experiment. There is the possibility that the lyophilized cells have more difficulties to rehydrate and grow in the intestinal broiler tract. Researchers have already studied the effect of *B. amyloliquefaciens* SG1 as a directly-fed microbial on broilers. Beneficial effects have been reported on growth performance, caecal microbiota composition, antibody response, nutrient digestibility and intestinal health.[31-33] As such, *B. amyloliquefaciens* is shown to be able to pass the upper digestive tract, reach the small intestine and exert its beneficial effect. Once in the intestinal tract, it is possible that *B. amyloquefaciens* SG1 is unable to produce its antimicrobial peptides that act against *C.*

perfringens or that they are neutralized or broken down by digestive enzymes or other micro-organisms.

Another important factor to take into consideration is the probiotic production process. The probiotic properties are influenced by the way the strain is produced. The drying process (e.g. freeze-drying vs. spray-drying) plays an important role. Fuller reported that adhesion to the intestinal epithelium is influenced by the carbohydrate source used in the growth medium and changes during the growth cycle.[34] Although we obtained a high count of viable *B. amyloliquefaciens* SG1 cells after lyophilization, the experimental conditions for growth used might not be the most optimal. Since the probiotic was supplemented in the feed, it is exposed to many factors that might influence its stability and viability such as temperature or the presence of enzymes in the feed. In addition, the administration route (in-feed supplementation, addition to drinking water, spraying…) can determine the probiotics intestinal colonization capacity and influence the trial outcome.[25, 26, 28, 30]

Other important factors relate to the host species. Genetic variation between different poultry species and breeds, immunological status and age of the animals can all influence the results outcome. Results of the *in vivo* trial indicated higher average lesion scores and percentage NE positive birds than previously reported. Since a more severe form of NE was established, it is possible that beneficial effects are missed due to the severity of the infection. Alternative experimental NE models have been described that could lead to a form of NE that more closely resembles the observed situation in practice.[35] In addition, environmental stress, duration of the probiotic treatment and management of the birds can influence the efficacy of the probiotic.[25, 26, 28, 30]

As reported previously, *B. amyloliquefaciens* SG1 supernatant significantly inhibits the growth of different *C. difficile* ribotypes with little variation in the size of inhibition zone.[16] Results of our current *in vitro* work, however, indicate that growth inhibition of *C. perfringens* by *B. amyloliquefaciens* SG1 is strain dependent with a large variation in the size of the inhibition zone. There is no obvious explanation as to why virulent NetB positive *C. perfringens* isolates are more sensitive to the supernatant of *B. amyloliquefaciens* SG1 as compared to avirulent NetB negative *C. perfringens* isolates. Further research is necessary to identify the antimicrobial compound responsible for the observed antibacterial activity and its mode of action.

In conclusion, although we were not able to demonstrate the beneficial effect of *B. amyloliquefaciens* SG1 in controlling NE in broilers despite its high *in vitro* antibacterial activity, it cannot be excluded that the *B. amyloliquefaciens* SG1 strain used in the current work still provides protection against broiler necrotic enteritis when e.g. the probiotic production process is optimized or another experimental disease model is used.

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GENERAL DISCUSSION

CLOSTRIDIUM PERFRINGENS AND CLOSTRIDIUM DIFFICILE: INTESTINAL PATHOGENS OF MEN AND ANIMALS

The genus *Clostridium* encompasses many pathogenic species that are harmful for men and animals. *Clostridium difficile* and *Clostridium perfringens* are two well-known pathogens associated with severe intestinal disease and linked with the use of broad spectrum antibiotics. *C. perfringens* is the cause of necrotic enteritis in broiler chickens. This disease was long constrained due to the use of antibiotics as growth promoters. However, public concern on the use of in-feed antibiotics and the alarming rise of antibiotic resistance has led to a ban on the use of antibiotics as growth promoters in Europe.[1] This has created the need to find alternatives to improve animal performance and prevent disease. *C. difficile* is an opportunistic pathogen that is able to cause severe intestinal inflammation when the normal gut microbiota is disturbed due to the consumption of antibiotics. Currently, infections are treated with the antibiotics metronidazole or vancomycin which seems contradictory since disease outcome results from the disruptive effect of these molecules on the gut microbiota.[2] In addition, antibiotic treatment is not always successful since high recurrence rates and limited treatment success for severe disease are being reported.[3] Moreover, *C. difficile* strains with elevated MIC values have already been reported for both antibiotics.[4-7] It is clear that there is a great need to look for efficient treatments that do not further disrupt the already altered indigenous microbiota, help to restore the complex balance of the normal gut microbiota and limit the use of additional antibiotic therapy.[8]

Although the discovery of antibiotics has been a major breakthrough in the history of medicine, its imprudent use has led to the emergence of antibiotic resistant pathogens.[9] Fear exists that the industry will not be able to develop new effective antibiotics at a sufficient rate to counteract the development of antibiotic resistance. There is a great need to find alternative treatment strategies to treat infectious diseases and to constrain the emergence of antibiotic resistance.

THE QUEST FOR ANTIBIOTIC ALTERNATIVES

Probiotics as promising agents for disease prevention

Probiotics are defined as live microorganisms that confer a health benefit to the host when consumed in appropriate amounts.[9] A good probiotic is able to maintain or improve the indigenous intestinal microbiota and prevent pathogen colonization.[10] As such, they are promising alternatives to prevent disease. For *C. perfringens* and *C. difficile*, probiotics have been proposed as a promising alternative for disease prevention and treatment. Probiotics have gained interest as potential prevention tools with the intent of modulating the intestinal microbiota and thereby protecting the chickens from *C. perfringens* induced NE.[11] In poultry, administration of probiotics is often associated

with a beneficial effect on broiler performance, modulation of intestinal microbiota, inhibition of pathogens, intestinal histological changes and immunomodulation.[12] Research on the use of probiotics for the prevention of *C. difficile* associated disease has gained much interest in the past years. A probiotic that can prevent CDI would help to lower the incidence of CDI, leading to reduced health care costs. Probiotics can help to maintain the gut microbiota and limit the risk of pathogen colonization by competitive exclusion. Different studies indicate the beneficial effects of probiotic administration on CDAD.[13-15] Most studies however, focus on commercially available probiotics and are not selected based on specific antibacterial activity.

Bacillus amyloliquefaciens SG1 – a promising probiotic

During laboratory practice, we have selected a *B. amyloliquefaciens* strain with high *in vitro* antibacterial activity against *C. perfringens* and *C. difficile*. Since the EFSA granted *B. amyloliquefaciens* a QPS status, it shows great promise as a probiotic.[16] In addition, *B. amyloliquefaciens* possesses several characteristics which would make it a promising probiotic, including its tolerance to low pH and bile salts, and its ability to produce an array of antimicrobial compounds.[17, 18] Therefore, our research focused on the potential probiotic use of *B. amyloliquefaciens* SG1 for the prevention of *C. perfringens* and *C. difficile* associated diseases.

To study *C. perfringens* induced necrotic enteritis, a well described and commonly used broiler model is available.[19-21] Beneficial effects have been reported for in-feed supplementation of *Bacillus*-based probiotics on broiler performance and the control of NE.[22-24] In addition, probiotic *B. amyloliquefaciens* administration in broilers has already been associated with beneficial effects on growth performance, caecal microbiota composition, antibody response, nutrient digestibility and intestinal health.[25-27] Vegetative *B. amyloliquefaciens* SG1 cells were lyophilized and mixed with the feed for practical reasons. Determination of the number of colony forming units of the lyophilized product at regular time intervals revealed a high stability and viability of the *B. amyloliquefaciens* SG1 cells during the whole trial. Unfortunately, no beneficial effects of in-feed administration of vegetative *B. amyloliquefaciens* SG1 cells on necrotic enteritis were found in the experimental broiler model used. Similar results were obtained for in-feed supplementation of *B. amyloliquefaciens* spores.[28]

Although several clinical trials indicate the beneficial use of probiotics for the prevention of *C. difficile* associated disease, little information is available from animal studies to support this idea. To study *C. difficile* associated disease, the mouse model described by Chen et al. is the most widely used.[29] Results from our *in vivo* trial indicated that oral administration of live *B amyloliquefaciens* cells successfully protected mice from *C. difficile* induced disease.

It is not easy to compare the results of both trials because we are working with two different pathogens and two different animal models. A multitude of differences between both experimental set ups can explain the different trial outcomes including differences in intestinal environment and gut microbiota between mice and broilers, different site of infection and duration of probiotic treatment. In addition, the mouse trial used live vegetative *B. amyloliquefaciens* SG1 cells while lyophilized *B. amyloliquefaciens* SG1 cells were used for the broiler trial. Lyophilization or freeze-drying is a process in which water is removed from bacterial cells enabling long-term storage. The bacteria first need to take up water to become active again and multiply which requires more time. However, in the broiler experiment, the probiotic was administered during the entire duration of the trial achieving a constant intake of the strain. On the other hand, *B. amyloliquefaciens* SG1 was directly administered to the mice so we know that each animal received the same high dose. In the broiler trial, *B. amyloliquefaciens* SG1 was administered in the feed. Therefore, it cannot be estimated how much each animal consumed of the probiotic strain. In addition, no data is available on the stability of lyophilized *B. amyloliquefaciens* SG1 cells in the feed. However, commercially available probiotics used to improve broiler performance or protect broilers from necrotic enteritis mainly consist of lyophilized microbial cells that need to be mixed with the feed. As a consequence, we have tested this *B. amyloliquefaciens* SG1 strain in a way that is most attractive for commercial purposes.

B. amyloliquefaciens SG1 successfully protected mice from *C. difficile* induced disease but failed to protect broilers from *C. perfringens* induced necrotic enteritis. It would have been interesting to analyze if the probiotic could also protect broilers from *C. difficile* infection to determine if the observed effect is pathogen or host related. Unfortunately, *C. difficile* is not a pathogen for broilers. The only studies available analyze the prevalence of *C. difficile* in poultry or poultry meat to study if it is a source of infection for community acquired CDI.[30, 31] Vice-versa, no mouse models are known to study *C. perfringens* induced intestinal inflammation. Mice are mainly used to study the effect of *C. perfringens* toxins on intestinal and systemic inflammation.[32] As a consequence, we are unable to draw any conclusion on this subject.

C. difficile and *C. perfringens* are able to cause intestinal disease in many different animal species. As such, it could be interesting to analyze if *B. amyloliquefaciens* SG1 can provide protection in other animals as well. For example, *C. difficile* is a well-known pathogen of piglets and horses. Unfortunately, the information concerning this subject is little. Only a few animal trials have been conducted that study the preventive effect of probiotic administration on CDAD in these animal species. In piglets, administration of a probiotic containing *Lactobacillus* spp. did not yield beneficial effects after *C. difficile* challenge. On the other hand, administration of a non-toxigenic *C. difficile*

strain lowered the prevalence of toxin-positive feces, mesocolonic edema and microscopic lesions as compared to positive control piglets.[33] Schoster et al. administered a probiotic containing 4 *Lactobacillus* strains and 1 *Bifidobacterium* strain to healthy neonatal foals and analyzed the incidence of diarrhea and fecal shedding of *C. difficile* as well as *C. perfringens*. Although initial tests indicated that these probiotic strains were able to inhibit the growth of *C. difficile* and *C. perfringens in vitro*, no beneficial effects were observed on the incidence of diarrhea or fecal shedding of the pathogens.[34] The authors however, postulate that the lack of protection could also be explained by the choice of the probiotic strains or the treatment dose.[35] *C. perfringens* causes enteritis in many different animal species but most research is focused on necrotic enteritis in broilers. The inhibitory effect of several potential probiotic bacterial species against *C. perfringens* has been described *in vitro*. Different studies in mice and chickens indicate that probiotic administration has a beneficial effect on intestinal *C. perfringens* colonization and can possibly cause a reduction in the number of necrotic lesions whether or not after challenge with *C. perfringens*.[36] However, *C. perfringens* is also able to cause enterotoxaemia in rabbits, cattle, sheep and horses which requires further research.[37] Candidate probiotics are commonly selected based on *in vitro* antagonism tests in which the pathogens are exposed to either the probiotic micro-organism itself or its extracellular compounds.[38] It is clear that well-performed animal studies are an absolute necessity to characterize the full *in vivo* probiotic potency.

It is of great importance to distinguish between the prophylactic or therapeutic use of a probiotic. Current research mainly focusses on the use of probiotics as a preventive measure for CDAD. We would most benefit from a probiotic that can prevent *C. difficile* colonization and as such, eliminates the need for additional antibiotic therapy. The probiotic could be administered to patients that are at high risk for developing CDAD and receive antibiotics to treat a certain disease. This could help to lower the incidence of CDI and reduce health care costs. It would however require that the probiotic strain can withstand the antibiotic administered. It has been demonstrated previously that the combined therapy of an antibiotic and probiotic led to a 73% reduction in CDI.[39] Results of the mouse trial in this study, indicated that *B. amyloliquefaciens* SG1 was able to colonize the gut and protect mice from developing CDI after a single intraperitoneal injection of clindamycin. However, the effect of prolonged treatment with different antibiotics on *B. amyloliquefaciens* SG1 survival requires further research.

Studies on the therapeutic use of probiotics for the treatment of severe diarrhea have been shown to be unsuccessful. Pillai and Nelson demonstrated that there is no evidence that supports the use of probiotics for the treatment of CDAD.[40] Studies on the use of probiotics in conjunction with conventional antibiotic treatment only provide weak evidence for its efficacy to treat CDAD. In

general, there is a lack of well-conducted clinical studies with sufficiently large sample sizes. The problem is that *C. difficile* is only responsible for approximately one third of all cases of antibiotic associated diarrhea, which makes it not easy to conduct clinical studies with large sample sizes.[41, 42] Moreover, for treatment purposes, the administered compound needs to be able to quickly control the infectious agent. CDI is generally associated with a sudden disease onset. After accurate diagnosis of CDI, the probiotic first needs to pass through the gastrointestinal tract and colonize the gut before it can exert its antibacterial effect. In view of efficient treatment of CDI, this requires too much time and potential therapeutic effects will be displayed too slow.[40, 43]

B. amyloliquefaciens SG1 has proven its effectiveness in controlling *C. difficile* associated disease in mice. Although *C. difficile* is the best known cause of antibiotic associated diarrhea, it is not the only cause. Other pathogens such as *C. perfringens*, *S. aureus* and *Salmonella* spp. have also been linked to antibiotic associated diarrhea.[44] We have demonstrated that *B. amyloliquefaciens* SG1 does not inhibit the growth of *S. aureus* or *Salmonella* spp. Therefore, its prophylactic use can possibly not be expanded to other infective causes of antibiotic associated diarrhea. For the prevention of antibiotic associated diarrhea in general, a multi-strain probiotic could be more interesting. Incorporation of several bacterial strains in one probiotic allows us to tackle several pathogens at once. Since *C. difficile* is one of the main causes of antibiotic associated diarrhea, *B. amyloliquefaciens* SG1 can be the compound responsible for the control of *C. difficile*.

A treatment strategy for CDAD that recently gained a lot of interest is fecal microbiota transplantation (FMT). It involves the administration of a stool sample from a healthy donor to a patient with a disease caused by an altered gut microbiota.[45] Studies indicate that FMT is superior to conventional antibiotic treatment for recurrent CDI and is well tolerated by the patients.[46] It has been recommended as a first line treatment in patients suffering from recurrent CDI.[47, 48] Although this treatment shows success and is easy to conduct, it has some disadvantages. Despite its effectiveness, the idea of receiving someone else's stool is not very appealing and is mostly used as a last resort treatment for severe cases. In addition, the exact composition of the donor stool is not known, which might lead to the risk of spreading infectious diseases such as HIV, hepatitis A and B, Epstein-Barr virus and *Campylobacter jejuni*. Since most donors usually are relatives of the patient, it can possibly be hard to find donors for patients with a family history of infectious disease or unhealthy gut microbiota. Despite its easiness to implement, there is no standardized experimental protocol for FMT making it not yet common practice.[49-52] The major advantage of probiotics as compared to FMT is the fact that the composition of the probiotic is known and can be controlled. In addition, probiotic consumption is more appealing than FMT.

Probiotics are defined by the World Health Organization as live micro-organisms that confer a health benefit to the host when consumed in appropriate amounts. The benefits of probiotics have been recognized by health authorities and evidence for their beneficial effects arises from many peer reviewed scientific publications. Probiotics have a long history of safe use in food products or as food supplements but it has become obvious that probiotics also have a therapeutic potential. The progress in science creates the necessity of regulatory updates. Unfortunately, science progresses faster than regulatory offices, creating a regulatory challenge. Regulators need to make a distinction between probiotics used as food supplements that serve a healthy population and probiotics with a medical purpose that only relate to a population of patients. For both probiotics, a specific guidance is of uttermost importance.[53, 54] There is a great need for an approach to define the health benefits of probiotics. Binnendijk and Rijkers looked at what the EFSA recognizes as a health claim. These included a.o. maintenance of the intestinal microbiota balance in order to decrease pathogenic micro-organisms, improvement of bowel function, maintenance of normal bowel function with reduced intestinal transit and increased bowel movements' frequency and preservation of an individual's microbiota receiving antibiotic therapy.[55] Of all applications, 78% had a correct health claim but all of them were turned down by the EFSA because of a lack of scientific evidence.[55] Only recently, the EFSA published an update on the scientific requirement of probiotic use related to gut health. It highlights the need for human intervention studies showing an effect on clinical outcomes related to gastrointestinal infections (e.g. improvement in the incidence, severity or duration of diarrheal episodes). In addition, they require that the cause of diarrhea should be established and accurate diagnosis is performed by a physician. Criteria used to exclude patients from the clinical trial need to be stated and results should encompass microbiological data.[56] Although several clinical trials indicate the beneficial effect of probiotic administration, there is not yet a consensus on their use to prevent CDAD due to a multitude of flaws in study design such as small sample sizes, differences in inclusion and exclusion criteria and clinical end points In addition, differences in probiotic duration, length of follow-up, combined use with antibiotics, combined data of primary and secondary CDI,… makes it difficult to compare results of clinical trials and draw definite conclusions. It is not straightforward to obtain large sample sizes because only a subset of patients on antibiotics will develop diarrhea and only a certain percentage of these cases are caused by *C. difficile*. In addition, it cannot be predicted in advance if the untreated, positive control group will be sufficiently large to detect significant effects as compared to the probiotic treatment group. Some studies were stopped early due to the low percentage of CDAD in the positive control group which makes the trial outcome not easy to interpret.[47, 57-60] For these reasons, most studies focus on the secondary prevention of CDAD because the rate to develop a relapse is high and even rises with subsequent

relapses. As such, there is a greater security to establish a sufficiently large control population. There is definitely a great need for large, well-designed clinical trials.[61, 62]

For probiotic purposes, the strain needs to be suitable for large-scale production processes. Use of *B. amyloliquefaciens* SG1 as a probiotic requires a range of safety studies to address its possible toxicity such as acute toxicity and repeated oral dose toxicity.[63] *In vivo* animal trials can be performed to determine if there is a dose dependent effect on CDI. We obtained a protective effect of the administration of vegetative *B. amyloliquefaciens* SG1 cells on CDAD in mice. It would be interesting to analyze if administration of *B. amyloliquefaciens* SG1 spores yields the same beneficial effect. Since spores are very stable, it would provide an advantage in terms of survival during gastrointestinal transit and stability of the final product. We have demonstrated that *B. amyloliquefaciens* SG1 encompasses a narrow spectrum of activity *in vitro* but the impact of *B. amyloliquefaciens* SG1 on the resident gut microbiota still needs to be analyzed. When used for the prevention of antibiotic associated diarrhea, a prerequisite is its ability to colonize the gut despite the concomitant use of antibiotics. We proved that *B. amyloliquefaciens* SG1 was able to prevent an initial episode of CDI. The possibility to prevent or cure recurrent CDI could be addressed. When results of all *in vitro* and *in vivo* tests yield good results, large clinical trials should be conducted to determine its safety in and efficacy in humans.

Antimicrobial metabolites as novel therapeutics

One reason that probiotics could provide protection against invading pathogens is by the production of antimicrobial metabolites. *Bacillus* species are widely used as probiotics and known for their ability to produce an array of secondary metabolites with antibacterial activity. *B. amyloliquefaciens* FZB42 devotes up to 8.5% of its genome to antimicrobial metabolites.[64] Most antibiotics encompass a broad spectrum of activity and cause a significant disturbance of the resident gut microbiota making the host susceptible for intestinal diseases. Additional unintended consequences include the selection of antibiotic resistance genes, promotion of horizontal gene transfer between bacteria, changes in metabolic activity and immune responses.[65] All of these deleterious effects could be avoided by using narrow spectrum antimicrobials. A few narrow spectrum antibiotics are known that specifically target *C. difficile*. Fidaxomicin is the only FDA and EMA approved narrow spectrum antibiotic that can be used for the treatment of CDI but is associated with a high production cost.[66, 67] Promising results were obtained from *in vitro* and *in vivo* experiments with cadazolid and SMT19969 but well performed clinical trials still need to be conducted to evaluate their safety and efficacy.[68-71]

Bacteria also produce compounds other than antibiotics with antimicrobial activity: ribosomally synthesized bacteriocins and non-ribosomally synthesized lipopeptides. These molecules have

recently gained interest as promising therapeutics since many of them encompass a narrow spectrum of activity. Only two bacteriocins with a narrow spectrum of activity against *C. difficile* have yet been described: thuricin CD, produced by *Bacillus thuringiensis*, and the R-type bacteriocins, produced by some *C. difficile* strains.[72-75] Due to their peptide nature, bacteriocins are sensitive to digestion when passing the gastrointestinal tract. Researchers encountered many problems concerning the bioavailability of thuricin in the gut since it undergoes proteolytic digestion. However, rectal delivery of thuricin caused a fast and significant reduction in the numbers of *C. difficile* indicating that rectal administration is a promising mode of delivery of thuricin CD to the colon to control CDI.[76] The only lipopeptide with reported anti-*C. difficile* activity is surotomycin. Although results of clinical trials shows its great promise as a therapeutic, its antibacterial activity is not limited to *C. difficile* but also directed against other Gram-positive bacteria and certain Gram-negative bacteria.[77-79]

We would greatly benefit from the use of narrow spectrum antimicrobials for different reasons. First of all, their high specific activity minimizes collateral damage to the indigenous gut microbiota which helps the host to restore its intestinal microbial ecosystem. In addition, they encourage a better antimicrobial stewardship. Since they act against specific pathogens, they can only be used to treat specific diseases. This automatically constrains overuse or incorrect prescription of antibiotics limiting the risk of resistance development. Finally, antimicrobials that are amenable to bio-engineering might possibly yield more active or less toxic derivatives.

As with all antimicrobials, the risk of resistance development should be taken into consideration. Resistance against bacteriocins has not yet been widely studied. Since bacteriocins are not yet used in a clinical setting, the only available information on resistance development arises from *in vitro* experiments. It gives us an idea on what resistance mechanisms could be possible but it is impossible to predict what will happen when they are used as therapeutics or at what rate resistance would arise. However, researchers believe that bacteriocin resistance is less likely to occur due to their rapid antibacterial action. In addition, nisin has already been used as a food preservative for a long time and no nisin resistance is yet detected. A major problem is that bacteriocin resistance is not yet defined. There is no consensus on what can be seen as low, medium or high level resistance. Differences in experimental set ups and terminology makes it difficult to compare the results obtained from different experiments.[80-82] Information on lipopeptide resistance mainly arises from studies with daptomycin. In a recent review, it was postulated that daptomycin resistance is a rare event. Only a few clinical cases have been reported. Resistance development was associated with hot spots in the genome of target bacteria but the exact link between both is unclear. The greatest difficulty is that, although it is known that daptomycin targets the cell membrane, the exact

mechanism of action is not yet well understood.[83] CB-183,315 or surotomycin is a structural analogue of daptomycin with antibacterial activity against *C. difficile*. Based on *in vitro* serial passage, spontaneous resistance and multistep resistance studies, it was hypothesized that development of resistance against CB-183,315 is rare. In addition, results of a phase 2 clinical trial indicated that the fecal concentration of CB-183,315 was higher than 1 mg/g which is significantly higher than the highest MIC value reported suggesting the unlikelihood of resistance development.[84, 85] Results of the currently available studies are promising but more studies are definitely necessary. Surfactin directly targets the bacterial cell membrane and causes a rapid desintegration of the bacterial membrane.[86] Classical antibiotics function as inhibitors of nucleic acid, protein or cell wall synthesis.[87] Resistance mechanisms are associated with mutations in the target protein, acquisition of resistance genes from other bacteria or expulsion of the antimicrobial from the cell. Since surfactin has a distinct mode of action, it is not susceptible to the known mechanisms of resistance development. Nevertheless, it cannot be excluded that novel mechanisms of resistance might be induced by repeated or long term use of the drug. Since surfactin is not yet used as a therapeutic, studies on possible resistance mechanisms are sparse. More knowledge on this issue is essential to counteract resistance development. A good antimicrobial stewardship is essential to control resistance development. The exposure of organisms to surfactin should be limited and confined to relevant therapeutic purposes. Surfactin should only be used to treat *C. difficile* infections if the pathogen is identified by diagnostic tests which automatically requires the need for fast and accurate diagnostic tools. Since lipopeptides are biodegradable, they are readily eliminated from the environment. All in all, this limits the exposure of bacteria to the antimicrobial and constrains the opportunity of bacteria to develop resistance.

Surfactin – a promising therapeutic for the treatment of CDAD

We have demonstrated that the inhibitory effect of the *B. amyloliquefaciens* SG1 strain, used in this work, on *C. difficile,* is caused by the production of the lipopeptide surfactin. Analysis of the purified samples showed that this surfactin had a very high activity against *C. difficile*. This is a first step to a possible antibiotic alternative for the treatment of *C. difficile* associated disease. Since *B. amyloliquefaciens* SG1 CFSN demonstrated a narrow spectrum of activity, we are hopeful that our surfactin encompasses a narrow spectrum of activity. However, we would need to determine the antimicrobial spectrum of the purified surfactin to draw any definite conclusions. As reported previously, the antibacterial activity of surfactin is dependent on the producer strain.[88] Therefore, the possibility exists that other *B. amyloliquefaciens* SG1 strains have an even higher anti-*C. difficile* activity or encompass an even narrower antibacterial spectrum. In addition, surfactin is produced as a mixture of homologues with differences in the number of carbon atoms of the lipid chain and

surfactin variants exist that have amino acid substitutions in the cyclic peptide part. Each variation can have an impact on the antimicrobial activity. It has been reported that the bactericidal activity of lipopeptides increases with the length of the carbon chain. Further increases in carbon length increases antifungal activity in addition to antibacterial activity.[89] Due to the high molecular similarities, it is not straightforward to separate the different homologues. We cannot predict in advance which homologues or variants have the highest antibacterial activity. Which homologues or variants are produced is not only strain dependent but can be influenced by the growth conditions of the bacteria. The most optimal growth conditions can be determined by comparison of the amount of antibacterial activity produced using different growth media or incubation conditions. As such, bacteria can be influenced towards lipopeptide synthesis.

For treatment purposes, surfactin need to be able to reach the intestinal tract and resist the harsh conditions of the gastrointestinal tract including the acidity of the stomach and proteolytic digestion. Since surfactin precipitates at low pH, it will lose its activity when entering the acidic environment of the stomach. However, we observed that an increase in the pH is able to fully recover its antibacterial action. When passing in the intestinal tract, the gradual rise in pH could potentially restore surfactins activity. Another hurdle encountered are digestive proteases. Surfactin only contains 7 amino acids and has a cyclic structure which makes it more resistant to proteolytic digestion. It is however sensitive to the action of lipases that are secreted by e.g. the pancreas. All of these problems can be easily overcome by encapsulation of surfactin to achieve a targeted release in the colon. Rectal administration might be another alternative to avoid these problems. As mentioned before, rectal administration of thuricin CD caused a fast and significant reduction in the numbers of *C. difficile* indicating it is a useful route of administration.[76] Rectal administration might, however, not be the most desirable way in patients suffering from severe diarrhea due to the risk of rapid elimination before the compound is released. As determined for *B. amyloliquefaciens* SG1 supernatant, it would be interesting to analyze hemolytic and cytotoxic activity of the purified surfactin. When used for *in vivo* studies, we have to address several important properties including bioavailability, toxicity, pharmacodynamics and pharmacokinetics. In addition, the purified surfactin needs to remain stable over a long period of time.

Currently, surfactin is only used for research purposes and very expensive to buy. In view of its use as a treatment, it needs to be obtained in pure form in adequate amounts. Its purification process is however associated with a high cost and yields are generally low which is a major restriction concerning its commercial use. Purification of a biosurfactant such as surfactin is associated with a 3 to 10 times higher cost than a chemical surfactant. Researchers need to tackle several problems to reduce the purification cost. A first issue is the carbon source used to grow the bacteria. There is a

need for inexpensive substrates to culture the bacteria in large volumes. Mutant *Bacillus subtilis* strains have already been constructed that overproduce surfactin.[90] As mentioned earlier, the amount of surfactin produced can be influenced by the composition of the growth medium.[91] This might help to increase the production yield. In addition, extensive foaming during culture makes recovery and purification difficult but can be controlled e.g. by applying a right agitation rate and aeration.[92] Combined with improvements in the downstream costs, the total cost to obtain high amounts of surfactin can be controlled.[93]

B. amyloliquefaciens SG1 – a source of antimicrobial metabolites?

B. amyloliquefaciens is known for its ability to produce a wide array of antimicrobial compounds.. During our research, we found that *B. amyloliquefaciens* SG1 produces at least 1 other compound than surfactin with antibacterial activity against *C. difficile*. In-gel detection of antibacterial activity from concentrated supernatant revealed the presence of a peptide smaller than 5 kDa with antibacterial activity against *C. difficile*. After SDS-PAGE, the compound could be visualized using silver-staining which confirms its peptide nature. Unfortunately, several attempts to purify this compound using a combination of different chromatographic techniques were unsuccessful (unpublished data). More research is necessary to identify this antibacterial compound. In addition, we are still unaware which compound is responsible for the antibacterial activity against *C. perfringens*.

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SUMMARY

The intestinal tract of all living species harbors a diverse and complex microbial ecosystem which plays an important role in human and animal health. Disruption of this complex ecosystem has been linked with gastrointestinal diseases such as cancer, inflammatory bowel disease and colitis. The gut microbiota has not only an important metabolic, nutritional and immune function but also forms an effective barrier that protects the host from invading pathogenic microorganisms. Consumption of antibiotics leads to a disturbance of the gut microbiota and makes the host susceptible to gastrointestinal infections. In addition, inappropriate and extensive use of antibiotics has led to the emergence of multidrug resistant bacterial pathogens.

Clostridium difficile is an antibiotic resistant pathogen of humans and animals able to colonize the gut after disruption of the intestinal microbiota due to antibiotic consumption. It is one of the main causes of antibiotic associated diarrhea and the main cause of pseudomembranous colitis. Current treatment strategies involve the administration of the broad spectrum antibiotics metronidazole or vancomycin. Unfortunately, these treatment strategies often fail and consequently high recurrence rates are being reported and strains with elevated MIC values have already been isolated. In addition, it seems contradictory that standard treatment involves the administration of antibiotics when disease outcomes results from the disruptive effect of antibiotics on the colonic microbiota. It is of great importance to look for alternative treatments that do not further disrupt the altered indigenous microbiota, help to restore the complex balance of the normal gut microbiota and restrict the need for additional antibiotic therapy.

During laboratory practice, we isolated a *Bacillus amyloliquefaciens* strain with growth inhibiting properties towards *C. difficile*. In a first study, we analyzed the *in vitro* antibacterial properties of *B. amyloliquefaciens* supernatant against *C. difficile.* The antibacterial activity was relatively resistant to changes in pH and temperature. Loss of activity was only observed at low pH or prolonged exposure to elevated temperatures. The antibacterial activity was not affected by addition of catalase, urea, bile salts and proteases, with the exception of some minimal loss of activity due to trypsin treatment. On the other hand, lipase, tween-80, lysozyme and pancreatine impaired the antibacterial activity. Increase in antibacterial activity coincided with growth of *B. amyloliquefaciens* and reached its maximum during the stationary growth phase. The supernatant of the bacterial culture showed no hemolytic activity and was not cytotoxic for intestinal epithelial cells. In addition, the supernatant only had a narrow spectrum of activity. No activity was detected against the Gram negative bacteria *Escherichia coli*, *Salmonell*a sp., *Pasteurella* sp. and *Pseudomonas aeruginosa*. Only the growth of *Yersinia tuberculosis* was inhibited to a minor extent. Moreover, the growth of several Gram positive bacteria belonging to *Clostridium* cluster I, IV, XIVa and XVI, isolated from the normal

gut microbiota, 7 out of 8 tested *Lactobacillus* strains and all tested *Staphylococcus* sp. was not affected by the supernatant. Only *Clostridium perfringens*, *Enterococcus cecorum* and two *Streptococcus* species were inhibited by the supernatant. In a second phase, the anti-*C. difficile* compound was purified and identified. Mass spectral analysis of the purified sample indicated the presence of a compound with a major peak at 1036.65 Da. MSMS analysis of this peak indicated a spectrum characteristic for the lipopeptide surfactin. These results were confirmed by mass spectral analysis of commercially available surfactin. In addition, we proved that commercially available surfactin possessed antibacterial activity against *C. difficile*. As such, *B. amyloliquefaciens* produces at least one compound, the lipopeptide surfactin, with growth inhibiting properties towards *C. difficile*.

Nowadays, the use of probiotics to control *C. difficile* has gained more and more attention. Several studies indicate the beneficial effect of probiotics for the prevention of antibiotic associated diarrhea or recurrent *C. difficile* infection. Our *B. amyloliquefaciens* strain was specifically selected for its anti-*C. difficile* activity. Since *Bacillus* species are commonly used probiotics, we investigated the possible prophylactic use of the strain to protect mice against *C. difficile* associated disease. First, the *in vitro* antibacterial activity of *B. amyloliquefaciens* supernatant was tested against a collection of 24 different *C. difficile* ribotypes. The supernatant was able to inhibit the growth of all *C. difficile* ribotypes in the same extent. Subsequently, a mouse trial was conducted to test if oral administration of *B. amyloliquefaciens* was able to prevent *C. difficile* associated disease. The effect of *B. amyloliquefaciens* treatment in mice was compared to *S. boulardii* treatment, commonly used as a probiotic, and an untreated control group. Results indicated that *B. amyloliquefaciens* treatment improved clinical signs and survival rates and protected mice from severe weight loss. The colon content of *B. amyloliquefaciens* treated mice showed a significantly lower amount of *C. difficile* toxin A and B levels. In addition, a significantly lower degree of colon damage was detected in *B. amyloliquefaciens* treated mice as compared to *S. boulardii* treated and untreated control mice, based on histopathological analysis of colon samples, colon weight/length and colon/body weight ratios. In conclusion, *B. amyloliquefaciens* successfully protected mice from *C. difficile* associated disease in mice.

Antibiotics have long been used as in-feed additives in farm animals to improve nutrient availability and growth performance. However, public concern on the use of in-feed antibiotics and the alarming rise of antibiotic resistant pathogens have led to a ban on the use of antibiotics as growth promoters in Europe. *Clostridium perfringens* is an intestinal pathogen associated with necrotic enteritis in broilers. In-feed supplementation of antibiotics has long constrained this disease but the ban on in-feed antibiotics caused a flare in the incidence of broiler necrotic enteritis. During our initial *in vitro*

work, we observed that *B. amyloliquefaciens* also possessed growth inhibiting properties towards *C. perfringens*. In a third study, we first analyzed the antibacterial activity of *B. amyloliquefaciens* supernatant against a collection of NetB-negative and NetB-positive *C. pefringens* isolates. Interestingly, virulent NetB-positive *C. perfringens* strains were significantly more inhibited than avirulent NetB-negative *C. perfringens* strains. In a second phase, an experimental broiler model was used to test the efficacy of in-feed supplementation of lyophilized vegetative *B. amyloliquefaciens* cells in preventing necrotic enteritis. Unfortunately, no beneficial effect of *B. amyloliquefaciens* supplementation could be detected since no differences in lesion score, percentage of necrotic enteritis positive animals or body weights were noted between the treatment groups and untreated control groups.

Although the discovery of broad spectrum antibiotics has definitely been a major breakthrough for medicine, we have evolved into an era in which its excessive use has led to the emergence of antibiotic resistant pathogens. Nowadays, the search for alternatives to broad spectrum antibiotics is gaining more and more attention. One possible alternative is the use antibacterial compounds with a narrow spectrum of activity. *C. difficile* and *C. perfringens* are two important intestinal pathogens of humans and animals that are linked with antibiotic consumption and would benefit from alternative treatments to control disease. We demonstrated that *B. amyloliquefaciens* showed significant *in vitro* antibacterial activity against both pathogens. In-feed supplementation of vegetative *B. amyloliquefaciens* cells could however not protect broilers from *C. perfringens* induced necrotic enteritis. On the other hand, prophylactic administration of *B. amyloliquefaciens* to mice successfully conferred protection against *C. difficile* associated disease which might be in part caused by the production of the lipopeptide surfactin. Administration of a *B. amyloliquefaciens* based probiotic to people at risk for developing *C. difficile* associated disease could be an interesting alternative for standard antibiotic therapy.

SAMENVATTING

De darm van alle levende organismen omvat een divers en complex microbieel ecosysteem dat een belangrijke rol speelt in de gezondheid van mens en dier. Een verstoring van dit complexe ecosysteem is geassocieerd met diverse gastrointestinale ziekten zoals kanker, inflammatoire darmziekten en colitis. De microbiota in de darm heeft niet alleen een belangrijke metabole, voedings- en immuunfunctie maar vormt ook een efficiënte barrière die de gastheer beschermt tegen de invasie van pathogene micro-organismen. Het gebruik van antibiotica verstoort de darmmicrobiota, vernietigt deze beschermende barrière en maakt de gastheer gevoelig voor gastrointestinale infecties. Daarnaast heeft het onverantwoorde en overvloedige gebruik van antibiotica geleid tot het ontstaan van multidrug resistente bacteriële pathogenen.

Clostridium difficile is een antibioticumresistente pathogeen van mens en dier. Door het gebruik van antibiotica treedt een verstoring op van de normale darmmicrobiota en kan deze bacterie de darm koloniseren. Het is één van de belangrijkste oorzaken van antibioticumgeassocieerde diarree en de belangrijkste oorzaak van pseudomembraancolitis. Momenteel wordt een infectie met deze bacterie behandeld met de breedspectrumantibiotica metronidazole en vancomycine. Deze behandelingen blijken echter niet altijd even succesvol aangezien een hoge mate van terugkerende infecties wordt vastgesteld. Er werden ook reeds *C. difficile* stammen met verminderde gevoeligheid ten opzichte van beide antibiotica geïsoleerd. Daarnaast lijkt het ook heel tegenstrijdig dat de behandeling gebeurt met breedspectrumantibiotica aangezien deze net de oorzaak zijn van de verstoorde darmmicrobiota waardoor *C. difficile* de darm kan koloniseren. Het is van groot belang om onderzoek uit te voeren naar alternatieve behandelingsmogelijkheden die de reeds verstoorde darmmicrobiota niet verder beschadigt, die het complexe microbiaal ecosysteem in de darm helpt te herstellen en die de noodzaak aan bijkomende antibioticumtherpie beperkt.

In het labo werd een bacterie geïsoleerd die in staat was om de groei van *C. difficile* te inhiberen. Deze bacterie werd geïdentificeerd als *Bacillus amyloliquefaciens*. In een eerste studie hebben we de *in vitro* antibacteriële activiteit van *B. amyloliquefaciens* cultuursupernatans tegen *C. difficile* geanalyseerd. Deze antibacteriële activiteit was relatief resistent aan wijzigingen in pH en temperatuur. Bij een lage pH en langdurige blootstelling aan verhoogde temperaturen werd een verlies van activiteit vastgesteld. De antibacteriële activiteit werd niet aangetast door behandeling met katalase, ureum, galzouten en proteasen. Enkel trypsinebehandeling leidde tot een minimaal verlies van activiteit. Er werd wel een verlies van activiteit vastgesteld na toevoeging van lipase, tween-80, lysozyme en pancreatine. Naarmate de groei van *B. amyloliquefaciens* vorderde, nam de mate van antibacteriële activiteit en werd een maximum bereikt in de stationaire groeifase. Het cultuursupernatans was niet cytotoxisch voor intestinale epitheelcellen en vertoonde geen hemolytische activiteit. Daarnaast bleek het supernatans een beperkt activiteitsspectrum te bezitten.

Er werd immers geen antibacteriële activiteit vastgesteld tegen de Gram-negatieve bacteriën *Escherichia coli*, *Salmonella* sp., *Pasteurella* sp. en *Pseudomonas aeruginosa*. Enkel de groei van *Yersinia tuberculosis* werd in beperkte mate geïnhibeerd. Het supernatant vertoonde ook geen antibacteriële activiteit tegen een reeks Gram-positieve bacteriën die behoren tot de *Clostridium* clusters I, IV, XIVa en XVI, 7 van de 8 geteste lactobacillen en alle geteste staphylococcen. Antibacteriële activiteit werd vastgesteld ten opzichte van *Clostridium perfringens*, *Enterococcus cecorum* en twee streptococcen. In een tweede fase van deze studie hebben we de component met antibacteriële activiteit tegen *C. difficile* opgezuiverd uit het supernatans en geïdentificeerd. Het resultaat van de massaspectrometrische analyse van de opgezuiverde fractie toonde de aanwezigheid van een grote piek bij 1036.65 Da. MSMS analyse van deze piek bewees dat het massaspectrum karakteristiek was voor het lipopeptide surfactine. Deze resultaten kwamen overeen met de massaspectra van commercieel beschikbaar surfactine. Bovendien bleek commercieel surfactine eveneens antibacteriële activiteit tegen *C. difficile* te bezitten. Uit deze studie kunnen we besluiten dat *B. amyloliquefaciens* minstens 1 antimicrobiële component, het lipopeptide surfactine, produceert dat actief is tegen *C. difficile*.

Het gebruik van probiotica is de laatste jaren zeer populair geworden. Verschillende studies tonen aan dat probiotica een gunstig effect hebben op het voorkomen van antibioticumgeassocieerde diarree en terugkerende infecties. De *B. amyloliquefaciens* stam gebruikt in dit onderzoek werd specifiek geselecteerd voor zijn antibacteriële activiteit tegen *C. difficile*. Er zijn reeds verschillende *Bacillus* species die gebruikt worden als probioticum. In een tweede studie hebben we onderzocht of het mogelijk is om *B. amyloliquefaciens* te gebruiken als profylactische behandeling om *C. difficile* geassocieerde ziekte te voorkomen in een muismodel. Eerst werd de *in vitro* antibacteriële activiteit van *B. amyloliquefaciens* cultuursupernatans getest tegen 24 verschillende *C. difficile* ribotypes. Het supernatans bleek alle ribotypes in dezelfde mate te inhiberen. Vervolgens werd een muizenproef uitgevoerd om te bepalen of orale toediening van *B. amyloliquefaciens C. difficile* geassocieerde ziekte kon voorkomen. Daarnaast kreeg een groep muizen ook *Saccharomyces boulardii* toegediend. Deze gist is reeds een veel gebruikt probioticum en werd meegenomen als positieve controle. Het effect van beide behandelingen werd vergeleken met een niet-behandelde controlegroep. Na afloop van de proef bleek dat een behandeling met *B. amyloliquefaciens* de klinische symptomen en overlevingskans van de muizen verbeterde. *B. amyloliquefaciens* behandelde muizen hadden ook significant minder gewichtsverlies en lagere *C. difficile* toxine A en B gehaltes in de coloninhoud. Op basis van histopathologische analyse en de verhouding colongewicht/lengte en colon/lichaamsgewicht werd significant minder darmschade vastgesteld voor *B. amyloliquefaciens* behandelde muizen in vergelijking met *S. boulardii* en onbehandelde muizen. Uit al deze resultaten

kunnen we besluiten dat de orale toediening van *B. amyloliquefaciens* muizen beschermde tegen *C. difficile* geassocieerde ziekte.

Antibiotica zijn lang gebruikt als voederadditief om de beschikbaarheid van nutriënten en de groei van dieren te bevorderen. De publieke bezorgdheid over het gebruik van antibiotica als voederadditief en de toename van het aantal antibioticumresistente pathogenen heeft in Europa geleid tot een verbod op het gebruik van antibiotica als groeipromoter. *Clostridium perfringens* is een darmpathogeen die necrotische enteritis veroorzaakt in vleeskippen. Het gebruik van antibiotica als voederadditief heeft deze ziekte lange tijd kunnen intomen. Dit verbod heeft echter geleid tot een toename in de incidentie van necrotische enteritis in vleeskippen. *In vitro* onderzoek toonde aan dat *B. amyloliquefaciens* ook de groei van *C. perfringens* kon inhiberen. In een derde studie hebben we de antibacteriële activiteit van *B. amyloliquefaciens* cultuursupernatans getest tegen een verzameling NetB-negatieve en NetB-positieve *C. perfringens* stammen. Hieruit bleek dat virulente NetB-positieve stammen significant meer geïnhibeerd werden dan avirulente NetB-negatieve stammen. Vervolgens werd een experimenteel diermodel gebruikt om na te gaan of het toevoegen van vegetatieve *B. amyloliquefaciens* cellen aan het voeder bescherming opleverde tegen *C. perfringens* geïnduceerde necrotische enteritis bij vleeskippen. Er kon echter geen voordelig effect worden vastgesteld van *B. amyloliquefaciens* supplementatie vermits er geen verschillen werden gedetecteerd in letselscore, percentage dieren positief voor necrotische enteritis en lichaamsgewicht tussen de behandelde en niet-behandelde groepen.

Hoewel de ontdekking van antibiotica zeker en vast een grote doorbraak betekende voor de geneeskunde, heeft het overmatig gebruik ervan uiteindelijk geleid dat tot het ontstaan van antibioticumresistente pathogenen. Het onderzoek naar alternatieven voor breedspectrum antibioticumtherapie krijgt tegenwoordig meer en meer aandacht. Een mogelijk alternatief is het gebruik van antibacteriële componenten met een beperkt activiteitsspectrum. *C. difficile* en *C. perfringens* zijn twee belangrijke darmpathogenen van mens en dier die geassocieerd zijn met het gebruik van antibiotica. Alternatieve behandelingsmethoden zouden voor beide voordelig zijn om de ziekte te controleren. We hebben aangetoond dat *B. amyloliquefaciens in vitro* antibacterieel werkt tegen beide pathogenen. Nochtans leverde de toediening van *B. amyloliquefaciens* via het voeder geen voordeel op in de bescherming van vleeskuikens tegen necrotische enteritis veroorzaakt door *C. perfringens*. Anderzijds werkte de orale toediening van *B. amyloliquefaciens* wel beschermend voor *C. difficile* geassocieerde ziekte in muizen. Dit beschermend effect kan mogelijk worden verklaard door de productie van het lipopeptide surfactine. Er werd immers aangetoond dat de antibacteriële activiteit van *B. amyloliquefaciens* ten opzichte van *C. difficile* werd veroorzaakt door dit lipopeptide. Het toedienen van een probioticum dat *B. amyloliquefaciens* bevat aan mensen met

een verhoogd risico op *C. difficile* geassocieerde ziekte kan een veelbelovend alternatief zijn voor standaard antibioticumtherapie.

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CURRICULUM VITAE

Sofie Geeraerts werd geboren op 4 juni 1985 te Aalst. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Wetenschappen – Wiskunde, aan het Instituut voor Katholiek en Secundair Onderwijs te Denderleeuw, startte ze in 2004 met de opleiding Bachelor in de Biochemie en Biotechnologie aan de Universiteit Gent. In 2009 behaalde ze het diploma Master in de Biochemie en Biotechnologie, afstudeerrichting Microbiële Biotechnologie, met onderscheiding.

Daarna trad ze in dienst bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde en deed onderzoek naar de bescherming van vleeskuikens tegen een breed spectrum *Salmonella* serotypes door toepassing van kolonisatie-inhibitie met gedefinieerde levend verzwakte stammen. In december 2010 behaalde zij een specialisatiebeurs toegekend door het Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie (IWT) in Vlaanderen. Daarop startte ze in januari 2011 haar doctoraatsonderzoek aan dezelfde vakgroep dat handelde over alternatieve behandelingsmethoden voor klassieke antibioticumtherapie tegen *Clostridium* geassocieerde ziekten. Promotoren tijdens het doctoraatsonderzoek waren Prof. Dr. Ir. F. Van Immerseel van bovengenoemde vakgroep en Prof. B. Devreese van de vakgroep Biochemie en Microbiologie aan de Faculteit Wetenschappen. Verder begeleidde zij verschillende studenten in het behalen van hun masterproef en vervolledigde ze in 2016 het programma van de Doctoral Schools of Life Sciences and Medicine aan de Universiteit Gent.

Sofie Geeraerts is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Ze nam deel aan verschillende nationale en internationale congressen en presenteerde de resultaten van haar onderzoek in de vorm van posters en voordrachten.

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- Over and Out -