Nisin is an effective inhibitor of *Clostridium difficile* vegetative cells and spore germination

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*Clostridium difficile* is the most frequently identified enteric pathogen in patients with nosocomial antibiotic-associated diarrhoea and pseudomembranous colitis. Several clinically isolated *C. difficile* strains are resistant to antibiotics other than metronidazole and vancomycin. Recently, bacteriocins of lactic acid bacteria have been proposed as an alternative or complementary treatment. The aim of this study was to investigate the inhibitory effect of nisin, a bacteriocin produced by several strains of *Lactococcus lactis*, against clinical isolates of *C. difficile*. Nisin Z obtained from culture of *L. lactis* subsp. *lactis* biovar. *diacetylactis* was tested along with commercial nisin A. The effect of nisin A on *C. difficile* spores was also examined. Nisin A and Z both inhibited the growth of all *C. difficile* isolates, and MICs were estimated at 6.2 μg ml\(^{-1}\) for nisin Z and 0.8 μg ml\(^{-1}\) for nisin A. In addition, *C. difficile* spores were also susceptible to nisin A (25.6 μg ml\(^{-1}\)), which reduced spore viability by 40–50%. These results suggested that nisin and hence nisin-producing *Lactococcus* strains could be used to treat *C. difficile*-associated diarrhoea.

INTRODUCTION

*Clostridium difficile*, a Gram-positive anaerobic spore-forming bacterium, is an emerging pathogen capable of causing severe gastrointestinal illness in individuals undergoing antibiotic therapy (Kelly & LaMont, 2008; Rupnik et al., 2009; Stanley et al., 2013). Infection with *C. difficile* may produce a wide spectrum of outcomes that range from asymptomatic colonization to acute diarrhoea and pseudomembranous colitis, which can result in colonic perforation and death if untreated (Kelly & LaMont, 2008; Rupnik et al., 2009; Stanley et al., 2013). Whilst the vegetative form of *C. difficile* is responsible for producing the toxins that cause illness, the spore is the principal transmitted form (Sorg & Sonenshein, 2008). Many antibiotics have been implicated in *C. difficile*-associated diarrhoea, including clindamycin, ampicillin and amoxicillin, as well as the cephalosporins and fluoroquinolones (Wiström et al., 2001; Stevens et al., 2011; Slimings & Riley, 2014).

Current treatment for *C. difficile*-associated diarrhoea is limited mainly to administration of the antibiotics metronidazole or vancomycin (Surawicz et al., 2013). Furthermore, treatment failure and recurrence of infection have also been reported in 2–38% and 8–50% of cases, respectively (Aslam et al., 2005). Based on its in vitro activity, its efficacy by either the oral or the intravenous route and its low cost, metronidazole was commonly used for treatment of mild *C. difficile* infection. However, some studies reported the emergence of *C. difficile* isolates with a reduced susceptibility to metronidazole (Baines et al., 2008; Peláez et al., 2008). Vancomycin is considered a second-line therapy, as prolonged treatment is associated with higher risk of selecting vancomycin-resistant *Enterococcus* in addition to its high cost.

It is clear that the health sector would benefit from efficacious alternatives that could be used instead of or in combination with antibiotics to combat *C. difficile*. Bacteriocins of lactic acid bacteria appear to offer one of the most promising alternatives in the fight against enteric pathogens (Hammami et al., 2013). Bacteriocins are proteinaceous antimicrobial molecules that are synthesized by a ribosomal mechanism in many bacteria (Drider & Rebuffat, 2011). Their inhibitory activity may be bactericidal or bacteriostatic and is generally directed against bacteria phylogenetically close to the producer strain (Tagg et al., 1976).

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; GRAS, generally recognized as safe.
Lactocin 3147, a two-component lantibiotic produced by a strain of *Lactobacillus lactis* isolated from an Irish kefir grain (McAuliffe et al., 1998), and thuricin CD, a unique two-component narrow-spectrum bacteriocin produced by a strain of *Bacillus thuringiensis* isolated from human intestinal flora (Rea et al., 2010), are reportedly effective against *C. difficile* (Rea et al., 2007, 2010). However, these two bacteriocins do not yet have GRAS (generally recognized as safe) status and remain to be approved by regulatory authorities before use for humans (or even animals). Nisin is a polypeptide of 34 amino acid residues produced by several strains of *L. lactis* subsp. *lactis*, and is inhibitory to a wide range of pathogens (Breukink & de Kruijff, 1999) and food spoilage agents (Ávila et al., 2014). It has GRAS status and is currently the only bacteriocin approved as a food additive. Nisin is now used as a food preservative in many countries and for the prevention of bovine mastitis. Nisin-producing strains of *L. lactis* have been isolated recently from the faeces of elderly subjects in Ireland (Lakshminarayanan et al., 2013). To date, few studies have shown in vitro activity of nisin against *C. difficile* (Bartoloni et al., 2004; Field et al., 2010). The objective of the present study was to investigate the inhibitory effect of nisin against vegetative cells and spores of clinical isolates of *C. difficile*.

**METHODS**

**Bacterial strains and growth media.** *L. lactis* subsp. *lactis* biovar. diacetylactis UL719 isolated from raw milk cheese (Ali et al., 1995) was used as the nisin Z-producing strain (Meghrout et al., 1997). *Pediococcus acidilactici* UL5, from our collection, was used as the indicator organism for nisin Z activity (Bouksaim et al., 1997). Clinical isolates of *C. difficile* were obtained from the Université Laval Infectious Disease Research Center Culture Collection and are listed in Table 1. The bacterial cultures were maintained in 20 % glycerol stock at −80 °C. Strains UL719 and UL5 were reactivated in de Man–Rogosa–Sharpe (MRS) broth obtained from Difco and were grown overnight at 30 °C. *C. difficile* isolates were reactivated in brain heart infusion (BHI) broth obtained from Difco and were grown overnight anaerobically at 37 °C. Prior to each experiment, each bacterial culture was subcultured at least three times (1 % volume transfer) at 24 h intervals.

**Antibiotics and antimicrobial substances.** Erythromycin, tetracycline hydrochloride, chloramphenicol, vancomycin, metronidazole and ciprofloxacin were all obtained from Sigma. Solutions of each antibiotic at a concentration of 1 mg ml⁻¹ were prepared in water or ethanol (70 %, v/v) depending on the solubility index and microfiltered (Cameo 25N 0.22 µm membrane; MSI).

Nisin A was obtained from Nisaplin (Danisco). The standard procedure used for the production and the purification of nisin Z has been described previously (Le Lay et al., 2008).

**Measurement of bacteriocin activity.** The agar diffusion test described previously (Le Lay et al., 2008) was used. Briefly, MRS broth containing 0.75 % (w/v) agar was cooled to 47 °C and seeded at 1 % (v/v) with an overnight culture of *P. acidilactici* UL5. The medium (25 ml) was then poured into a sterile Petri dish and allowed to solidify at room temperature. Wells (7 mm diameter) were cut in the solidified agar using a sterile metal cork borer and filled with 80 µl nisin solution. The plates were left at 4 °C for 2 h to allow diffusion of the tested aliquot and then incubated at 30 °C. The diameter of the inhibition zones (Fig. 1) was measured after 18 h.

**Susceptibility of *C. difficile* isolates to antibiotics and to nisins A and Z.** The MIC of the antimicrobial agents was determined using a microplate assay as described previously (Mota-Meira et al., 2000). The OD₆₅₀ of a mid-exponential-phase culture of *C. difficile* grown in BHI broth was adjusted to 0.1 with fresh BHI broth using a Spectronic 20 spectrophotometer (Bausch & Lomb). The viable count in this inoculum was determined using 10-fold dilutions (in 0.1 %, w/v, peptone water) plated on BHI agar and incubated anaerobically at 37 °C for 24–48 h. These counts were found to range from 5 × 10⁶ to 1 × 10⁷ c.f.u.ml⁻¹. A twofold serial dilution of antimicrobial agent or antibiotic in BHI broth (125 µl) in a polystyrene microplate (Becton Dickinson Labware) was inoculated with bacterial suspension (50 µl) to obtain ~2.5–5.0 × 10⁸ c.f.u. per well, as per the Clinical and Laboratory Standards Institute (CLSI) standard method (CLSI, 2007). The microplates were incubated anaerobically at 37 °C for 24 h. The OD₆₅₀ was read using a Thermomax microplate reader (Molecular Devices). Positive controls (inoculated wells without antimicrobial agent) and negative controls (un-inoculated wells containing broth medium and antimicrobial agent) were included. The MIC was the lowest concentration of the tested agent giving complete inhibition of growth (OD₆₅₀ equal to the OD₆₅₀ of the negative control). The microplate assay was repeated four times for each combination of antimicrobial agent and bacterial strain, and the MIC was determined as the median of the four repetitions. In accordance with the guidelines established by the Clinical and Laboratory Standards Institute for anaerobic bacteria (CLSI, 2007) and European Society of Clinical Microbiology and Infectious Diseases, the breakpoints used were 2 µg ml⁻¹ for vancomycin, 8 µg ml⁻¹ for erythromycin, 16 µg ml⁻¹ for tetracycline, and 32 µg ml⁻¹ for metronidazole and chloramphenicol. For antimicrobial agents to which no standard breakpoints to *C. difficile* have been defined, breakpoints were considered as follows: ciprofloxacin ≥8 µg ml⁻¹ (Mutlu et al., 2007).

**Transmission electron microscopy.** *C. difficile* ATCC 630 was fixed for 1 h at 4 °C in cacodylate buffer (pH 6.9) containing 1 % (v/v) paraformaldehyde and 2 % (v/v) glutaraldehyde. The suspensions were centrifuged and dehydrated by successive washes with ethanol for 30 min. The *C. difficile* pellets were embedded in Spurr resin, and ultrathin sections were obtained (Ultratome III; LKB), mounted on a 0.5 % Piloform-coated copper grid (3.05 mm diameter) and stained with 3 % aqueous uranyl acetate and 0.1 % lead citrate prior to imaging using a JEOL transmission electron microscope (model 1200).

**Spore preparation.** A BHI agar plate was inoculated with an overnight culture of *C. difficile* ATCC 630 and incubated at 37 °C under anaerobic conditions for 5 days. Spores were recovered from the agar using sterile distilled water. For spore purification, spores were cleaned by repeated centrifugation and washing with sterile distilled water until they were 99 % free of sporulating cells, cell debris and germinated spores, as assessed by phase-contrast microscopy. Cleaned spores were suspended in sterile distilled water (Paredes-Sabja et al., 2008) and stored at −20 °C until further use. The number of spores ml⁻¹ was counted directly by phase-contrast microscopy using a counting chamber (Petroff-Hauser Bacteria Counter; Hauser Scientific).

**Spore viability in the presence of nisin.** A solution of nisin A prepared at 3.2, 12.8 or 25.6 µg ml⁻¹ was added to a suspension containing *C. difficile* at 2 × 10⁸ spores ml⁻¹, and the mixture was incubated for 1 or 24 h at 37 °C and then centrifuged. The pellet was washed, plated on BHI agar containing 0.1 % sodium taurocholate (as germinating agent) and incubated at 37 °C for 24 h under anaerobic
Table 1. MIC (μg ml⁻¹; median of four repetitions) of bacteriocins and antibiotics against strains and isolates of C. difficile

<table>
<thead>
<tr>
<th>Strain/isolate*</th>
<th>Bacteriocin</th>
<th>Antibiotic†</th>
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<tr>
<td></td>
<td>Nisin A</td>
<td>Nisin Z</td>
</tr>
<tr>
<td>ATCC 630</td>
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*ATCC, American type Culture Collection; CCR1, Université Laval Infection Research Center Culture Collection.
†Cip, Ciprofloxacin (breakpoint ≥8 μg ml⁻¹); Van, vancomycin (breakpoint ≥2 μg ml⁻¹); Met, metronidazole (breakpoint ≥32 μg ml⁻¹); Ery, erythromycin (breakpoint ≥8 μg ml⁻¹); Chl, chloramphenicol (breakpoint ≥32 μg ml⁻¹); Tet, tetracycline hydrochloride (breakpoint ≥16 μg ml⁻¹). S, sensitive; R, resistant.

conditions. The resulting vegetative cells were counted and compared to a control not treated with nisin.

Effect of nisin on C. difficile spore germination. To determine the effect of nisin on spore germination, a suspension containing C. difficile at 2 × 10⁵ spores ml⁻¹ was plated on BH1 agar containing 0.1% sodium taurocholate and 0.1, 0.2, 0.4, 0.8 1.6 or 3.2 μg nisin A ml⁻¹. The plates were incubated at 37 °C for 24 h under anaerobic conditions. The resulting colonies were counted and compared to a control not treated with nisin.

Statistical analyses. The data were analysed using the SAS version 9.2 (SAS Institute). Experimental values are reported as mean ± SD. The statistical significance of the differences between treatments was evaluated using a one-way ANOVA t-test. The level of significance was P ≤ 0.05.

RESULTS

Susceptibility of clinical isolates of C. difficile to antibiotics and nisin

The susceptibilities of clinical isolates of C. difficile to antibiotics and nisin A and Z are summarized in Table 1. All tested strains (n=23) were susceptible to metronidazole, vancomycin and chloramphenicol according CLSI breakpoints, with MICs of <1, <1–1.5 and 1–6 μg ml⁻¹, respectively. Of the tested strains, 22 isolates were resistant to at least one antibiotic. In particular, a high number of isolates resistant to ciprofloxacin was recorded

Fig. 1. Inhibitory activity of bacteriocins nisin A and nisin Z against P. acidilactici UL5 (sensitive strain) and C. difficile ATCC 630 as revealed by zones of clearing in the agar diffusion assay.

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(21/23) with a MIC range of 8–128 μg ml⁻¹. To a lesser extent, a total of 8/23 and 1/23 isolates were resistant to erythromycin and tetracycline, respectively. In comparison, C. difficile isolates were more sensitive to nisin A than to nisin Z, with susceptibilities ranging from 0.8 to 51.2 μg ml⁻¹ and from 6.4 to 51.2 μg ml⁻¹, respectively. We also noted that strain CCRI 9238 was the least sensitive, with MIC values of 51.2 μg ml⁻¹ for both nisins, whilst strain CCRI 9242 was the most sensitive, with MICs of 0.8 and 6.4 μg ml⁻¹ for nisins A and Z, respectively.

Transmission electron microscopy

Fig. 2 shows the electron micrographs of nisin-treated and untreated C. difficile vegetative cells. Untreated cells suspended in BHI without nisin exhibited a completely intact cell structure (Fig. 2a). However, treatment with nisin A for 1 min at concentrations of 16 and 64 μg ml⁻¹ (Fig. 2b, c) was associated with significant holes in the cell membrane and release of cytoplasmic contents, likely causing cell death. This is widely believed to be the basis for the antibacterial activity of nisin. Similar results were observed with nisin Z at 32 and 128 μg ml⁻¹ (Fig. 2e, f).

Effect of nisin A on C. difficile spores

The effect of nisin A on C. difficile spores was evaluated by counting germination at different concentrations. Fig. 3 shows that the impact on spore viability was weak at 3.2 μg ml⁻¹. Viability ranged from 82 to 99 % after 1 and 24 h of contact with nisin. At higher concentrations, nisin appeared to cause a statistically significant decrease in viability to 79–81 % at 12.8 μg ml⁻¹ (P≤0.05) and to 40–50 % at 25.6 μg ml⁻¹ (P≤0.05).

Effect of nisin A on C. difficile spore germination

Fig. 4 shows the effect of nisin A on taurocholate-induced germination of C. difficile spores. Germination dropped by 0.3±0.08 and 0.8±0.4 log in the presence of nisin at concentrations of 0.1 and 0.2 μg ml⁻¹, respectively. Significant decreases in germination (>1.8 log) were obtained at a higher concentration (0.4 μg ml⁻¹).

DISCUSSION

In recent years, bacteriocins have attracted increasing attention in medicine, with their highly specific activity at the nanomolar range, non-toxicity to humans, unique mechanism of action and low propensity to generate resistance. Many bacteriocins have been assessed for potential application as therapeutic agents (Hammami et al., 2013). In this study, we investigated the potential of nisin as an anti-infective agent for treating C. difficile by examining the susceptibility of 20 clinical isolates and three ATCC strains to nisin A and Z as well as six antibiotics, including vancomycin and metronidazole – two drugs commonly used for the treatment of C. difficile infection.
In this study, nisin A was more potent than its Z variant, and thus residue 27 (His for nisin A, Asn for nisin Z) (Mulders et al., 1991) is likely important for bacteriocin action on C. difficile cells. Other studies have shown that the MIC differed between nisin A and nisin Z for the same target organism (Le Blay et al., 2007). Our results for nisin A are comparable to the MICs obtained for lacticin 3147, a bacteriocin that shows great promise for the treatment of C. difficile infection. Lacticin 3147 MIC values ranging from 0.95 to 15 μg ml⁻¹ have been found for a bank of C. difficile isolates using the agar dilution test method (Rea et al., 2007). Another bacteriocin inhibitory to C. difficile is thuricin CD, for which MICs of 0.125–0.5 μM (the range for nisin A in the present study is 0.238–15.2 μM) have been reported for 19 strains, including 13 clinical isolates (Mathur et al., 2013). The nisin A MICs determined in the present study compare favourably to the range (0.5–4.0 μg ml⁻¹) reported previously for vancomycin or metronidazole (Drummond et al., 2003), indicating that this bacteriocin is at least as effective as the antibiotics used commonly to treat C. difficile infection.

Unlike vegetative C. difficile, spores are highly resistant to chemical disinfectants and antibiotics. By altering the normal composition of the protective colonic microbial flora, antibiotic treatments create favourable conditions for colonization and hence infection by C. difficile (Wilson & Perini, 1988; Tannock et al., 2010). However, spores require the presence of certain compounds in order to undergo germination. It has been shown that C. difficile spores germinate in the presence of the secondary bile salt taurocholate, which acts as a co-germinant with glycine (Sorg & Sonenshein, 2008). We therefore investigated the antimicrobial effect of nisin on C. difficile spore viability and germination in a medium containing taurocholate. At a concentration of 3.2 μg ml⁻¹, nisin did not inhibit spores significantly. Nerandzic & Donskey (2013) reported similar results with no inhibition of dormant spores at the same nisin concentration (3.2 μg ml⁻¹). In this study, a concentration of ≥12.8 μg ml⁻¹ was required for nisin to inhibit C. difficile spores. In instance, the germination of C. difficile spores dropped by 40–50 % after treatment with a high concentration (25.6 μg ml⁻¹) of nisin A. We have shown that nisin is able to inhibit the outgrowth of C. difficile (log reduction >4 at 3.2 μg ml⁻¹) after germination is initiated. Other bacteriocins have been shown to inhibit the germination of bacterial spores, e.g. lacticin 3147 produced by L. lactis IFPL 3593 against Clostridium tyrobutyricum (Martinez-Cuesta et al., 2010), bacteriocin AS-48 produced by Enterococcus faecalis A-48-32 against Bacillus cereus (Abriouel et al., 2002), and nisin against B. cereus (Pol et al., 2001) and Bacillus anthracis (Gut et al., 2008, 2011).

Nisin inhibits Gram-positive vegetative bacterial cells by two mechanisms: membrane pore formation and interfering with cell wall biosynthesis (Ruhr & Sahl, 1985; Wiedemann et al., 2001). Gut et al. (2008, 2011) have studied the inhibition of B. anthracis spore outgrowth by nisin. They first showed that nisin had an inhibitory action on the germinated spores and then generated nisin molecules coupled to fluorescein for epifluorescence microscopy, which allowed them to locate the site of action at the poles of the spores. The binding site corresponded to the localization of lipid II, which is required for the biosynthesis of new cell wall during the outgrowth process. They also demonstrated that the effect on lipid II alone was not sufficient to inhibit growth, and concluded that the inhibitory mechanism of nisin on the outgrowth of B. anthracis spores was the combination of binding lipid II and membrane disruption.
Several studies on the emergence of \textit{C. difficile} isolates have been reported, with a reduced susceptibility to metronidazole (Baines \textit{et al.}, 2008; Pelaez \textit{et al.}, 2008) and high risks of selecting resistant vancomycin-resistant \textit{Enterococcus}. With treatment failures and recurrence of infections (Aslam \textit{et al.}, 2005), there is an urgent need for novel antimicrobial treatments. In this study, we have shown the capacity of nisin to inhibit \textit{in vitro} both \textit{C. difficile} vegetative cells and spore germination. Further \textit{in vivo} experiments are required before the possible application of nisin as a therapeutic agent for the treatment of \textit{C. difficile} infection.

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\section*{References}


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