Antimicrobial activity of lacticin 3147 against clinical Clostridium difficile strains

Mary C. Rea,1,2 Evelyn Clayton,1,2 Paula M. O’Connor,1,2 Fergus Shanahan,2,3 Barry Kiely,4 R. Paul Ross1,2 and Colin Hill2,5

Correspondence
R. Paul Ross
paul.ross@teagasc.ie
1Teagasc, Biotechnology Centre, Moorepark Food Research Centre, Moorepark, Fermoy, Cork, Ireland
2Alimentary Pharmabiotic Centre, University College, Cork, Ireland
3Department of Medicine, University College, Cork, Ireland
4Alimentary Health, Kinsale, Cork, Ireland
5Department of Microbiology, University College, Cork, Ireland

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Clostridium difficile-associated diarrhoea (CDAD) is the most common hospital-acquired diarrhoea, and is a major type of gastroenteritis infection in nursing homes and facilities for the elderly. In this study the antimicrobial activity of the two-component lantibiotic, lacticin 3147, against a range of genetically distinct C. difficile isolates was studied. The bacteriocin exhibited an MIC50 of 3.6 μg ml⁻¹ for 10 genetically distinct C. difficile strains isolated from healthy subjects, inflammatory bowel disease patients and culture collection strains. In time-kill studies, 10⁶ c.f.u. ml⁻¹ C. difficile ATCC 42593 and CDAD isolate DPC 6220 were killed within 120 or 20 min incubation, respectively, at a concentration of 6 μg lacticin ml⁻¹. Interestingly, addition of lacticin 3147 to exponentially growing cells of C. difficile ATCC 43593 caused rapid lysis of the cells after an initial lag phase, as measured by the concomitant release of the intracellular enzyme, acetate kinase. The addition of a food-grade, milk-based lacticin containing powder to faecal fermentation demonstrated that lacticin is effective in completely eliminating 10⁶ c.f.u. C. difficile ml⁻¹ from a model faecal environment within 30 min when present at concentrations as low as 18 μg ml⁻¹. While other culturable microflora such as total anaerobes, bacteroides, total non-spore-forming anaerobes and total Gram-negative anaerobes were unaffected, populations of lactobacilli and bifidobacteria were reduced by 3 log cycles at bacteriocin levels sufficient to eliminate over 10⁶ C. difficile. In light of these findings, the potential of lacticin 3147 for treatment of CDAD is discussed.

INTRODUCTION

First described in 1935, Clostridium difficile was not recognized as the causative agent of nosocomial diarrhoea until the 1970s (George et al., 1978; Hall & O’Toole, 1935). One of the main predisposing factors for the acquisition of C. difficile-associated diarrhoea (CDAD) is antibiotic therapy. The Health Protection Agency in the UK reported 42 625 cases of C. difficile infection in patients aged 65 years and over in England for the first 9 months of 2006, which showed a 5.5 % increase over the same period in 2005 according to figures from the mandatory surveillance reporting scheme for CDAD. Outbreaks of CDAD due to the hyper-virulent strain C. difficile PCR ribotype 027 have been reported in the USA and Europe (Joseph et al., 2005; Pepin et al., 2004; Valiquette et al., 2004).

Abbreviations: AU, activity unit; CDAD, Clostridium difficile-associated diarrhoea; WDA, well diffusion assay.

Current treatments for CDAD are oral metronidazole and vancomycin (Aslam et al., 2005; Bricker et al., 2005). However, treatment failures and recurrence of infections have been reported to range from 2 to 38 % and from 8 to 50 %, respectively, when these antibiotics have been administered singly or in combination (Aslam et al., 2005). Where possible, metronidazole would be the drug of choice because of the danger of colonization of the gut with vancomycin-resistant enterococci (Bricker et al., 2005; Ohl et al., 2005) or the spread of vancomycin resistance to pathogenic bacteria within the hospital environment, such as Staphylococcus aureus.

Alternative and novel strategies need to be developed for the treatment of this disease. It has been reported that nisin, a cationic peptide produced by Lactococcus lactis, was more active against C. difficile than vancomycin or metronidazole (Bartoloni et al., 2004). Lacticin 3147 is a two-component lantibiotic produced by a strain of L. lactis.
that was originally isolated from an Irish kefir grain (McAuliffe et al., 1998). Recently, the structure and mode of action, including binding to lipid II, have been determined in this d-alanine containing lantibiotic (Cotter et al., 2005; Martin et al., 2004; McAuliffe et al., 2000; Wiedemann et al., 2006). Unlike nisin, which is poorly soluble and therefore less active at pH 7.0, lacticin is soluble and active at physiological pH, and therefore may be more suitable for human clinical applications.

The aim of this study was to investigate the efficacy of lacticin 3147 as an antimicrobial agent against pathogenic C. difficile isolates, and to determine its effect on gut microflora in a model faecal environment that contained C. difficile.

**METHODS**

**Bacterial strains used.** C. difficile ATCC 43593 and clinical isolate DPC 6220 were used for testing bacteriocin sensitivities in time-kill studies. L. lactis MG1363(pOMO2)(pMRCO1), a lacticin overproducing strain (Cotter et al., 2005), was used to produce the purified lacticin preparation. L. lactis subsp. lactis HP was used as the target strain in well diffusion assays (WDAs) to determine the concentration of lacticin as an activity units ml⁻¹ (AU ml⁻¹) value. L. lactis subsp. lactis HP(pMRCO1), a lacticin-insensitive strain of L. lactis HP, was used to verify that inhibition of target strain (L. lactis spp. lactis HP) was due to lacticin 3147 activity.

**Isolation and characterization of C. difficile strains.** To establish a bank of C. difficile isolates, strains were isolated from the stool samples of healthy volunteers, and inflammatory bowel disease and CDAD patients using standard methods (Jousimies-Somer et al., 2002), and confirmed as C. difficile by 16S sequencing (Simson et al., 2003). Isolates, including two ATCC strains (43593 and 43600), were genetically fingerprinted using PFGE as described by Gal et al. (2005). The DNA bands were visualized using an Alphalager 3400.

**Preparation of purified lacticin 3147**

**Preparation of lacticin.** Purified bacteriocin was prepared as follows: L. lactis MG1363(pOMO2)(pMRCO1) was subcultured twice in GM17 broth at 1 % at 30 °C before use. Two litres of tryptone yeast broth (TYB) were inoculated with the culture at 0.5 % and incubated at 30 °C overnight. TYB was used as follows: 2.5 g trypitone (Oxoid); 5.0 g yeast extract (Oxoid); 0.25 g MgSO₄; 7H₂O; 0.05 g MnSO₄. 4H₂O dissolved in 900 ml distilled H₂O. The medium was clarified, after autoclaving at 121 °C for 15 min, by passing through a column containing 2-propanol washed XAD beads (Sigma-Aldrich). Before use, filter-sterilized glucose and β-glycerophosphate were added to give a final concentration of 10 g and 19 g l⁻¹, respectively, and a final volume of 1 l. Following fermentation the culture was centrifuged at 8280 g for 15 min. The cell pellet and supernatant were retained. The cells were then resuspended in 200 ml 70 % 2-propanol pH 2.0 per l broth and stirred at 4 °C for 4 h. The culture supernatant was passed through XAD beads, pre-washed with 1 l distilled H₂O. The column was then washed with 500 ml 40 % ethanol and the inhibitory activity eluted in 400 ml 70 % 2-propanol pH 2.0 and retained (S₁). The cells, which had been resuspended in 70 % 2-propanol pH 2.0, were centrifuged at 8280 g for 15 min and the supernatant (S₂) retained. S₁ and S₂ were combined. The 2-propanol was then evaporated using a rotary evaporator (Buchi) and the sample applied to a 6 g (20 ml) Phenomenex C-18 column pre-equilibrated with methanol and water. The column was washed with 2 column volumes of 40 % ethanol and the inhibitory activity eluted in 1.5 column volumes of 70 % 2-propanol pH 2.0. The preparation was freeze-dried and resuspended in 50 mM phosphate buffer pH 6.5. The AU ml⁻¹ value was calculated (Ryan et al., 1996) using L. lactis subsp. lactis HP as the indicator strain.

**Preparation of powdered fermentate of lacticin.** A powdered skimmed milk-based preparation of lacticin 3147 was prepared as outlined by Morgan et al. (1999) with the following modifications: after 20 h fermentation the pH was allowed to drop naturally to pH 6.1 with constant agitation. Once the fermentate reached pH 6.1, it was maintained at this pH. The fermentate was evaporated to 50 % solids (with a heat-treatment step on passage into the evaporator of 85 °C for 10 s) and spray dried (Niro Tall Form Dryer) with an air inlet temperature of 185 °C and an outlet temperature of 85 °C.

**Determination of the MIC of lacticin against C. difficile**

**Preparation of lacticin standard curve.** Purified lacticin 3147 was prepared as described above and the purity of the two peptides (LtnA1 and LtnA2) determined using matrix-assisted laser desorption ionization-time of flight MS as described by Cotter et al. (2005). Activity was determined using the WDA. This preparation was then diluted to give activities of 80 000, 40 000, 20 000, 10 000 and 5000 AU ml⁻¹. Each concentration was aliquoted in 100 μl amounts, in triplicate, into 8 mm clear conical crimp vials and freeze-dried overnight in an Advantage freeze-drier (Virtilis). Samples were then hydrolysed to their amino acid constituents via vapour phase hydrolysis in a MARS 5 microwave (CEM) equipped with a protein hydrolysis accessory kit. The hydrolysis conditions used were as follows: microwave output 300 W, ramp to 150 °C in 20 min, hold for 5 min and cool to 30 °C before removing samples. Following hydrolysis the samples were resuspended in 125 nmol norleucine ml⁻¹, the internal standard, and the amino acids quantified using a JLC-500/V amino acid analyser (Jeol) fitted with a Jeol Na⁺ high-performance cation-exchange column.

A standard curve was then obtained by plotting the concentration of lacticin, calculated in μg ml⁻¹, against AU ml⁻¹.

**Determination of the MIC of lacticin.** C. difficile strains were grown overnight in reinforced Clostridium medium (RCM). Subsequently, they were subcultured in RCM again for ~5 h and diluted to give ~10⁵ cfu ml⁻¹. Five microlitres of culture was spotted onto the surface of Wilkins-Chalgren agar plates containing lacticin. Plates containing lacticin were prepared using the agar dilution test method as described by Jousimies-Somer et al. (2002). MICs were determined after incubation of the plates for 48 h in an anaerobic chamber at 37 °C. MIC₅₀ was defined as the lowest concentration of lacticin 3147 at which 50 % or more inhibition of the test strains occurred.

**Demonstration of activity of lacticin 3147 against C. difficile using kill curves.** One millilitre of double strength RCM was added to a sterile container and inoculated with an overnight culture of C. difficile at 1 %. Lacticin, either the purified preparation or lacticin powder (stock suspension of 20 %), was added to give the required concentration and the volume brought up to 2 ml with sterile H₂O. The bacteriocin was omitted from the control and the volume replaced with sterile H₂O. When lacticin powder was used reconstituted skimmed milk was the control. All media were pre-conditioned under anaerobic conditions before use. Samples were removed at intervals and plated on reinforced Clostridium agar (RCA) for the purified lacticin and/or on C. difficile selective agar (Oxoid) for the lacticin powder, and incubated at 37 °C for 24–48 h.

**Demonstration of lysis of C. difficile by lacticin.** C. difficile ATCC 43593 was subcultured overnight in fresh RCM and then inoculated...
into fresh RCM at 2%. Growth was followed by measuring the optical density at 600 nm until it reached ~0.5. The culture was then split and lacticin added to one portion to give a final concentration of 15 μg ml⁻¹. Optical density measurements were taken at intervals from both the test and control samples. Samples (500 μl) were centrifuged at 20 800 g for 5 min, and the supernatant retained on ice for measurement of acetate kinase, as described by Rose (1955). Activity was determined in cell-free supernatants using acetohydroxy acid as a standard. Experiments were carried out in triplicate. The AU values were calculated as the amount of enzyme that produced 1 μmol acetohydroxy acid per 15 min at 30 °C.

**Determination of the effect of lacticin on culturable gut flora in faecal fermentations without pH control.**  
Fermentation medium (Fookes & Gibson, 2003) containing 1% starch was placed in a boiling water bath for 30 min, and transferred immediately to an anaerobic cabinet and stirred overnight to ensure anaerobic conditions. Fresh faecal samples were obtained on three occasions from healthy adults who had not been prescribed antibiotics in the previous 3 months, and a fresh 10% slurry prepared in anaerobic 0.1 M sodium phosphate buffer pH 7.0 containing 0.05% cysteine. Two vessels of fermentation medium were inoculated with the faecal slurry (10%) in the anaerobic chamber. Samples were withdrawn at 0 h and after 5 h anaerobic incubation at 37 °C for microbial analyses. At 5 h, skimmed-milk powder was added to one vessel (control) and lacticin powder to the other vessel (test) to give a final lacticin concentration of 5000 AU ml⁻¹ (74 μg ml⁻¹) in the fermentation medium. At 90 min after lacticin addition samples were removed for microbiological analyses.

To determine the optimal concentration of lacticin 3147 required to kill *C. difficile*, with the least effect on lactobacilli or bifidobacteria, the medium was spiked with a fresh culture of *C. difficile* ATCC 43593 to give initial numbers of ~10⁶ cells ml⁻¹, and aliquoted into five 100 ml amounts. Lacticin powder was added to give concentrations of lacticin of 320, 640, 1280, 2500 and 5000 AU ml⁻¹ (which was equivalent to 4.7, 9.5, 19, 37 and 74 μg ml⁻¹ lacticin, respectively). No lacticin 3147 was added to the control. Samples were withdrawn at intervals for microbiological analysis of *C. difficile*, *Lactobacillus* and *Bifidobacterium*.

The concentration of lacticin in the fermentation was determined by WDA using *L. lactis* subsp. *lactis* HP as the target strain. *L. lactis* subsp. *lactis* HP(pMRCo1), a lacticin 3147-insensitive strain of HP, was used as a control to ensure that inhibition of HP was due to lacticin and not to some inhibitory compound produced. Serial dilutions of the samples were made in anaerobic diluent in an anaerobic chamber and enumerated on selective media incubated at 37 °C anaerobically for 5 days, unless otherwise specified, as follows: enterococci on kanamycin acsulcin azide agar (Merck) for 24 h; lactobacilli on *Lactobacillus* selective agar (Difco), bifidobacteria on MRS (de Man–Rogosa–Sharpe) medium (Difco) modified by the addition of 0.05% cysteine and 100 μg mupirocin ml⁻¹ (Oxoid) for 3 days, bacteroides on bacteroides bile esculin agar (Jousimies-Somer et al., 2002), total anaerobes on Wilkens–Chalgren anaerobic agar containing Tween 80 and 5% defibrinated horse blood microbiological analysis. At 5 h, skimmed-milk powder was added to one vessel (control) and lacticin powder to the other vessel (test) to give a final lacticin concentration of 5000 AU ml⁻¹ (74 μg ml⁻¹) in the fermentation medium. At 90 min after lacticin addition samples were removed for microbiological analyses.

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**RESULTS AND DISCUSSION**

Since the main purpose of this study was to investigate lacticin 3147 as a potential therapeutic agent against intestinal *C. difficile* infection, the initial work involved the isolation of a range of genetically distinct *C. difficile* strains from a variety of clinical conditions and from healthy individuals. This gave us clinically relevant target strains on which to test the bacteriocin. Genetic relatedness was determined by PFGE. Eight genetically different *C. difficile* strains were isolated from CDAD patients (five strains), inflammatory bowel disease patients (two strains) and a healthy adult (one strain); two ATCC strains were also used in the study.

While the inhibitory activity of bacteriocins is frequently measured by WDAs and expressed as AU ml⁻¹ in this study, in order to determine MIC values in μg ml⁻¹ (allowing direct comparison to other antimicrobial treatments), a standard curve converting AU ml⁻¹ to μg ml⁻¹ was generated by plotting the former against the latter. The existence of a linear relationship between AU and μg ml⁻¹ was established by linear regression analysis, which showed an R² of 0.998 when the line went through zero (Fig. 1). By extrapolation, the concentration of lacticin in μg ml⁻¹ in the subsequent experiments was calculated. MIC values for the strains tested were in the range 0.95–15 μg ml⁻¹. The MIC values for lacticin determined for a range of clinical isolates in this study compare favourably with those reported for vancomycin or metronidazole (0.5–4.0 μg ml⁻¹) (Drummond et al., 2003) versus 3.6 μg ml⁻¹ for lacticin, indicating that lacticin 3147 is at least as effective as the commonly used antibiotics. These strains had previously been shown not to be resistant to vancomycin or metronidazole (E. Clayton, M. C. Rea, F. Shanahan, E. M. Quigley, B. Kiely, C. Hill & R. P. Ross, unpublished results). Future work will require the testing of a larger number of wild-type *C. difficile* isolates, particularly those that exhibit antibiotic resistance, e.g. PCR ribotype 027.

Kill curves were carried out in triplicate using both purified lacticin and lacticin powder. *C. difficile* ATCC 43593 (toxin negative) and the clinical isolate of *C. difficile* from a CDAD patient, designated DPC 6220 (toxin positive), were both tested. No viable cells of ATCC 43593 or DPC 6220 were detected on RCA, following incubation for 120 and 20 min, respectively, in the presence of 6 μg lacticin ml⁻¹ (400 AU ml⁻¹) (Fig. 2). Moreover, no viable cells were detected after overnight incubation of the culture containing lacticin with either strain, suggesting that either there was no outgrowth of spores or those spores that did germinate were killed during prolonged incubation. During the kill-curve experiments it was observed that addition of lacticin 3147 to cultures of *C. difficile* resulted in the clearing of the broth over time. Cell numbers at the start of the experiments for both strains were ~10⁶ c.f.u. ml⁻¹, indicating that lacticin 3147 was having a potent effect on the culture. When lacticin 3147 was added to exponentially growing cells there was an initial lag period with a small reduction in OD₆₀₀ followed by a dramatic decrease in OD₆₀₀ from ~0.45 to 0 in 45 min – this decrease in OD₆₀₀ was paralleled by the concomitant release of the intracellular enzyme acetate kinase into the growth
medium. No acetate kinase activity was detected in the supernatant of the control culture in the absence of lacticin (Fig. 3). Studies on the mode of action of lacticin 3147 have shown that it has a dual mechanism that involves initial binding of the A1 peptide to the cell wall precursor lipid II, followed by binding of the A2 peptide, which leads to pore formation. Most likely, this blocking of cell wall synthesis and pore formation explains the ultimate lysis of the cells in the case of C. difficile (Cotter et al., 2005; Wiedemann et al., 2006).

A food-grade powdered preparation of lacticin 3147 was also effective in killing C. difficile (~ 5 log reduction of counts of C. difficile in a broth culture within 120 min) (Fig. 4). In this respect, powdered lacticin is comparatively cheap to prepare in reasonably large quantities and activity in the powder remains stable for at least 1.5 years (Morgan et al., 1999).

The effect of lacticin 3147 on the culturable gut microbiota was investigated using a simple anaerobic faecal-based fermentation. In these experiments, the food-grade powdered lacticin 3147 was used as the source of the bacteriocin. While lacticin 3147 did not affect the culturable flora, such as non-sporing anaerobes and non-spore forming Gram-negative anaerobes or bacteroides, Gram-positive organisms, such as enterococci, lactobacilli and bifidobacteria, were greatly reduced (at least a 4 log cycle reduction) 90 min after addition of lacticin (74 μg ml⁻¹). At lower concentrations of the bacteriocin this...
potentially negative effect on lactobacilli and bifidobacteria was lessened. For example, Fig. 5 shows that the addition of 19 mg lacticin ml⁻¹ resulted in *C. difficile* being undetectable 30 min later; however, there was a 2 log cycle reduction of *Lactobacillus* sp. and *Bifidobacterium* sp. in the same time period at that concentration. It has been reported that infected patients excrete ~10⁴–10⁷ *C. difficile* (g faeces)⁻¹ but information regarding the level of *C. difficile* in the infected region of the gut of patients suffering from CDAD is sparse (Mulligan *et al.*, 1979). The results presented here demonstrate that lacticin 3147 can kill (at least 5 log cycles) *C. difficile* in a model faecal environment.

Antibiotic treatment has been shown to be a predisposing factor in the acquisition of CDAD; patients suffering from CDAD are mainly treated with oral vancomycin or metronidazole (Bricker *et al.*, 2005; Ohl *et al.*, 2005). However, recurrent diarrhoea has been shown in 5–20 % of patients receiving antibiotic treatment for CDAD (McFarland, 2005; Poutanen & Simor, 2004). While vancomycin is one of the drugs of choice for the treatment of CDAD, this drug was once considered as one of the last lines of defence in the treatment of some microbial infections. The emergence of antibiotic resistance in strains of pathogenic bacteria, i.e. vancomycin resistance in *S. aureus* (Hiramatsu, 2001) and enterococci (Depardieu *et al.*, 2004; Franz *et al.*, 1999), and the appearance of vancomycin- (Pelaez *et al.*, 2002) and metronidazole-resistant (Brazier *et al.*, 2001) strains of *C. difficile* is now a cause of great concern worldwide.

Alternative strategies that have been suggested for the management of CDAD include the use of probiotic cultures such as *Lactobacillus*, bifidobacteria and *Saccharomyces boulardii* (Can *et al.*, 2006; Goossens *et al.*, 2003; Marteau, 2002; Plummer *et al.*, 2004), non-toxigenic *C. difficile* (Seal *et al.*, 1987) nisin (Bartoloni *et al.*, 2004), and prebiotics such as non-digestible oligosaccharides (Hopkins & Macfarlane, 2003) and bovine antibody-enriched whey (van Dissel *et al.*, 2005).

Our results would indicate that lacticin 3147 has potential for the treatment of *C. difficile* infections due to its lytic mode of action, and its activity at low concentrations and at physiological pH. We have found that when the pH of a cell-free supernatant of a lacticin-producing culture is adjusted through the pH range 2–9 no loss of activity is observed. In contrast, when cell-free supernatants of a nisin-producing culture are treated in the same way a 50 % loss of activity is observed at pH 7.0 (M. C. Rea, unpublished results).

When assessing peptides such as nisin and lacticin 3147 for treatment or prevention of the disease it should be emphasized that bioavailability needs to be considered. In this respect, orally administered bacteriocins would not be expected to survive gastric transit given that they are sensitive to proteolysis, and so would need to be encapsulated to target specific areas of the gut, i.e. the sigmoid colon, to be effective for oral administration.
Alternatively, the bacteriocin could be administered as an enema via the anal route where it could be highly effective based on our in vitro results. In addition, any negative effect of lacticin 3147 on beneficial lactobacilli and bifidobacteria populations could be counteracted by using probiotic strains that have been made resistant to the bacteriocin. Indeed, a similar approach for controlling Lactobacillus populations in cheese was used by our group previously where combinations of lacticin-producing starters and lacticin-tolerant lactobacilli were employed to beneficially control cheese flora (Ryan et al., 2001).

In conclusion, we have demonstrated that this two-component bacteriocin is highly active against C. difficile and may provide a basis for alternative therapies for treatment of CDAD.

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