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Authors	O'Reilly, Catherine;O'Connor, Paula M.;O'Sullivan, Orla;Rea, Mary C.;Hill, Colin;Ross, R. Paul
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ORIGINAL ARTICLE

Impact of nisin on *Clostridioides difficile* and microbiota composition in a faecal fermentation model of the human colon

Catherine O'Reilly[1,2,3] | **Paula M. O'Connor**[1,3] | **Órla O'Sullivan**[1,3] | **Mary C. Rea**[1,3] | **Colin Hill**[2,3] | **R. Paul Ross**[1,2,3]

[1]Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, Ireland

[2]School of Microbiology, University College Cork, Cork, Ireland

[3]APC Microbiome Ireland, University College Cork, Cork, Ireland

Correspondence
R. Paul Ross, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
Email: p.ross@ucc.ie

Abstract

Aims: Nisin is a bacteriocin with a broad spectrum of activity against Gram-positive bacteria. The aims were to assess nisin activity against *Clostridioides difficile* in a complex microbial environment and determine the minimum inhibitory concentration at which *C. difficile* growth is suppressed whilst having minimal impact on the faecal microbiota.

Methods and Results: Faecal slurries were prepared from fresh faecal samples and spiked with *C. difficile* (10^6 CFU per ml). Nisin was added to each fermentation at a range of concentrations from 0 to 500 μM. Following 24 h, 16S rRNA gene sequencing was performed, and the presence of viable *C. difficile* was assessed. There was no viable *C. difficile* detected in the presence of 50–500 μM nisin. There was a decrease in the diversity of the microbiota in a nisin dose-dependent manner. Nisin predominantly depleted the relative abundance of the Gram-positive bacteria whilst the relative abundance of Gram-negative bacteria such as *Escherichia Shigella* and *Bacteroides* increased.

Conclusions: Using an *ex vivo* model of the colon, this study demonstrates the ability of purified nisin to selectively deplete *C. difficile* in a faecal microbial environment and establishes the minimum concentration at which this occurs whilst having a minimal impact on the composition of the microbiota.

Significance and Impact of the Study: This study opens up the potential to use nisin as a therapeutic for clostridial gut infections.

KEYWORDS

16S rRNA gene sequencing, *C. difficile*, *ex vivo* faecal fermentation, nisin

INTRODUCTION

Clostridioides difficile is a Gram-positive anaerobic spore-forming bacterium that can cause severe gastrointestinal illness in individuals undergoing antibiotic treatment (Kelly & La Mont, 2008; Rupnik et al., 2009; Stanley et al., 2013). Antibiotic exposure generally leads to disruption of the normal colonic microbiota. This can result in reduced resistance against intestinal colonization by *C. difficile* and result in an increased risk of *C. difficile* infection

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(CDI). Additional risk factors for CDI are inflammatory bowel disease, immunodeficiency and related illnesses (Ananthakrishnan et al., 2008; Kyne et al., 2002). Many antibiotics have been linked with CDI including ampicillin, amoxicillin, clindamycin, cephalosporins and fluoroquinolones (Stevens et al., 2011; Wiström et al., 2001). In 2016, a total of 7711 reported cases of CDI were recorded in 20 countries by the European Centre for Disease Prevention and Control (ECDI), 5756 of which (74.6%) were healthcare-associated (HA) CDI and 1955 (25.4%) of which were either community-associated (CA) or of unknown origin *C. difficile* infections (European Centre for Disease Prevention and Control, 2016). *Clostridioides difficile* infection can cause acute diarrhoea and pseudomembranous colitis, which can lead to colonic perforation and, if untreated, ultimately death (Kelly & La Mont, 2008; Rupnik et al., 2009; Stanley et al., 2013). The antibiotics vancomycin and metronidazole are well-established treatments for CDI (Surowiec et al., 2006). Their use is controversial, however, due to an increase in the number of reported treatment failures and recurrence of CDI (Aslam et al., 2005). The use of vancomycin has been associated with the development of vancomycin-resistant enterococci for instance (Lagrotteria et al., 2006). Similarly, *C. difficile* isolates that are resistant to metronidazole have been identified (Freeman et al., 2015). Fidaxomicin is the most recent medication approved for the treatment of CDI. Fidaxomicin is expensive, however, and the cost far exceeds that of metronidazole or vancomycin. It is recommended only to be used in populations who are at a high risk of relapse and those in which fidaxomicin has proven more effective than other treatments (Mullane, 2014). Due to the increase in antibiotic resistance and hospital-acquired infections, there is an urgent need to develop new treatments for CDI.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacterial species which can exhibit either a narrow spectrum of activity (targeting members of the same species) or a broad activity spectrum (targeting other species and genera) (Cotter et al., 2013). They are generally produced by bacteria within their ecological niches and serve as a competitive advantage and self-preservation tool. Most of the currently recognized bacteriocins are produced by Gram-positive bacterial species with lesser known producers among Gram-negative bacteria (Ghodhbane et al., 2015; Meade et al., 2020). Several bacteriocins have proven activity against *C. difficile* such as lacticin 3147 (Rea et al., 2007), microbisporin (Castiglione et al., 2008), thuricin CD (Rea et al., 2010) and nisin (Le Blay et al., 2007).

Nisin is of interest for several reasons. Nisin A is produced by *Lactococcus lactis* subsp. *lactis* and has a broad spectrum of activity against most Gram-positive bacteria and other lactic acid producing bacteria (Abee & Delves-Broughton, 2003). Nisin A was first discovered in 1928 and is the most intensively studied lantibiotic (Rogers & Whittier, 1928). Nisin has been approved as safe for use in the food industry (as a natural food preservative, E234) by the US Food and Drug Administration (FDA), World Health Organization (WHO) and the European Union (EU) (Chen & Hoover, 2003; Delves-Broughton, 1996; Guinane et al., 2005). Nisin has been shown to effectively kill *C. difficile in vitro* at concentrations comparable to antibiotics such as metronidazole and vancomycin (Le Lay et al., 2016). Because nisin is proteinaceous in nature, digestive proteases will break it down (albeit to a lesser extent than less modified bacteriocins in our experience). Delivery to the colon for the treatment of *C. difficile* could be achieved through rectal administration, encapsulation or removal of the proteolytic sites. In this study, we used an *ex vivo* model of the colon to investigate the ability of purified nisin to selectively deplete *C. difficile* in a faecal microbial environment and establish the minimum concentration at which this occurs whilst having a minimal impact on the composition of the microbiota.

MATERIALS AND METHODS

Donor recruitment for faecal fermentation studies

Donor recruitment and enrolment were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol no. APC055). All donors completed and signed a consent form to acknowledge their participation in the study. All human subjects were healthy females and males between 23 and 65 years of age. Participants had no antibiotic use in the 6 months prior to participation in the study.

Micro-Matrix™ cassette setup and parameters and faecal medium

Six healthy volunteers of varying ages (23–62) and gender (3 male and 3 female) provided fresh faecal samples on the day of the experiment. The micro-Matrix™ (Applikon Biotechnology) mini-fermentation system was used to model the distal colon as described previously (O'Donnell et al., 2018).

The micro-Matrix™ provides simultaneous individual measurement and control of temperature, pH and dissolved oxygen concentration (dO_2) in 24 parallel micro-bioreactors (wells) with working volumes of up to 5 ml each. Each well of the micro-Matrix cassette operates as a stand-alone

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bioreactor. The precalibrated integrated pH and DO sensors remove the need to calibrate each well prior to beginning the fermentation (O'Donnell et al., 2018). The faecal medium was prepared according to Fooks and Gibson (2003). In brief, the fermentations were carried out in three sealed micro-Matrix cassettes (24 wells/cassette) with 2 subjects per run. Each condition was carried out in duplicate wells of the micro-Matrix plate. The nisin A used in this study was a high content powder form (95% content/ultrapure; % weight/weight; hydrous potency ≥38,000 IU/mg) which was purchased from Handary. Fermentations were carried out using the fermenter as follows: 1 mM stock of nisin A was made on the day of the experiment. The 1 mM stock was added to wells at varying volumes for final concentrations of 25, 50, 100, 250 and 500 μM in a total volume of 5 ml. Control with 0 μM nisin was also included for each donor sample. *C. difficile* (Ribotype 027) was spiked in at 10^6 CFU per ml. The 20% faecal slurry was prepared under anaerobic conditions using 50 mM phosphate buffer pH 7.0 containing 0.5% cysteine which had previously been boiled and cooled anaerobically. About 1 ml was added to each well of the micro-Matrix® plate.

The time 0 h (T0) sample containing 20% slurry and 80% faecal medium was taken during the setup of the cassette in the anaerobic chamber in 1 ml aliquots. One millilitre of sample was snap frozen at −80°C immediately until used for DNA extraction. A sample taken at 0 h (and also T24 h) was serially diluted and plated on CCEY agar (Lab M) (with 1% lysed horse blood) and incubated at 37°C for 48 h to detect viable *C. difficile* growth and determine the minimum concentration of nisin at which *C. difficile* was successfully inhibited to the extent that the pathogen could not be detected. The presence of nisin in the samples was determined using mass spectrometry as described below.

The cassette was then clamped and sealed using the transport clamps and transferred to the micro-Matrix. Samples were incubated anaerobically for 24 h at 37°C at 250 rpm.

Anaerobic conditions were established in the fermenters by sparging the system with Nitrogen which was under direct control in the machine set at 40%. The installation of the filled cassette and the attachment of the parts were performed according to the micro-Matrix manual. CO_2 was used for downward pH control, 20% (v/v) sodium hydroxide (NaOH) was used as upward pH control, the orbiter was set to 250 rpm. The control set-points parameters on the micro-Matrix were pH 6.8 (6.6–7.2 low and high alarm points respectively), dO_2 0% (high alarm 20%).

DNA extraction

Samples (1 ml) were taken at T0 and T24 from each well of the micro-Matrix cassette and snap frozen at −80°C until use. The Zymo Research ZR faecal DNA kit (Cambridge Biosciences) was used to extract DNA from samples. Extractions were carried out according to the manufacturer's specifications.

Library preparation

The V3-V4 variable region of the 16S rRNA gene was amplified from 30 faecal DNA extracts using the 16S metagenomic sequencing library protocol (Illumina Saffron). Samples were quantified, and libraries were prepared for sequencing as described previously (Fouhy et al., 2015). In brief, two PCR reactions were completed on the template DNA for the 16S rRNA amplicon sequencing. The DNA template was amplified initially with primers specific to the V3–V4 region of the 16S rRNA gene. These primers also incorporate the Illumina overhang adaptor (forward primer 5′ TCGTCGGCAGCGTCAG ATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5′ GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC). Following this, a second PCR reaction with two indexing primers (Illumina Nextera XT indexing primers, Illumina) was added to allow for demultiplexing. Samples were run on the Illumina Miseq sequencer with a Miseq 500 cycle kit on the Illumina Miseq sequencer at the Teagasc Sequencing facility at the Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. Following sequencing, data were analysed bioinformatically to establish the effect of the antimicrobials on the composition of the gut microbiota.

Bioinformatics and statistical analysis of compositional sequencing data

Raw Illumina 250 bp paired-end sequence reads were merged using Flash (FLASH: fast length adjustment of short reads to improve genome assemblies) (Magoč & Salzberg, 2011) and quality checked using the split_libraries script from the Qiime package (Caporaso et al., 2010). Reads were then clustered into operational taxonomic units (OTUs) and chimeras removed with the 64-bit version of USEARCH (Edgar, 2010). Subsequently, OTUs were aligned and a phylogenetic tree was generated within Qiime (Caporaso et al., 2010). Taxonomical assignments were reached using the SILVA 16S specific database (version 116) (Quast et al., 2013).

Statistical analysis

Calypso online software (version 8.84) (Zakrzewski et al., 2017) and SPSS V8 (IBM) were used for statistical

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analysis as appropriate. Cyanobacteria and chloroplast signatures as well as OTUs with a relative frequency below 0.01% were removed. Cumulative sum scaling was used and data were log2 transformed to account for non-normal distribution of taxonomic count data for alpha and beta diversity testing. Multivariate analysis including both redundancy analysis (RDA) and CCA methods were used to investigate the complex associations between microbiota composition and treatment groups. Alpha diversity was measured using Shannon diversity, Simpson diversity and evenness. Beta diversity was measured based on Bray–Curtis distance matrices. ANCOM was used to study the gut microbiota of subjects in each treatment group. ANCOM compares the log ratio of the abundance of each taxon to the abundance of all remaining taxa at one time and then Mann–Whitney U is calculated on each log ratio. ANCOM accounts for compositional constraints of metagenomic data to reduce false discoveries in detecting differentially abundant taxa (Weiss et al., 2017). Kruskal–Wallis and Mann–Whitney U tests were performed to identify taxonomical differences between treatment groups at the family and genus level. LEfSe: Linear Discriminant Analysis Effect Size algorithm was used to identify predominant taxa between treatment groups considering biological consistency and effect relevance (Segata et al., 2011). The threshold for the logarithmic LDA score was set at 3.0 for biomarker discovery. All p values were corrected for multiple testing using the false discovery rate (FDR) correction. Significance was accepted as $p < 0.05$ following FDR correction. Barcharts showing average relative abundance across treatment groups were created in Excel (Microsoft).

Mass Spectrometry

Samples from the faecal fermentations (1 ml) were centrifuged for 60 s at 21 000 *g*. The supernatant was filtered using a 0.45 μM filter and was removed for analysis. The mass spectrometry in all cases was performed with an Axima TOF^{2}MALDI TOF mass spectrometer (Shimadzu Biotech). A 0.5 μl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid [CHCA], 10 mg ml^{-1} in 50% acetonitrile-0.1% [v/v] trifluoroacetic acid) was placed onto the target and left for 1–2 min before being removed. The residual solution was then air-dried and the sample solution (resuspended lyophilized powder or CMS supernatant) was positioned onto the precoated sample spot. Matrix solution (0.5 μl) was added to the sample and allowed to air-dry. The sample was subsequently analysed in positive-ion linear mode.

RESULTS

In this study, we used an *ex vivo* human colonic model with *C. difficile* spiked into faecal slurries at concentrations representative of a colonic infection. Purified nisin A was added at a range of concentrations and fermented anaerobically for 24 h at 37°C and constant pH to assess the impact of nisin on the gut microbiota and *C. difficile* growth.

Diversity decreases with increasing concentrations of nisin

16S rRNA gene sequencing resulted in >49,729 reads per sample. The gut microbial composition of the six donor subjects was assessed using OTUs at time 0 (baseline) samples and after 24 h fermentation without nisin. These were compared to nisin-treated samples from the same donor with varying concentrations of nisin from 25 to 500 μM after 24 h fermentation. There was a significant decrease in alpha diversity in the presence of nisin in a dose-dependant manner across several measures including Shannon Index, Simpson Index and evenness ($p < 0.001$). The groups with the highest alpha diversity were the baseline (time 0) and no treatment control groups after 24 h as shown in Figure 1. RDA plots were used to explore the association between treatment with increasing nisin concentrations on the compositions of the microbial community compared to no treatment and baseline microbiota composition. There was a clear separation between the groups treated with nisin and the untreated groups ($p < 0.05$). Similarly, canonical correspondence analysis (CCA) was used to summarize the data set and to evaluate the expected relationships and differences between groups. As with RDA analysis plots, the CCA analysis showed that nisin treatment had an impact on the microbial composition. Figure 2 shows the RDA and CCA plots at the OTU level where distinct clustering is observed depending on treatment with nisin or no treatment. The samples clustered together in groups that were dependent on the dose of nisin. Beta diversity measures the differences in diversity between samples. Bray–Curtis distance matrices showed a notable separation based on either treatment with nisin or no treatment in samples from the same donor. The PCoA plot shown in Figure 3 shows each sample from each individual donor. Each treatment group is represented by a different colour. The untreated baseline samples were taken at time 0 and the no treatment control group after 24 h fermentation cluster together whilst the nisin-treated samples cluster together.

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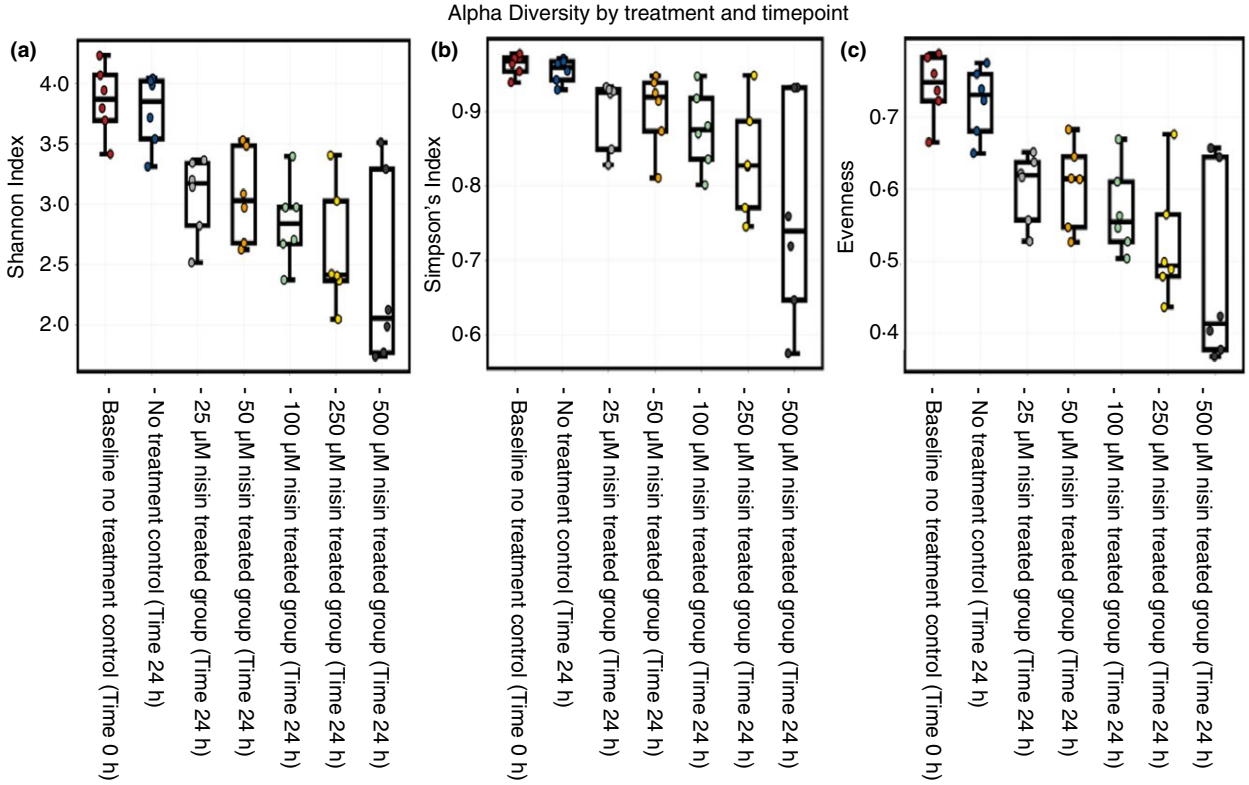


FIGURE 1 Alpha diversity decreases with increasing nisin concentration. Alpha diversity shown as (a) Shannon Index, (b) Simpson's Index and (c) Evenness ($p < 0.001$)

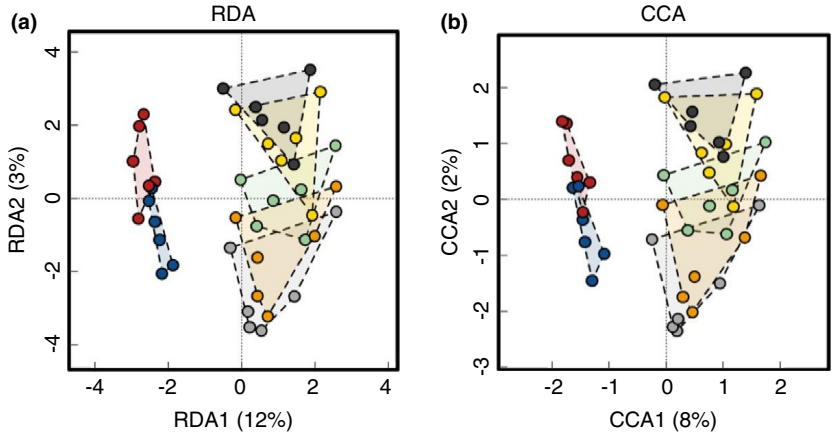


FIGURE 2 (a) Redundancy analysis (RDA) of operational taxonomic units (OTUs) showing separation of samples by presence or absence of nisin. (b) Canonical correspondence analysis (CCA) on OTUs. Samples separate based on nisin treatment or no nisin treatment with the no treatment at 24 h most similar to the baseline microbiota. Red = baseline no treatment control (time 0 h); blue = no treatment control (time 24 h); grey =25 μM nisin-treated group (time 24 h); orange =50 μM nisin-treated group (time 24 h); Green =100 μM nisin-treated group (time 24 h); yellow =250 μM nisin-treated group (time 24 h); black =500 μM nisin-treated group (time 24 h)

Gut microbiota composition changes in the presence of nisin

At the phylum level, 10 out of 15 taxa were included in the analysis. At the family level, 72 taxa were identified, 33 of which were included in the analysis. At the genus level, 188 taxa were identified, 103 of which were included in the analysis. After 24 h of fermentation, the nontreatment control (NTC) group was most similar to the baseline group (samples taken at time 0 h). The dominant phyla present in the baseline and NTC groups were the Firmicutes (Average [Av]

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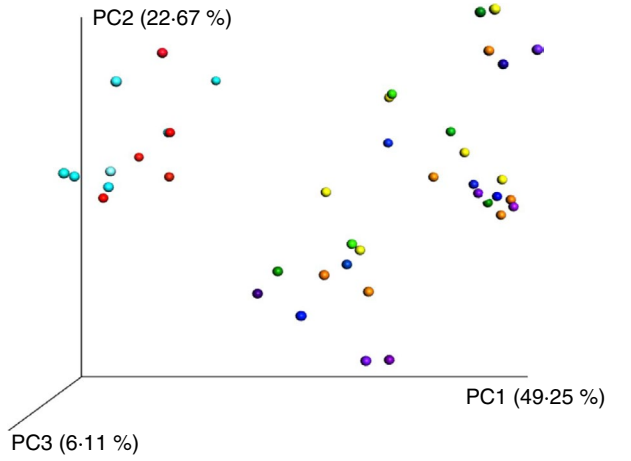


FIGURE 3 Principle coordinates of analysis (PCoA) plot showing separation of samples based on nisin treatment. Samples separate based on nisin treatment or no nisin treatment with the no treatment at 24 h most similar to the baseline microbiota ($p < 0.05$). Light blue = no treatment control (time 0 h); red = no treatment control (time 24 h); green =25 μM nisin-treated group (time 24 h); dark blue = 50 μM nisin-treated group (time 24 h); yellow =100 μM nisin-treated group (time 24 h); orange =250 μM nisin-treated group (time 24 h); purple = 500 μM nisin-treated group (time 24 h)

relative abundance 60.15% and 58.75% respectively) and Bacteroidetes (28.75% and 27.75% respectively), followed by Actinobacteria (4.4% and 9.1% respectively) and Proteobacteria (4.76% and 3.53% respectively) (see Figure 4a). The dominant bacteria present in the nisin-treated groups were the Bacteroidetes, Firmicutes and Proteobacteria. The Actinobacteria phyla had decreased to ~1% in all nisin-treated groups. There was a significant increase in the relative abundance of the Gram-negative Proteobacteria and Bacteroidetes groups between the NTC and nisin-treated groups ($p < 0.05$ ANCOM). There was a significant decrease in Actinobacteria and Firmicutes between the NTC and nisin-treated groups ($p < 0.05$ ANCOM). The abundance of Proteobacteria increased from 3.5% in the NTC group after 24 h to 24.2% and 23.9% in the group treated with 25 and 50 μM nisin, respectively, after 24 h incubation. There was a further increase in Proteobacteria to 31.6% in the group treated with 100 μM nisin, 33.3% in the group with 250 μM nisin, and then up to 40.8% in the group treated with 500 μM nisin. Following 24 h, the Actinobacteria decreased in the no treatment control from 9.1% to 1% in the nisin-treated groups. The average relative abundance of the Firmicutes phyla decreased as the concentration of nisin increased. Firmicutes represented 58.8% of all phyla in the untreated control group after 24 h. This decreased two-fold to 27.5% in the group treated with 25 μM nisin. The Firmicutes decreased steadily to 18.6% in the group treated with 500 μM nisin.

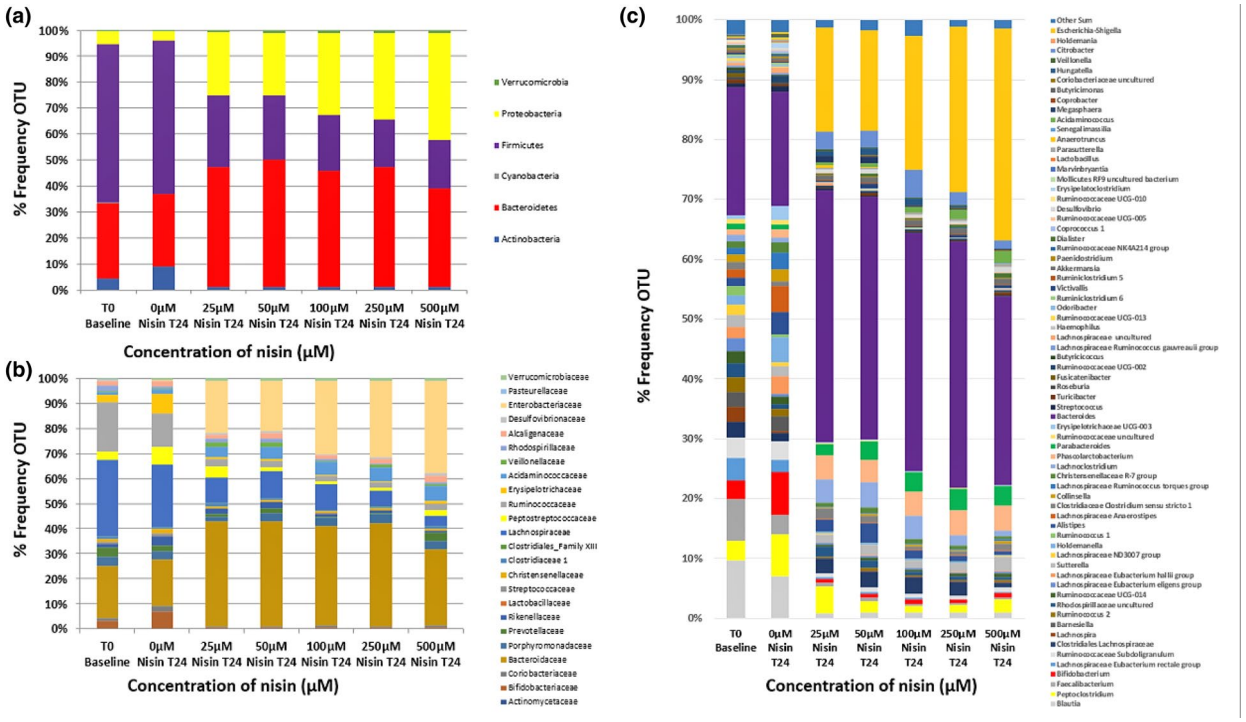


FIGURE 4 Relative abundance at the phylum level (a), family level (b) and genus level (c) across treatment groups (average $n = 6$ subjects)

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The most prevalent families present in the baseline and the NTC group after 24 h fermentation were *Lachnospiraceae*, *Bacteroidaceae* and *Ruminococcaceae* followed by *Erysipelotrichaceae*, *Peptostreptococcaceae* and *Bifidobacteriaceae*. *Bacteroidaceae* and *Enterobacteriaceae* were the most abundant families present in the nisin-treated groups followed by *Lachnospiraceae* (see Figure 4b). Of the 33 taxa included in the analysis, there was a significant increase in the relative abundance of *Enterobacteriaceae* and *Bacteroidaceae* between the NTC and the nisin-treated groups ($p < 0.05$). In contrast, there was a significant decrease in the relative abundance of *Ruminococcaceae*, *Lachnospiraceae* and *Clostridoides Family X III* between the NTC and the nisin-treated groups ($p < 0.05$). There was also an observed reduction in the relative abundance of *Erysipelotrichaceae*, *Bifidobacteriaceae*, *Coriobacteriaceae*, *Peptostreptococcaceae* and *Rikencellaceae* in the nisin-treated groups which increased as the concentration of nisin increased compared to the NTC and nontreatment control group (not significant after FDR adjustment). The relative abundance of *Enterobacteriaceae* in the baseline samples and after 24 h in the NTC group was negligible and increased as the nisin concentration increased. In the group treated with 25 μM nisin, the abundance of *Enterobacteriaceae* was 20%. This increased steadily to 36% in the group treated with 500 μM nisin (see Figure 4b). The average relative abundance of *Bacteroidaeceae* was 20.1% at time 0 h across the six subjects. After 24 h, the relative abundance of *Bacteroideaceae* was 18% in the NTC group. This increased to 40% (twofold) in the groups treated with 25–250 μM nisin after 24 h. In the nisin group treated with 500 μM, the abundance of Bacteroideaceae was 30%. The *Peptostreptococcaceae* (which include *C. difficile*) decreased from 7% to 1% when nisin was added. The abundance of *Ruminococcaceae* decreased from 13.5% in the NTC after 24 h to 2.9% or below in the nisin-treated groups. *Lachnospiraceae* decreased from 24.6% in the NTC group after 24 h to 9.7% in the group treated with 25 μM and decreased in a dose-dependent manner further to 4.05% in the group treated with 500 μM of nisin. The *Erysipelotrichaceae* decreased from 7.64% in the NTC group to negligible levels in the nisin-treated groups. The *Bifidobacteriaceae* decreased from 6.94% in the NTC group after 24 h to below 1% in the nisin-treated groups.

Following 24 h, the most dominant genera in the no treatment control group were the *Bacteroides* (18.4%) followed by *Bifidobacterium* (6.94%), *Blautia* (6.75%) and *Peptoclostridium* (6.75%). Other genera present were *Lachnospira anaerostipes* (4.2%), *Holdemanella* (4%), *Alistipes* (3.6%), *Feacalibacterium* (3.11%), Ruminococcaceae *Subdoligranulum* (2.97%) and *Barnisella* (2.3%) (Figure 4c). The no treatment control group and the baseline group were most similar compared to the nisin-treated groups. At the genus level, there was a significant difference in 36 out of the 103 genera included in the analysis between the nisin-treated groups and the NTC. The predominant genera in the nisin-treated groups were *Bacteroides* and *Escherichia Shigella*. There was a notable decrease in diversity in the nisin-treated groups with the loss in relative abundance in groups such as *Bifidobacterium*, *Blautia* and also *Peptoclostridium* (which includes the spiked in *C. difficile* spp.). There was an increase in the relative abundance of *Escherichia Shigella* in a dose-dependent manner from negligible levels (below 1%) in the NTC group and baseline to 16.8% in the group treated with 25 μM up to 34.1% in the group treated with 500 μM nisin. The *Bacteroides* genera, which is Gram-negative, increased over twofold from the no treatment control and baseline group to approximately 40% in the groups treated with 25–250 μM nisin and 30% in the group treated with 500 μM nisin. The lower abundance of *Bacteroides* in the group with the highest concentration of nisin may be reflective of the *Escherichia Shigella* being the most abundant genera in that group.

To determine whether bacterial composition in the faecal fermentates was different between nisin and non-nisin treatments after 24 h, we used a feature selection analysis (LDA effect size [LEfSe]) approach which determines features (either phylum, family or genus in our study) most likely to explain differences between the treatment groups. The LDA score was set to 3.0. There were four phyla, 12 families and 50 genera that discriminated between nisin treatment and no nisin treatment after 24 h (see Figure 5). The Proteobacteria and Bacteroides phyla were the discriminative taxa in the nisin-treated groups whilst the Firmicutes and the Actinobacteria were the discriminative phyla in the no treatment control samples. The discriminative genera in the nisin-treated groups were *Escherichia Shigella*, *Bacteroides*, *Phascolarctobacterium*, *Parabacteroides*, *Prevotella 7*, *Klebsiella* and *Eubacterium fissicatena* group. The discriminative genera in the no treatment control group were the *Blautia*, *Feacalibacterium* and *Barnsiella* and 40 other genera (shown in Figure 5).

Effect of nisin on *C. difficile* growth

The 16S rRNA gene sequencing revealed that the relative abundance of Peptostreptococcaceae (which includes *C. difficile*) was 6.75% in the NTC after 24 h fermentation. This decreased to 4.4% in the group treated with 25 μM of nisin. There was a decrease in the RA of the family of between 1% and 2% in the groups treated with 50 μM nisin or above. Plating for viable *C. difficile* on CCEY agar revealed that there was no viable *C. difficile* present in any of the

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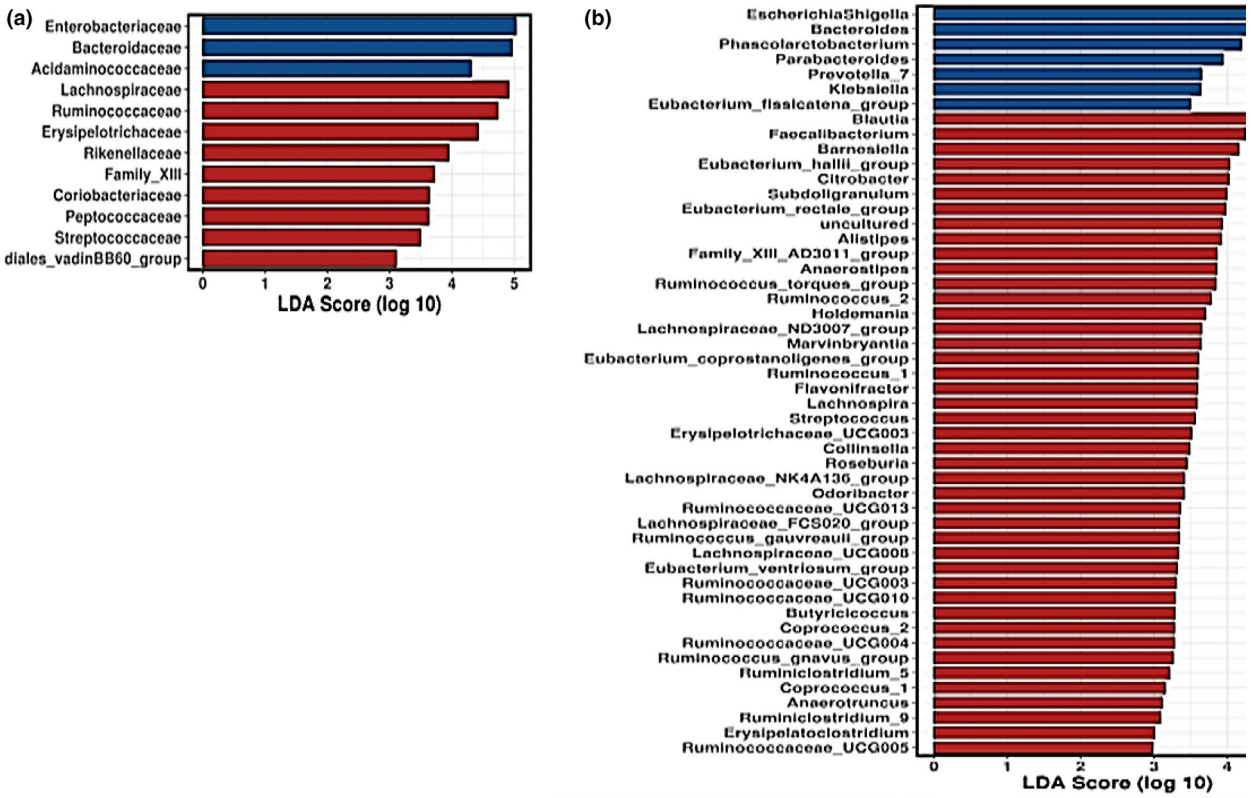


FIGURE 5 LEfSe analysis shows differentially abundant (a) family and (b) genera as biomarkers for nisin-treated samples (blue) and no nisin-treated samples (red) determined using Kruskal–Wallis test ($p < 0.05$) with LDA score >3

samples following 24 h fermentation in the groups treated with 50 μM nisin or above.

Detection of nisin in the faecal fermentate after 24 h fermentation

MALDI TOF MS of the faecal fermentate showed masses at (3354.46 Da) that correlated with intact nisin after 24 h as shown in Figure S1.

DISCUSSION

The aim of this study was to assess the ability of nisin A to kill *C. difficile* in a complex faecal environment and to establish the concentration of nisin at which no viable *C. difficile* was present whilst having the least impact on overall gut bacterial diversity. To our knowledge, this is the only study that has used compositional sequencing to assess the impact of pure nisin on the gut microbiota in a colon model. After 24 h fermentation, no viable *C. difficile* were detected at concentrations of 50 μM nisin or above across all subjects. A previous study used a colon model to assess the efficacy of nisin (in the form of Nisaplin®) to inhibit *C. difficile* and the impact on the composition of the gut microbiota. The study used qPCR coupled with propidium monoazide (PMA) treatment to enumerate six bacterial populations representative of gut commensals (Le Lay et al., 2015). These included Lachnospiraceae, Ruminococcaceae, *Bacteroidetes*, *Bifidobacteria*, *Enterobacteriaceae* and *Lactobacillaceae*/Leuconostocaceae groups. Although the colon model and the experimental setup differed in several ways compared to our study, we can draw some parallels. They found that nisin at 76 μmol/L concentration (20× MIC) was effective at killing *C. difficile*. In our study, nisin caused a decrease in the relative abundance of *Lachnospiraceae*, *Ruminococcaceae* and *Bifidobacteriaceae* whilst the *Bacteroidetes* and *Enterobacteriaceae* increased (see Figure 4b), which is consistent with previous findings. The 16s rRNA gene sequencing revealed that *Lactobacillaceae* were present in samples from five out of six subjects at very low abundance (between 1% and 0.001%) and decreased with nisin treatment in a manner that was dose dependent. This is consistent with previous studies where nisin treatment caused a decrease in *Lactobacillaceae*. Previous studies have demonstrated the susceptibility of other Gram-positive bacteria including pathogens to nisin (Le Blay et al., 2007). Several other bacteriocins

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such as lacticin 3147 (Rea et al., 2007), microbisporicin (Castiglione et al., 2008) and thuricin CD (Rea et al., 2010, 2011) have been shown to effectively kill *C. difficile*. Nisin is the only bacteriocin that has been given approval by the American Food and Drug Administration, the World Health Organization and the European Union as a natural food additive (Delves-Broughton, 1996). Le Lay et al. (2016) compared the susceptibility of 20 clinical isolates of *C. difficile* to nisin A and Z and compared that with six antibiotics (erythromycin, tetracycline hydrochloride, chloramphenicol, ciprofloxacin, vancomycin and metronidazole). They found that nisin A MICs were 0.5–4.0 mg ml^{-1} which were comparable with the MICs for vancomycin or metronidazole which were reported previously by Drummond et al. (2003). This suggests that nisin is just as effective as the antibiotics such as metronidazole and vancomycin used commonly to treat *C. difficile* infection (Le Lay et al., 2016). Bacteriocins like nisin are ribosomally synthesized and due to their proteinaceous nature are sensitive to proteolytic digestion in the digestive tract and so may require encapsulation for oral administration. This differs from most antibiotics which are secondary metabolites (Yang et al., 2014) and not protease sensitive. Bacteriocins have attracted much attention in recent years due to their unique mechanism of action and low propensity to generate resistance and hence could be a solution to some of the most challenging issues of multi-drug resistant pathogens caused by overuse of such antibiotics which cause diseases such as MRSA and VRE. Lantibiotics such as nisin bind to the pyrophosphate sugar moiety of the cell wall precursor lipid II which is in contrast to well-known glycopeptide antibiotics vancomycin and teicoplanin, which bind to the D-Ala-D-alanyl group of lipid II (Draper et al., 2015). For this reason, it is believed that new resistance mechanisms against lantibiotics are hard to establish for bacteria. Resistance against lantibiotics does exist however (Clemins et al., 2018). The CprABC transporter from *C. difficile* has been shown to confer resistance against nisin and gallidermin for instance (McBride & Sonenshein, 2011; Suárez et al., 2013). This is something that should be considered if lantibiotic compounds are to be used as antimicrobials for the treatment of *C. difficile* infections. How the induction of resistance is mediated and regulated is not completely evident. Some *in vivo* studies have been performed but the understanding of binding affinities of the lantibiotic toward the transporter as well as structural studies are needed (Clemins et al., 2018; Suárez et al., 2013).

A previous *ex vivo* study used faecal fermentations to investigate the effect of a broad spectrum bacteriocin, lacticin 3147 and a narrow spectrum bacteriocin, thuricin CD on the composition of the gut microbiota and *C. difficile* (Rea et al., 2011) They compared the effects of the bacteriocins to metronidazole and vancomycin and reported a dramatic impact on the composition of the bacteria present upon exposure to metronidazole, vancomycin and lacticin 3147. They also recorded a decrease in Firmicutes and an increase in Proteobacteria in the metronidazole, vancomycin and lacticin 3147 treated groups and a dramatic decrease in overall diversity and an increase in Enterobactericaeae. In our study, we found that nisin also caused a decrease in the Firmicutes and an increase in Proteobacteria (see Figure 4a) similar to another lantibiotic, lacticin 3147. An increase in Proteobacteria is associated with antibiotic use and has been proposed as a precedent for disease and an indication of an unstable microbial community (Shin et al., 2015). Proteobacteria blooms in humans have been observed in patients with low grade inflammatory conditions such as irritable bowel syndrome (Carroll et al., 2012). It has also been linked to diseases such as inflammatory bowel disease (Morgan et al., 2012), necrotizing enterocolitis (Normann et al., 2013) and colon cancer (Wang et al., 2012). For this reason, it can be seen as an unfavourable side effect of antibiotic use. Additionally, a reduction in health-promoting bacteria such as *Lactobacillus* and *Bifidobacterium* spp. can also be regarded as a negative outcome of antimicrobial treatments. We have observed a similar effect with nisin treatment in a dose-dependent manner that is comparable to that seen with antibiotic treatment. The ability of nisin to kill *C. difficile* in a colon model would suggest that this bacteriocin could be a potential alternative to traditional antibiotics.

Although antibiotics have revolutionized modern medicine, their overuse has become a problem due to the increased incidence of antibiotic resistance. Antibiotic overuse has also been linked with autoimmune and allergic diseases. An example of this is the increase in asthma in children in developing countries which has been linked to antibiotics (Iizumi et al., 2017). One such study conducted in Denmark found that there was an increased risk of childhood asthma and maternal use of antibiotics before pregnancy and postpartum (Stokholm et al., 2014). Other studies have linked prenatal exposure to antibiotics with an increased risk of obesity and weight gain in babies (Jepsen et al., 2003; Vidal et al., 2013). In contrast, there is increasing evidence to suggest that nisin may have an immunomodulatory role and has been shown to inhibit cancer cell growth by inducing apoptosis in cancer cell lines such as head and neck squamous cell carcinoma (Joo et al., 2012) and colon cancer (Ahmadi et al., 2017).

Oral administration of bacteriocins as a treatment for gastrointestinal ailments is a promising area of research and elucidating the optimum dose and delivery mode is

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necessary. Previous studies using animal models such as mice, rats and chickens with bacteriocins have shown promising results such as a reduction in the number of pathogens or an impact on the composition of intestinal microbiota. There are varying results, however, depending on whether the bacteriocin is delivered as a purified form or in prebiotic or probiotic form. Other influencing factors impacting the variable results include the animal model used and the dosage of the peptide. An example of this is a study by Bernbom et al. (2006) which investigated the effect of nisin-producing *Lactococcus lactis* strain CHCC 5826, non-nisin-producing *L. lactis* strain CHCH 2862 and pure nisin on the composition of intestinal microbiota in human flora-associated rats. They found that in the groups fed nisin-producing and non-nisin-producing *L. lactis* for eight consecutive days that there was a significant increase in *Bifidobacterium* in the faecal samples and a reduction in enterococci/streptococci in the duodenum, ileum, cecum and colon. This was not the case in the group fed purified nisin. *Lactococcus lactis* strains may affect the intestinal microbiota by competing for nutrients or adhesion sites. Therefore, the effect of changes in the composition of intestinal microbiota maybe not related to the presence of nisin in *L. lactis* (Yang et al., 2014).

This study has shown the effectiveness of purified nisin to kill *C. difficile* in a model colon system. In addition, an optimum concentration at which there is minimum collateral damage using 16s rRNA gene sequencing has been established in this system and will be useful for future *in vivo* work with nisin. The use of purified bacterial peptide or protein may be a preferred method of delivery, however, as nisin is subject to digestion it may not reach the lower GI tract intact. Encapsulating purified nisin may overcome this issue and future *in vivo* studies will be necessary to determine this. A previous study in mice by Gough et al. delivered nisin orally using starch matrices and found that this mode of delivery conferred some degree of protection on the nisin from digestion whilst having an impact on the gut microbiota (Gough et al., 2018).

A limitation of the 16S rRNA gene sequencing is that it can only reveal alterations in the abundance of OTUs relative to genus level relative to others present and we are unable to ascertain the absolute quantity of a particular bacterial strain in a sample. For instance, whilst we observed an increase in the relative abundance of Enterobacteriaceae with nisin treatment and a decrease in Bifidobacteriaceae, we are unable to deduce the actual numbers in CFU per ml.

Future studies using larger animal models such as a porcine model which has a digestive system more similar to humans would be beneficial to study this further. It is also not known whether the impact of nisin treatment on the diversity of the gut microbiota causes lasting or temporary changes compared to antibiotics and future *in vivo* work is necessary to determine the efficacy of using nisin as a treatment for *C. difficile* infection.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this work.

AUTHOR CONTRIBUTIONS

R Paul Ross and Mary C Rea conceived and planned the experiments. Catherine O'Reilly carried out the experiments, statistical analysis and wrote the manuscript with support from Mary C Rea, Paul Ross and Colin Hill. Órla O'Sullivan performed the bioinformatic analysis. Paula M O'Connor performed the mass spectrometry analysis. All authors discussed the results and contributed to and approved the final manuscript.

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SUPPORTING INFORMATION

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