scientific reports

OPEN

Leuconostoc lactis strain APC 3969 produces a new variant of cyclic bacteriocin leucocyclicin Q and displays potent anti-*Clostridium perfringens* activity

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***Clostridium perfringens* is an important foodborne pathogen that produces diverse toxins and is often associated with foodborne gastroenteritis. In this sense, novel biopreservatives with anti-*C. perfringens* activity are of interest. Among them, bacteriocins produced by lactic acid bacteria stand out as potential candidates. This study describes leucocyclicin C, a novel variant of the bacteriocin leucocyclicin Q, capable of inhibiting *C. perfringens*. The bacteriocin comprises 61 amino acids, has a molecular mass of 6,081.44 Da, and is produced by the strain *Leuconostoc lactis* APC 3969. Like many circular bacteriocins, leucocyclicin C has a broad spectrum of activity, is protease resistant, and has high stability against thermic and pH stresses. The leucocyclicin C genetic cluster comprises ten genes instead of the five genes previously described for leucocyclicin Q. Also, this genetic cluster seems to be part of a putative composite transposon. Leucocyclicin C has a minimum inhibitory concentration (MIC) of 3.288 µM against *C. perfringens*, comparable with other antimicrobial peptides. These results suggest that leucocyclicin C has the potential as a biopreservative for controlling *C. perfringens* in food.**

Keywords Circular bacteriocin, Leucocyclicin C, Variant, *Leuconostoc lactis*, *Clostridium perfringens*

Clostridium perfringens is an anaerobic, gram-positive, rod-shaped, spore-forming bacterium that is commonly associated with foodborne gastroenteritis[1]. This pathogen is ubiquitous and can be found in different sources such as raw materials in feed production, soil, mammalian gut, and plants, among others. However, *C. perfringens* can cause serious foodborne illness since its spores survive food processing and germinate in the gastrointestinal (GI) tract of humans. Indeed, the short germination time of the spores, along with their high resilience combined with inadequate hygiene conditions, all facilitate the survival of *C. perfringens*[2]. On entering the GI tract, *C. perfringens* can sporulate and produce enterotoxins, leading to illness symptoms, such as abdominal pain, diarrhoea, and gas production[3]. There are seven types of *C. perfringens* (A to G), which are categorized based on their patterns of toxin production [*Alpha, Beta, Epsilon, Iota*, enterotoxin CPE and necrotic enteritis B-like toxin (NetB)]. Among them, type A isolates produce only alpha toxins and are commonly reported in food-poisoning outbreaks; type B isolates (*Alpha*, *Beta* and *Epsilon* toxins) are usually associated with haemorrhagic enteritidis in sheep, foals and calves; type C isolates (*Alpha*, *Beta* toxins and may present CPE enterotoxin) may cause serious clostridial necrotizing enteritis; type D isolates (*Alpha*, *Epsilon* toxins and may present CPE enterotoxin) are associated with enterotoxaemia in goats, sheep and cattle; type E isolates (*Alpha*, *Iota* toxins and may present CPE enterotoxin) are commonly associated with enterotoxaemia in lambs and calves; type F isolates (*Alpha* toxin and CPE enterotoxin) may cause human food-poisoning and non-foodborne diarrhoea; and type G isolates (*Alpha* toxin and NetB toxin) are suggested to be the aetiological agents of avian necrotic enteritis[4,5]. To mitigate such risks, different approaches have been investigated and developed to inactivate vegetative cells and spores of *C. perfringens*, including the use of strict processing conditions[2], physical treatments (thermal and pressure), chemical additives (preservatives, organic acids) and biological compounds (plant extracts, animal

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lysozyme and microbial metabolites)[6]. Recently, studies have shown promising results using bacteriophages, bacteriocinogenic bacteria and purified bacteriocins with anti-*C. perfringens* activities[7–9].

Microbial secondary metabolites with antimicrobial properties, especially bacteriocins, have attracted attention as promising alternatives to traditional antibiotics and chemical food preservatives. Bacteriocins are small ribosomally-synthesized peptides that can demonstrate narrow- or broad-spectrum antimicrobial activity. They may be classified into two major classes: Class I, post-translationally modified peptides, such as head-to-tail circularization, and Class II, peptides without any modification[10]. One example of Class I bacteriocins are lantibiotics, small peptides with characteristic amino acids containing thioether bridges (lanthionine and β-methyllanthionine). Nisin, the most well-studied bacteriocin, is the principal representative of this class[11]. Class I also includes sactibiotics, linaridins, thiopeptides, lasso peptides and glycocins, circular bacteriocins[12], among others[10]. The Class II bacteriocins can be divided into five subgroups: (i) pediocin-like bacteriocins with a consensus YGNGVXC N-terminal sequence; (ii) two-component peptides where both are required for activity; (iii) linear, non-pediocin-like, non-two component bacteriocins; (iv) defensin-like bacteriocins[13]; and (v) leaderless bacteriocins[10,14]. The circular bacteriocins are an interesting subgroup due to their circular structure associated with a globular conformation, which limits the accessibility of proteases. In addition, one study indicated that the circular structure is not essential for bioactivity but reinforces the overall structural stability and inhibitory activity. It is important to highlight that the linear form of the bacteriocin enterocin AS-48 presented lower inhibitory activity when compared to its circular form[15]. Such structural advantages render these bacteriocins ideal scaffolds for the design of novel drugs and preservatives for food applications[16]. The widely studied circular bacteriocin, AS-48, exerts antibacterial activity through interactions of its charged and hydrophobic residues with phospholipids of the target bacterial membrane, thus integrating into and permeabilizing the cell membrane[17]. In addition, circular bacteriocins, such as circularin A and plantacyclin B21AG, exhibit high antibacterial activity on gram-positive bacteria, such as *C. perfringens*[9,18].

Bacteriocins produced by lactic acid bacteria (LAB) are particularly appealing to the food industry due to the GRAS (generally recognized as safe) status of LAB, which means that the bacteriocins can be applied directly, e.g. nisin, as fermentates, e.g. Microcin® or as safety cultures[19,20]. Despite this, very few bacteriocins are commercially available as food biopreservatives[10]. Yet, given the consumer demand for natural alternatives to chemical preservatives in food, there is a need for more bacteriocins with potent activity against foodborne pathogens and that exhibit stability during food processing, *Leuconostoc* species are members of the LAB group and are prevalent in various foods, such as plants, raw milk, wine, and fermented products. Due to their ability to coexist with *Lactobacillus* sp., their high tolerance to extreme conditions and their capacity to produce flavour compounds, they are commonly used as dairy starter cultures[21]. Like other LAB strains, the bacteriocin-producing capacity of *Leuconostoc* spp. provides the microbes with competitive advantages in different environments. Studies have shown that this genus can produce multiple bacteriocins with different antibacterial spectra. For example, *Leuconostoc mesenteroides* TA33a produces three leucocins (A-TA33a, B-TA33a and C-TA33a)[22]. *Leuconostoc pseudomesenteroides* QU 15 can produce leucocin A-QU 15, leucocin Q and leucocin N[23]. Previously, one study identified a cyclic bacteriocin, leucocyclicin Q, produced by *L. mesenteroides* TK41401. Leucocyclicin Q showed a broad inhibition spectrum against various gram-positive bacteria but weakly affected gram-negative strains[24].

In this study, we identified and characterized a new variant of leucocyclicin Q, termed leucocyclicin C, produced by *Leuconostoc lactis* APC 3969 isolated from raw bovine milk collected in bulk. We focused on its inhibitory spectrum against important foodborne and clinical pathogens including *C. perfringens*, its stability in temperature and pH extremes, and sensitivity to proteases, and we investigated the genes involved in its production. This new variant possesses a broad inhibition spectrum and high resistance to heat and pH adjustments similar to other circular bacteriocins. However, unlike leucocyclicin Q which is reported to be encoded by five genes[25], we showed that the biosynthetic gene cluster of leucocyclicin C is composed of 10 genes and is part of a putative composite transposon while the active peptides differ by a single amino acid substitution. This is the first report of a circular bacteriocin produced by *L. lactis*. Given its stability and potent activity against important foodborne and clinical pathogens, particularly *C. perfringens,* the characterisation of leucocylicin C in this study will contribute to its development as a novel food biopreservative in the future.

Results

The inhibitory spectrum of *Leuconostoc lactis* APC 3969

The *L. lactis* APC 3969 strain was obtained from a screening of antimicrobial-producing bacteria from raw bovine milk collected in bulk samples. Two techniques were used to assess the antimicrobial activity of the strain: the spot-on-lawn assay and the well diffusion assay (WDA). From the 26 indicator strains tested (see Table 1), 16 strains (61.54%) were inhibited as indicated by the presence of clear halos; 5 strains (19.23%) displayed growth reduction with the absence of clear halos; and five strains (19.23%) were not inhibited (Table 1). Both *Clostridium* strains (*Clostridium perfringens* EM124 and *Clostridium tyrobutyricum* DSM 663) were inhibited in the WDA using cell-free supernatant (CFSN) of the producer strain. *L. lactis* APC 3969 showed a broad inhibitory spectrum capable of inhibiting bacteria from different gram-positive genera, including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Listeria*, and *Clostridium*. It also inhibited gram-negative microorganisms *Escherichia coli* and *Pseudomonas aeruginosa*.

Sensitivity of *L. lactis* APC 3969 antimicrobial activity against different treatments

The sensitivity of the strain's antimicrobial activity to different treatments was evaluated using the agar-spot assay (proteases and 0.2 M NaOH) or well diffusion assay (temperatures and pHs), with *Lactococcus lactis* HP as the indicator strain. The results revealed that the antimicrobial activity produced by the strain is sensitive to proteinase K, which confirmed the proteinaceous nature of the antimicrobial activity. However, the activity

Species	Strains	Growth conditions			Inhibitory activity
		Temperature (°C)	Atmosphere	Media	
Staphylococcus epidermidis	DSM 3095	37	Aerobic	BHI	+
Staphylococcus caprae	DSM 20608	37	Aerobic	BHI	**GR**
Staphylococcus pseudointermedius	DK279	37	Aerobic	BHI	+
Staphylococcus aureus	DPC 5645	37	Aerobic	BHI	**GR**
S. aureus	A8M	37	Aerobic	BHI	+
S. aureus	B2M	37	Aerobic	BHI	+
S. aureus	C5M	37	Aerobic	BHI	**GR**
Enterococcus faecalis	ATCC 29200	37	Aerobic	BHI	–
E. faecium	DPC 3675	37	Aerobic	BHI	–
Enterococcus sp. (VRE)	APC 1026	37	Aerobic	BHI	**GR**
Enterococcus sp. (VRE)	APC 1027	37	Aerobic	BHI	–
Enterococcus sp. (VRE)	APC 1028	37	Aerobic	BHI	+
Enterococcus sp. (VRE)	APC 1029	37	Aerobic	BHI	–
Micrococcus luteus	APC 4061	37	Aerobic	BHI	–
Streptococcus agalactiae	35	37	Aerobic	TSA	**GR**
S. agalactiae	119	37	Aerobic	TSA	+
Lactococcus lactis	HP	30	Aerobic	GM17	+
L. lactis	ATCC 11454	30	Aerobic	GM17	+
Listeria innocua	DPC 3572	37	Aerobic	BHI	+
L. innocua	UCC	37	Aerobic	BHI	+
Listeria monocytogenes	EDGe	37	Aerobic	BHI	+
Clostridium perfringens	EM124	37	Anaerobic	RCM	+*
Clostridium tyrobutyricum	DSM 663	37	Anaerobic	RCM	+*
Escherichia sp.	UCC	37	Aerobic	BHI	+
Escherichia coli	APC 6054	37	Aerobic	BHI	+
Pseudomonas aeruginosa	PA-01	37	Aerobic	BHI	+

Table 1. Growth conditions of the indicator strains and inhibitory spectrum of the strain *L. lactis* APC 3969. VRE, vancomycin-resistant enterococci; MRS, de Man, Rogosa and Sharpe; BHI, brain–heart infusion; RCM, Reinforced clostridial media; GM17, Glucose M17. –, No activity; + , Inhibitory activity; **GR**, Growth reduction. *Inhibitory activity was evaluated using a well diffusion assay.

resisted treatment with 0.2M NaOH, trypsin and carboxypeptidase A. The cell-free supernatant stability of the strain was stable over a wide range of temperatures (60° to 121 °C) and pH (4.0 to 9.0; Table 2; Fig. S1).

Colony mass spectrometry analysis of *L. lactis* APC 3969

The colony mass spectrum of the strain *L. lactis* APC 3969 revealed a high-intensity peak of 6,081.45 Da (Fig. 1), suggesting that the antimicrobial activity may be associated with a compound with this mass.

Genomic analysis of *L. lactis* APC 3969

The whole genome of *L. lactis* APC 3969 was sequenced to identify the genetic cluster in APC 3969 responsible for the observed inhibitory activity. The genome was assembled using Unicycler and then annotated using PGAP. The final assembly resulted in a complete genome with a CG content of 42.5% and a size of 1.952 Mbp (sequence coverage: short reads 83.5x, long reads 63.2x). The final assembly resulted in nine circular elements: one chromosome (1,758,865 bp) and eight plasmid-like elements (49,992 bp, 45,108 bp, 40,861 bp, 18,883 bp, 17,022 bp, 12,732 bp, 7,140 bp and 1,811 bp). The circular extrachromosomal DNA elements were assigned as plasmids based on the best hits when analyzed on the BLAST online platform using nt datbase.

Afterwards, the genome sequence was then submitted to the online platforms Antismash 7.0 and BAGEL4. Both tools revealed the presence of three putative bacteriocin gene clusters: (i) lactococcin 972-related peptide (chromosome), (ii) enteroxin X-related peptide (plasmid-like element pLeuC03), and (iii) leucocyclicin Q-related peptide (plasmid-like element pLeuC01). Other putative core peptides found showed homology to bacteriocin-encoding genes, however they were not considered due to the lack of a putative complete gene cluster (data not shown).

Three gene clusters were analyzed to determine if any encoded a peptide that would be consistent with the highest intensity mass spectrum peak (6,081.45 Da) from the colony mass spectrum. Each core peptide was aligned with its closest related amino acid sequence using Clustal Omega. The alignment of the lactococcin 972-related peptide with lactococcin 972 revealed that both peptides share a similar leader sequence with an

Treatment	Activity
0.2 M NaOH	R
Proteinase K	S
Trypsin	R
Carboxypeptidase A	R
pH 4.0	R
pH 5.0	R
pH 6.0	R
pH 8.0	R
pH 9.0	R
pH 10.0	R
60 °C	R
70 °C	R
80 °C	R
90 °C	R
100 °C	R
121 °C	R

Table 2. Sensitivity of *L. lactis* APC 3969 to different treatments. R – Resistant; S – Sensitive.

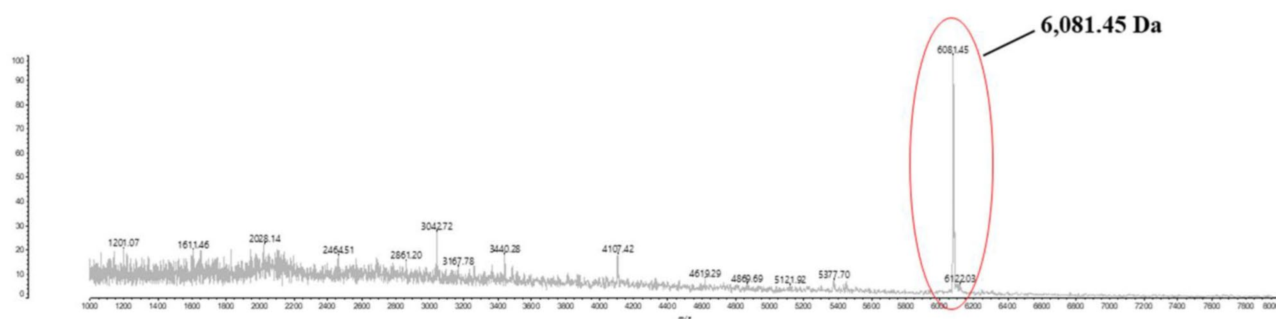


Fig. 1. Colony MALDI-TOF mass spectrum of the bacteriocin producing strain *L. lactis* APC 3969. The highest intensity peak (6,081.45 Da) is highlighted by the red circle.

Ala-X-Ala cleavage site (Fig. 2A)[26]. The predicted mature peptide of lactococcin 972 is 7,589.09 Da, which does not match any mass on the colony mass spectrum of APC 3969. The same was observed with the putative peptides found in the plasmid-like element pLeuC03, which were related to enterocin X[27], a two-component class II bacteriocin. Neither predicted mass correlated with masses present in the mass spectrum (Fig. 2B, C).

CLUSTALW alignment of the leucocyclicin Q-related peptide with leucocyclicin Q revealed that both peptides have a high identity (98.41%) and the same two-amino acid (MF) leader sequence [24]. The only difference between the mature peptides is a Threonine to Alanine substitution at position 48 of the leucocyclicin Q-related peptide (Fig. 3A). The predicted mass of the mature leucocyclicin Q-related peptide is 6,099.44 Da. However, since this putative peptide is a variant of leucocyclicin Q, we hypothesize that during the head-to-tail cyclization process, the peptide loses one H_2O molecule (18 Da), resulting in a final theoretical mass of 6,081.44 Da. This mass matches precisely with that of the colony mass spectrum and is hereafter named leucocyclicin C. Upstream of the gene encoding the core peptide, the same ribosome binding site (GGAGGA) to leucocyclicin Q was observed in leucocyclicin C (Fig. 3B).

Next, we investigated the genes responsible for leucocyclicin C production by comparing the genes near the core peptide to the leucocyclicin Q genetic cluster (AB795997.1). Both bacteriocins exhibited the same genetic organization, and the proteins encoded by these genes exhibited ≥ 97.6% identity (Table 3; Fig. 4). In order to differentiate the two genes encoding the core peptide, the gene encoding leucocyclicin C is hereafter named *llcA*.

However, the leucocyclicin C genetic cluster appears to have five additional genes downstream of *llcD* (*llcB2*, *llcI*, *llcE*, *llcF* and *llcG*) in comparison to the leucocyclicin Q gene cluster. These genes may be involved in the biosynthesis of the novel variant (Fig. 4). The first gene, *llcB2*, encodes a putative membrane protein with low similarity to other membrane proteins found in other genetic clusters of circular bacteriocins including garvicin ML, circularin A, enterocin AS-48, uberolysin, pumilarin, among others. The second gene, *llcI*, is proposed to encode a putative dedicated immunity protein present in the majority of gene clusters as it shares the same biochemical properties, i.e., small, cationic, and hydrophobic[14]. Lastly, the following three genes, *llcE*, *llcF* and *llcG*, appear to encode a multi-component ABC transporter, which can be found in other circular bacteriocin genetic clusters (enterocin AS-48, carnocyclin A, circularin A, garvicin ML, cerecyclin and aureocyclicin 4185).

A

```
APC3969       MNKFKKVVLTGVIASGIFSGISFAHADQVGGGDWRHGVGSYYVWSYYFHNYRNHSSSVSG   60
Lac972        -MKTKSLVL-ALSAVTLFS---AGGIVAQAEGTWQHGYGVSSAYSNYHHGSKTHSATVVN   55
                * *.:** .: *   :**     .      . * *:** *    .:* *.*. :.**::* .

APC3969       QYFASSGRTSP--GYDAQASAPKS-LFGNKAYYDFW  93
Lac972        NNTGRQGKDTQRAGVWAKATVGRNLTEKASFYYNFW  91
              :  .  .*: :    *   *:*:. :.      . **:**
```

Theoretical molecular mass: 7,589.09 Da

B

```
APC3969_1     MESNFKKLNNQELSSVVGGSNDGFWTGVGYYSRILYNTQKH----NAKMFGWIL-----   50
EntX_A        -MQNVKEVSVKEMKQIIGGSNDSLWYGVGQFMGKQANCITNHPVKHMIIPGYCLSKILG   58
                .*.*::. :*:..::*****.:* *** :      *   .:    :  : *: *
```

Theoretical molecular mass: 3,668.15 Da

C

```
APC3969_2     MIKTNLNSLAEDYSTLNNTELNNVSGGWWQIVAAYLGY---QAFEHSDQIVAGWEKAGNK 57
EntX_B        ---------MKKYNELSKKELLQIQGGIAPIIVAGLGYLVKDAWDHSDQIISGFKKGWNG 51
                           :.*. *.:.** ::.**   *:.* ***   :*::*****::*::*. *

APC3969_2     RL--        59
EntX_B        GRRK        55
```

Theoretical molecular mass: 3,736.21 Da

Fig. 2. Alignment of the precursor peptides of the putative bacteriocins with lactococcin 972 (**A**), enterocin Xα (**B**), enterocin Xβ (**C**). The leader sequences are highlighted in yellow and the recognition sites for cleavage are highlighted in bold.

A

```
APC3969       MFLVNQLGISKSLANTILGAIAVGNLASWLLALVPGPGWATKAALATAEAIVKHEGKAAA    60
lcyQ          MFLVNQLGISKSLANTILGAIAVGNLASWLLALVPGPGWATKAALATAETIVKHEGKAAA    60
              *************************************************:**********

APC3969       IAW        63
lcyQ          IAW        63
              ***
```

B

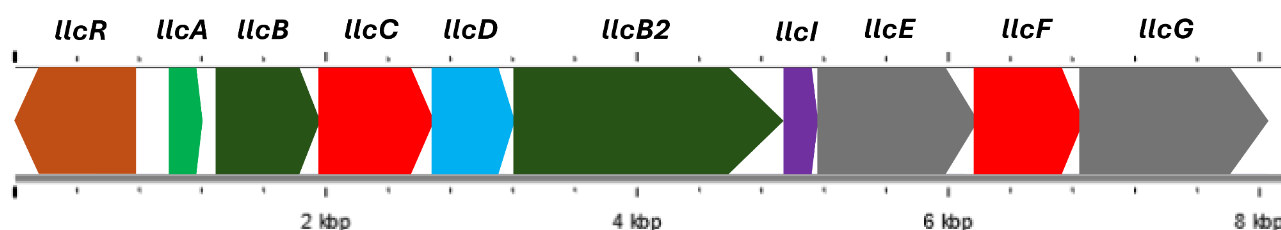
```
                                RBS
aaa   aaa   ttt        ttggaggatttaat   atg   ttc | ttg   gta   aac   cag   tta   ggg   att   tca   aaa
                                        M     F   | L     V     N     Q     L     G     I     S     K
tcg   tta   gct   aat   act   att      ctt   ggt   gca   att   gct   gtt   ggt   aat   ttg   gcc   agt
S     L     A     N     T     I        L     G     A     I     A     V     G     N     L     A     S
tgg   tta   tta   gct   ttg   gtt      cct   ggt   ccg   ggt   tgg   gca   aca   aaa   gca   gca   ctt
W     L     L     A     L     V        P     G     P     G     W     A     T     K     A     A     L
gcg   aca   gct   gaa   gca   att      gtg   aag   cat   gaa   gga   aaa   gca   gct   gct   att   gcg
A     T     A     E     A     I        V     K     H     E     G     K     A     A     A     I     A
tgg   taa
W     *
```

Fig. 3. (**A**) Alignment of the precursor peptides of leucocyclicin Q and leucocyclicin C showing the leader sequences are highlighted in yellow and a single threonine to alanine substitution in the structural peptide highlighted in blue. Identical amino acids (*) and conservative substitutions (:). (**B**) The nucleotide sequence of the leucocyclicin C propeptide. The cleavage site is highlighted by the dashed line between residues 2 and 3. RBS, putative ribosome binding site – 7 bp upstream of the start codon. *—stop codon.

Genes from leucocyclicin C	Genes from leucocyclicin Q	Identity with leucocyclicin C (%)
llcR	*lcyR*	97.6
llcA	*lcyQ*	98.4
llcB	*lcyB*	99.1
llcC	*lcyC*	99.2
llcD	*lcyD*	100.0

Table 3. Identity between the proteins encoded by the genetic cluster of both leucocyclicin producers. Data obtained from GenBank (AB795997.1) for leucocyclicin Q.

Leucocyclicin C



Leucocyclicin Q

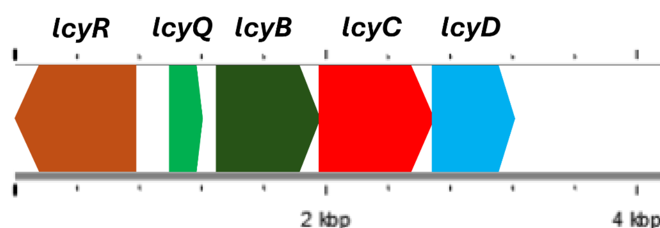


Fig. 4. Gene clusters of leucocyclicin C and leucocyclicin Q. Data obtained from GenBank (AB795997.1) for leucocyclicin Q.

The ~8.0 kb leucocyclicin C gene cluster contains ten genes, and the principal properties such as biochemical properties, intracellular localization and highest similarity hit are summarised in Table 4.

Analysis of a putative composite transposon encoding leucocyclicin C

The genetic cluster encoding leucocyclicin C was not detected in 44 other *L. lactis* genomes deposited in the NCBI database. Sequences upstream and downstream of the cluster were analyzed for mobile genetic elements (MGEs), and the results revealed that the cluster is flanked by two insertion site (IS) 6 family elements, which fits the features of a composite transposon (Fig. 5). Two inverted repeats were identified upstream (GGTTCTGGTG CAAAGTTTA) and downstream (GGTTCTGGTGCAAAGTTGA) of the cluster. In addition, three other open read frames (ORFs) were identified within the borders of the putative composite transposon: (i) the one encodes a putative ATP-binding protein, (ii) the second encodes a DNA invertase protein, and (iii) encodes a DNA/RNA helicase domain-containing protein.

Leucocyclicin C purification

To confirm that the strain's antimicrobial activity is due to the production of leucocyclicin C, the peptide was purified from the cell extract by C18 Solid Phase Extraction and Reversed Phase HPLC fractionation. Well

Gene	No. of aa	Similarity (reference)	Localization (confidence) by PSORTb	ProtParam results				Putative function
				p*I*	M_w	GRAVY	AI	
llcR	251	putative transcriptional regulator [*Leuconostoc mesenteroides*] BAP16063.1	Membrane (9.55)	9.13	29,558.54	−0.111	106.81	Response regulator protein
llcA	63	leucocyclicin Q [*Leuconostoc mesenteroides*] BAL14584.1	Membrane (9.55)	9.52	6,381.57	0.835	128.89	Leucocyclicin C precursor
llcB	217	membrane protein [*Leuconostoc mesenteroides*] BAP16065.1	Membrane (10.00)	9.49	24,922.07	1.103	150.88	Membrane protein
llcC	244	immunity protein [*Leuconostoc mesenteroides*] BAP16066.1	Membrane (9.51)	5.30	27,618.66	−0.171	98.28	ATP-binding protein
llcD	175	immunity protein [*Leuconostoc mesenteroides*] BAP16067.1	Membrane (10.00)	9.47	19,347.22	1.029	137.86	Membrane protein with DUF95 domain
llcB2	565	hypothetical protein [*Lactobacillaceae* bacterium] MDR3190324.1	Membrane (10.00)	9.59	64,595.06	0.703	134.71	Membrane protein
llcI	49	No significant match	Membrane (9.55)	9.86	5,504.88	1.353	171.02	Immunity protein
llcE	338	HlyD family efflux transporter periplasmic adaptor subunit [*Leuconostoc falkenbergense*] WP_282821363.1	Unknown (2.50)	8.67	37,141.85	−0.443	86.69	ABC transporter component
llcF	229	ABC transporter ATP-binding protein [*Leuconostoc falkenbergense*] WP_282855217.1	Membrane (9.99)	7.93	25,370.04	−0.182	101.27	ABC transporter ATPase
llcG	389	ABC transporter permease [*Leuconostoc gelidum* group] WP_097001799.1	Membrane (10.00)	9.63	41,445.97	0.315	112.80	ABC transporter component

Table 4. Bioinformatic analysis of the leucocyclicin C genetic cluster.

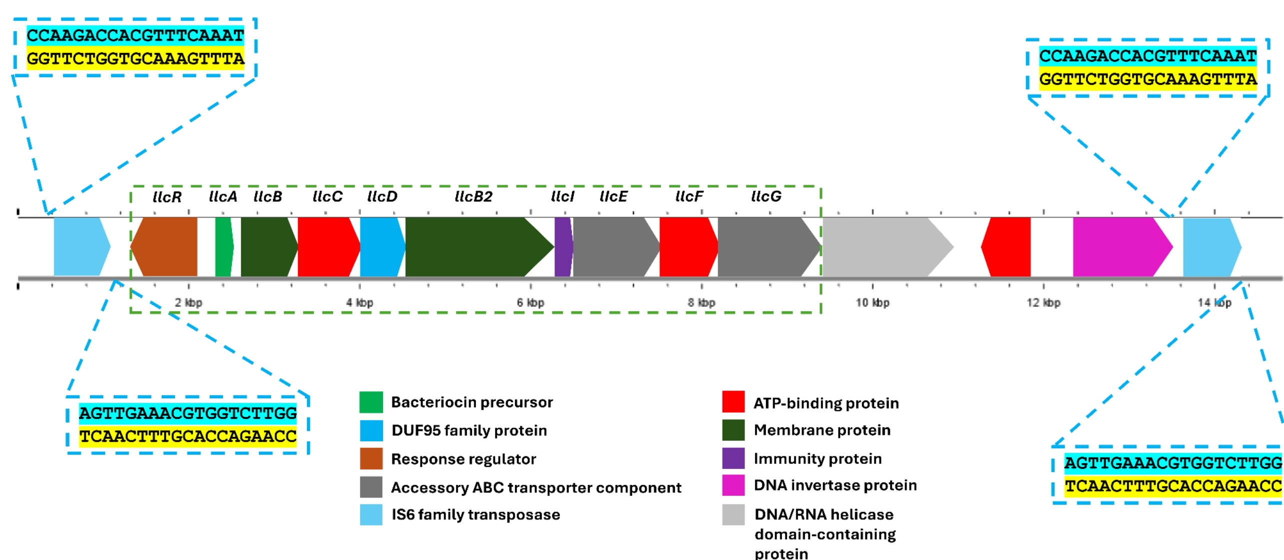


Fig. 5. Putative composite transposon that encodes the leucocyclicin C genetic cluster. The leucocyclicin C gene cluster is highlighted by a dashed green box. The inverted repeats are highlighted by a dashed blue box.

diffusion assays (WDA) with *L. innocua* DPC 3572 as the indicator strain were used to follow antimicrobial activity throughout purification. Following HPLC fractionation, the WDA assay revealed that the antimicrobial activity was present in fraction 51 (Fig. 6A), while MALDI-TOF mass spectrometry (Fig. 6B) confirmed the fraction contained a 6,081.06 Da mass that correlated with the mass from the colony mass spectrum (6,081.45 Da; Fig. 1), and the theoretical 6,081.44 Da mass of leucocyclicin C.

The minimal inhibitory concentration of leucocyclicin C against *C. perfringens* EM 124

The minimum inhibitory concentration (MIC) of purified leucocyclicin C was assessed against *C. perfringens* EM124 in a 23-hour growth experiment (Fig. 7). The MIC value was determined to be 3.288 µM. The growth experiment also revealed that concentrations above 0.822 µM of leucocyclicin C increased the lag phase of the strain. In comparison, concentrations less than 0.822 µM did not affect the growth of the strain when compared with the control (Fig. 7).

Discussion

The alarming rise of antimicrobial resistance in clinical isolates in the past few years and increased consumer demand for food products without artificial preservatives has boosted research into discovering and developing new antimicrobials[28,29]. Bacteriocins are perhaps one of the most promising natural antimicrobial alternatives, especially those produced by LAB, due to the GRAS status of the strains.

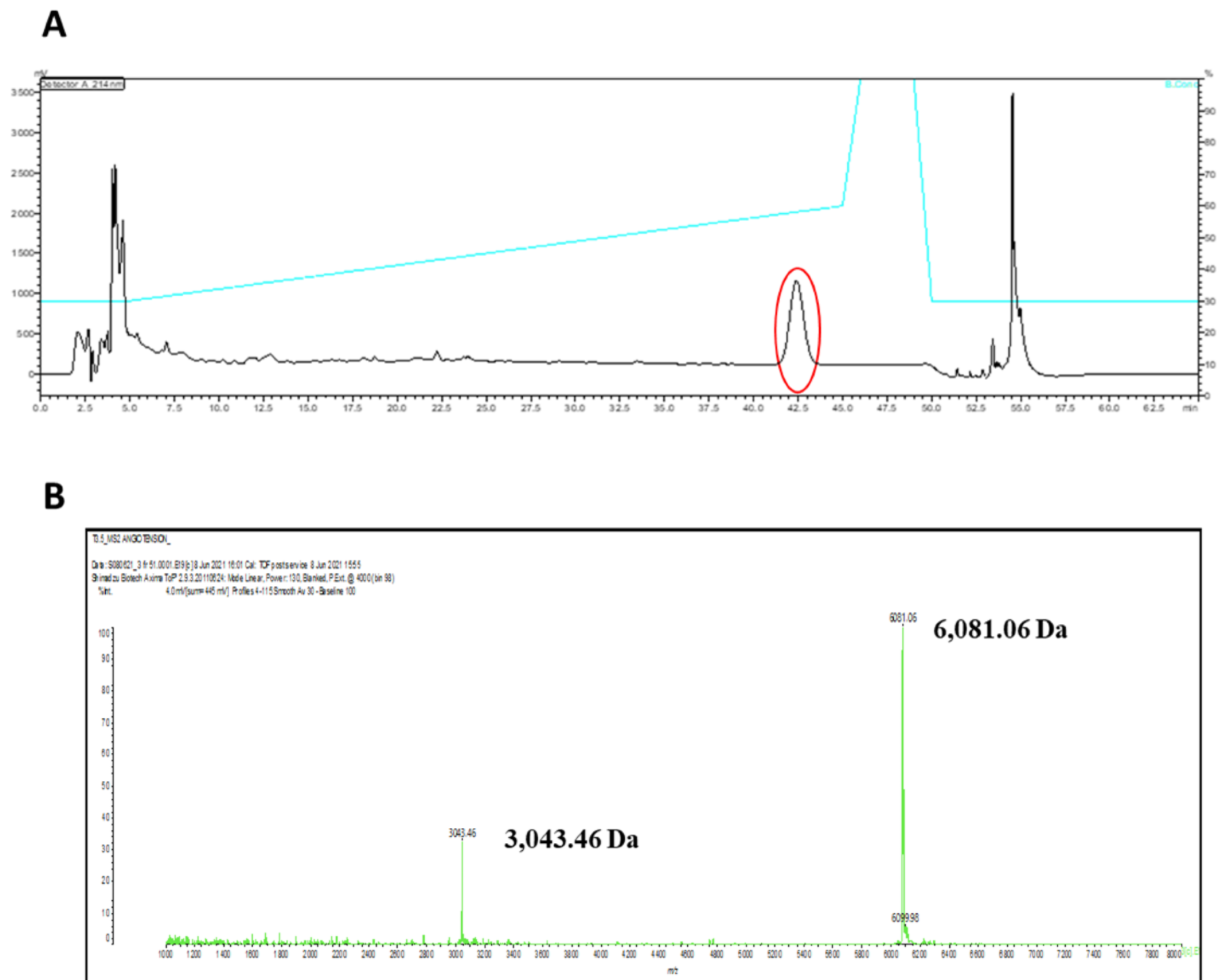


Fig. 6. (**A**) HPLC analysis of the cell extract of *Leuconostoc lactis* APC 3969 with fraction 51, highlighted by the red circle. (**B**) MALDI-TOS MS of fraction 51 showing a mass of 6081.06 Da which correlates with the theoretical mass of leucocyclocin C (6081.44 Da). Note that the 3043 Da mass is doubly charged leucocyclin C.

When compared to other groups of bacteriocins, such as lantibiotics and pediocin-like bacteriocins, circular bacteriocins remain poorly understood[14], yet offer particular promise as food biopreservatives due to their structural stability owing to their globular confirmation. In the present study, we identified and characterized leucocyclicin C, a new variant of the circular bacteriocin leucocyclicin Q, produced by the raw bovine milk collected in bulk isolate *L. lactis* APC 3969. The workflow of this study is presented in the Fig. S2.

In total, 16 of 26 indicator strains were inhibited by leucocyclicin C, and five exhibited reduced growth in its presence. Leucocyclicin C inhibited *Enterococcus*, *Streptococcus*, *Lactococcus*, *Listeria* and *Escherichia*, which was also reported for leucocyclicin Q[24]. In addition, leucocyclicin C could inhibit or reduce the growth of the four *S. aureus* strains tested[24] unlike leucocyclicin Q, which did not inhibit *S. aureus* subsp. *aureus* ATCC 12600[24]. Further studies are needed to verify if the single amino acid substitution enhanced the antimicrobial activity of leucocyclicin C as only one *Staphylococcus* isolate was assessed for leucocyclicin Q. Leucocyclicin C is thus a broad-spectrum bacteriocin with activity against important food and clinical pathogens (*Listeria*, *Clostridium, Staphylococcus*, *Enterococcus*, *Streptococcus*, *Escherichia*, *Pseudomonas*), which makes this novel variant a promising candidate for future both food biopreservative and even human therapeutic applications.

Leucocyclicin C activity was retained when treated with 0.2M NaOH, confirming that the antimicrobial activity is not due to organic acids. However, the activity was unaffected by carboxypeptidase A and trypsin, and it was sensitive to proteinase K, confirming the proteinaceous nature of the antimicrobial compound. Carboxypeptidases cleave the C terminal amino acid of peptides and so cannot act on circular bacteriocins as they have undergone head-to-tail cyclization. Resistance to carboxypeptidase has previously been used to confirm the circular nature of other bacteriocins, such as enterocin AS-48RJ[30] and leucocyclicin Q[24].

Lastly, the resistance to trypsin was expected as leucocyclicin Q is also trypsin-resistant. However, this resistance phenotype is not specific to all circular peptides, pumilarin presented partial sensitivity[31] and carnocyclicin A antimicrobial activity was eradicated in the presence of trypsin[32]. Resistance to trypsin is

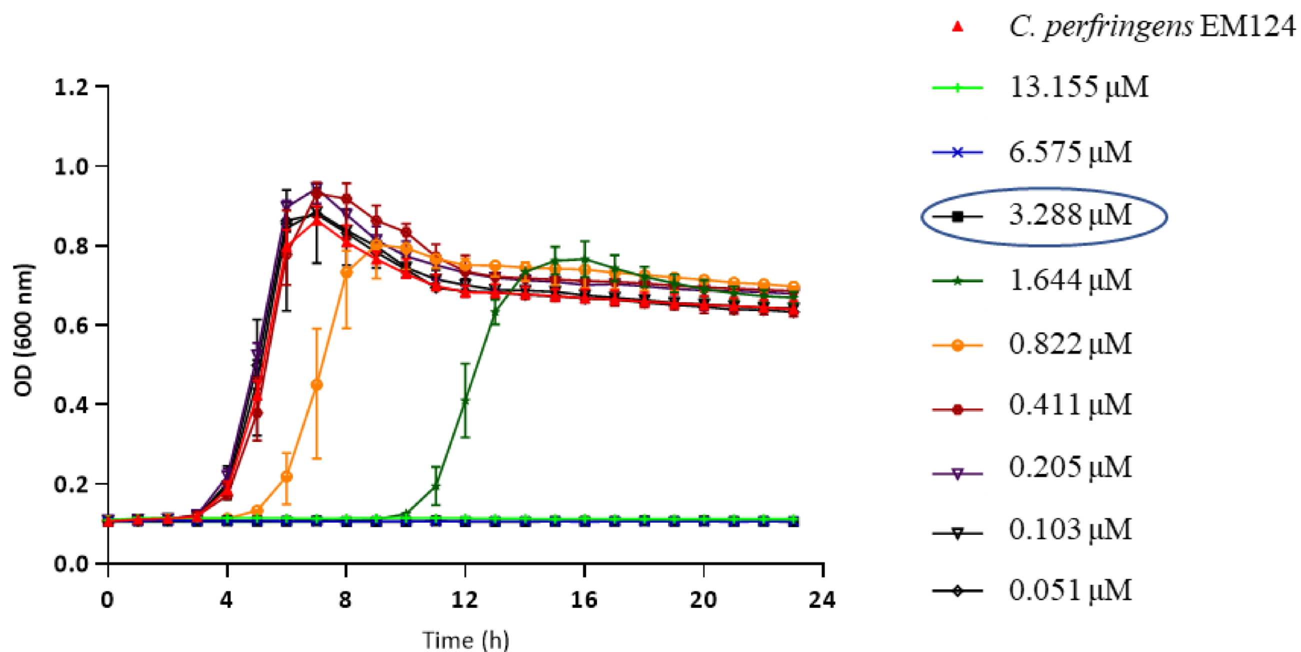


Fig. 7. Effect of different concentrations of leucocyclicin C on the growth of *Clostridium perfringens* EM124. The MIC value for the strain is highlighted by the blue circle.

presumably due to the globular conformation of the peptide, making any cleavage sites unavailable to the protease[14,33,34].

Leucocyclicin C retained all inhibitory activity after 2 h exposure to a range of pH values (4.0 to 9.0) and after 15 minutes exposure to different temperatures (60° to 121°C). This data agrees with the findings for leucocyclicin Q[24] and lactocyclicin Q[35]. This ultra-stability to thermal and pH stresses is a feature of all representatives of this group[14].

The genome analysis revealed that leucocyclicin C is encoded by a large bacteriocinogenic cluster of ten genes. The first five genes of leucocyclicin C share the same genetic organization as those encoding leucocyclicin Q, with high levels of identity between them (≥ 97.6%). The other five genes encode a putative membrane protein (*llcB2*), a putative dedicated immunity protein (*llcI*) and a putative multi-component ABC transporter (*llcEFG*). The gene clusters of most circular bacteriocins encode a small, cationic and hydrophobic dedicated immunity protein. The same biochemical characteristics were found for the encoded protein of the gene *llcI*[14]. It is important to highlight that a previous study with leucocyclicin Q, Mu and collaborators revealed that a *lcyD* knockout mutant of the bacteriocinogenic strain *L. mesenteroides* TK41401 still presents the same high level of immunity against leucocyclicin Q as the wildtype strain. Even if the presence of the gene *lcyD* alone confers immunity against the circular peptide, the results together suggest that another protein(s) could also confer immunity, in this case, a possible *llcI*-like gene not identified in the sequenced region of *L. mesenteroides* TK41401[25]. Three genes encode a multi-component ABC transporter downstream of the immunity gene, which is potentially related to an accessory function. This hypothesis is supported by studies performed with two other circular bacteriocins, enterocin AS-48 (as-48*EFGH*) and carnocyclin A (*cclEFGH*), where the deletion of the corresponding genes in those gene clusters led to a reduction of the immunity of the strain and a concomitant decrease in bacteriocin production[36,37]. The strain *L. mesenteroides* TK41401 was not fully sequenced, generating only a fragment of 3.7 kb flanking the core peptide. This may explain why the other five genes downstream of the gene *lcyD* were not found, as the fragment length did not encompass the entire genetic cluster[25]. The five extra genes in the leucocyclicin C gene cluster are most likely also present in the genetic cluster of leucocyclicin Q. Future studies are necessary in order to prove the contribution of these five genes in the biosynthetic and immunity of leucocyclicin C.

To determine if the leucocyclicin C genetic cluster is present in other *L. lactis* genomes, 44 unique genomes from this species were analyzed using the online platform BAGEL4. The results show that none of the 44 strains harbour the bacteriocinogenic cluster found in APC 3969. Thus, the putative function of the ORFs flanking the cluster was investigated against the NCBI database using the BLAST online platform (https://www.ncbi.nlm.nih.gov/)[38]. Downstream of the genetic cluster, three ORFs can be found: one encoding an ATP-binding protein (WP_216988100.1), the second encoding a DNA invertase Pin (recombinase family protein; WP_228934490.1) and the third encoding a DNA/RNA helicase domain-containing protein (WP_224132774.1). In addition, flanking those 13 genes are two insertion sequences (ISs) 6 family transposases (WP_064520495.1; WP_055307866.1) with respective inverted repeats (GGTTCTGGTGCAAAGTTTA and GGTTCTGGTGCA AAGTTGA). The presence of two ISs flanking other proposed genes is typically associated with a composite transposon[39]. These transposable elements are known for carrying antimicrobial resistance genes and other types of genes, such as ones related to antimicrobial resistance, in this case, a bacteriocin gene cluster[40]. However,

more studies are necessary to elucidate if this putative MGE is responsible for disseminating this uncommon genetic cluster.

Initially, the colony mass spectrum obtained for *L. lactis* APC 3969 revealed a mass of 6,081.45 Da, which agrees with the predicted 6081.44 Da mass for leucocyclicin C. The bacteriocin was purified using HPLC to confirm that the antimicrobial activity produced by the strain was indeed due to leucocyclicin C production. Mass spectrometry analysis of the active HPLC fraction verified that the mass of the purified bacteriocin (6,081.06 Da) matches with the mass obtained for the bacterial colony (6,081.44 ± 0.5 Da) and correlates with the theoretical mass of leucocylcin C. Comparison of the mass for leucocyclin C with the theoretical mass for the structural gene reveals a two amino acid leader sequence (MF). In contrast, the 18 Da difference between the theoretical and detected mass confirms that the peptide (6,099 Da) goes through head-to-tail cyclization (6,099 – 18 = 6,081 Da).

As *C. perfringens* is an important pathogen, the present study focused on the effectiveness of leucocyclicin C against *C. perfringens* EM124. To our knowledge, the inhibitory activity of circular bacteriocins such as plantacyclin B21AG[18], circularin A[9] and garvicin ML[33] against *C. perfringens* is unknown since not all bacteriocins were assessed against this strain, and no MIC values have been reported. Thus, the MIC obtained for leucocyclicin C (3.288 μM) against *C. perfringens* EM124 was compared to published values for bacteriocins from other classes and other antimicrobial peptides. Some show comparable results, such as plectasin, a defensin-like fungal peptide, with an MIC value of 3.64 μM[41]; sublancin, a glycocin, with an MIC value of 8 μM[42]; nisin, a lantibiotic, (0.29 – 1.14 μM), MP1102 – 0.91 μM[41]; thuricin A5, a leaderless bacteriocin, (2.0 μM)[43]; and ruminococcin C1, a sactibiotic, (0.4 – 0.8 μM)[44]. This comparison is somewhat limited as these studies assayed different *C. perfringens* strains, and thus, genetic variations between them could justify the MIC range. However, circular bacteriocins stand out from other bacteriocin classes due to their highly stable nature and desirable biotechnological properties. These desirable characteristics and its potent inhibitory activity make leucocyclicin C a possible new candidate for further investigation as a food biopreservative against *C. perfringens* and potentially other pathogens and spoilage organisms.

Conclusion

The genus *Leuconostoc* constitutes a large proportion of the LAB group of bacteria and possesses many desirable biotechnological properties, especially the capacity to produce bacteriocins. This work evaluated and characterized the antimicrobial activity of the strain *L. lactis* APC 3969, which was isolated from raw bovine milk collected in bulk and can produce leucocylicin C, a novel single-amino acid variant of leucocylicin Q. The circular peptide presented a broad spectrum of activity, inhibiting important pathogens (*Listeria*, *Staphylococcus* and *Clostridium* and gram-negative *E. coli* and *Pseudomonas aeruginosa*), high thermal and pH stability, and resistance to trypsin and carboxypeptidase A. Most of these characteristics are shared with leucocyclicin Q. The genetic cluster of leucocyclicin C is bigger than expected, being composed of 10 genes, which makes it, as far we are aware, the biggest genetic cluster described to date for a circular bacteriocin. Interestingly, the bacteriocinogenic cluster also seems to be part of a putative MGE, which may justify why only this genome of *L. lactis* encodes this bacteriocin. However, future studies will investigate the contribution of each of these genes to bacteriocin production and immunity and determine if the composite transposon can be mobilised to other strains. Lastly, the MIC obtained for this peptide against *C. perfringens* is compatible with the MIC values found for other antimicrobial peptides. Further investigation is necessary to assess the feasibility of the strain as a novel food biopreservative against *C. perfringens* and other foodborne pathogens in a food model.

Materials and methods

Antimicrobial activity

The antimicrobial activity of the strain *L. lactis* APC 3969 was assayed on MRS medium by the agar-spot assay as described by Giambiagi-deMarval et al. (1990) with minor modifications[45]. Five microliter of an overnight growth culture of the bacteriocinogenic strain was spotted on the surface of MRS agar, allowed to dry at room temperature, and then incubated for 20-24 h at 30°C. After incubation, chloroform was applied to inactivate the cells and the plate was overlaid with 3 mL of soft agar (0.75 % w/v) inoculated with the indicator strain (~7.0 log CFU/mL). The plates were incubated overnight at optimal growth conditions, as shown in Table 1. The inhibitory activity effect of the producer strain against the indicator was assessed by measuring the true halo of the inhibitory zone (total halo – spot size = true halo) and divided into five categories: growth reduction (hazy zone with reduction of cell density); weak inhibition (0.5–5 mm); moderate inhibition (>5–≤10 mm); strong inhibition (>10 mm) and no inhibition (0 mm). The experiment was conducted in triplicate.

Due to the lack of growth of the *Clostridium* indicators (*C. perfringens* EM124 and *C. tyrobutyricum* DSM 663) on the agar-spot assay, the well diffusion assay was applied as described by Twomey et al. (2021) with minor modifications[46]. The indicator strain was inoculated (~7.0 log CFU/mL) in 20 mL of molten agar and then poured into sterile petri dishes. After solidification of the agar, wells of 6 mm width were created in the agar and 50 μL of the neutralized (using NaOH) cell-free supernatant of *L. lactis* APC 3969 (24 h incubation) was added. Finally, the plates were incubated at the optimal growth conditions, as described in Table 1. The inhibitory effect of the strain was determined by the presence or absence of inhibitory halos around the wells where supernatants were transferred.

Evaluation of the effects of proteases and NaOH on the antimicrobial activity of *L. lactis* APC 3969

The sensitivity of the antimicrobial activity of the strain *L. lactis* APC 3969 was evaluated against proteinase K (1 mg/mL; Sigma-Aldrich, St Louis, USA), carboxypeptidase A (1 mg/mL; Sigma-Aldrich, St Louis, USA),

trypsin (1 mg/mL; Merck KgaA, Darmstad, DE) and 0.2 M NaOH on agar by the methodology previously described[45]. Briefly, an overnight growth culture of the strain *L. lactis* APC 3969 was spotted (5 μL) five times on an MRS (de Man, Rogosa and Sharpe) plate and incubated for 20-24 h at 30°C. Subsequently, the spotted cells were inactivated with chloroform and 40 μL of each treatment (0.2M NaOH or protease) was added around the bacteriocinogenic strain. For the control, 10 mM PBS (pH 7.4) was used as treatment. The plates were air-dried and incubated at 37°C for 4 hours. Lastly, the plates were overlaid with *L. lactis* HP and incubated overnight at 30°C. The resistance (R) or the sensitivity (S) of the antimicrobial activity against each treatment was determined by measuring the true halo and comparison to the control.

Evaluation of the stability of the antimicrobial activity of the strain *L. lactis* APC 3969 at different temperatures and pH

The stability of the antimicrobial activity of the strain *L. lactis* APC 3969 against different temperatures and pH was evaluated by WDA against *L. lactis* HP as described previously with minor modifications[47]. For this, a 24 h neutralized (pH 7) CFSN of the producer strain was treated with two different protocols. For the temperature evaluation, the CFSN was exposed to different temperatures (60°C, 70°C, 80°C, 90°C, 100°C and 121°C) for 15 min and then cooled to room temperature and immediately assayed. For the pH evaluation, the CFSN pH was changed to target conditions (pH 4, pH 5, pH 6, pH 8, pH 9, pH 10), using 1M HCl and 1M NaOH, and incubated at room temperature for 2 h. The sample's pH was then returned to its initial value (pH 7) and immediately assayed using well diffusion assay[46]. The effect of each treatment was determined by the maintenance or reduction of the size of the halo when compared to the control (neutralized CFSN without any thermic treatment). All the treatments were evaluated on the same Petri dish plate and the assay was performed in duplicate.

Colony MALDI-TOF mass spectrometry

In order to assess the molecular mass profile of peptides associated with *L. lactis* APC 3969 cells, colony MALDI-TOF (matrix-assisted laser desorption/ionization coupled to time-of-flight) mass spectrometry was applied as previously described by Field et al. (2012) with minor modifications[48]. Briefly, the strain was streaked on an MRS plate and incubated overnight at 30°C. Fresh colonies from the plate were mixed with 50 μL 70% propan-2-ol + 0.1% trifluoroacetic acid (TFA), vortexed and centrifuged at 21,000 *g* for 20 seconds. The supernatant was used for the MALDI-TOF mass spectrometry analysis, which was performed with an Ultraflex MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). A 0.5-μL aliquot of matrix solution [α-cyano 4-hydroxy cinnamic acid, 10 mg/mL in acetonitrile-0.1 % (v/v) TFA] was placed onto the target and left for approximately 20 seconds before being removed. The sample supernatant (0.5 μL) was applied to the pre-coated target spot. Afterwards, the matrix solution (0.5 μL) was added to the samples and air-dried. Sample was analyzed in positive-ion reflectron mode, and molecular masses from the spectrum were compared to antimicrobial databases, especially bacteriocin databases.

Genome sequencing and bioinformatics analysis

The bacterial genome of the bacteriocinogenic strain *L. lactis* APC 3969 was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St Louis, USA) as described by the manufacturer. A Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, USA) was applied to quantify the genomic DNA. A combination of Illumina and Oxford Nanopore platforms were used to obtain short and long reads. The genome was assembled *de novo* using the Unicycler 0.5.0v[49] from the short reads and long reads. The final assembly was annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP)[50]. In order to assess the coverage of the short and long reads, Bowtie2 (v2.3.4.1), minimap2 (v2.17) and Samtools (v1.7) were utilized[51–53]. The online platforms antiSMASH v.7.0 (https://antismash.secondarymetabolites.org)[54] and BAGEL4 (http://bagel.molgenrug.nl)[55] were used to identify the presence of genetic clusters related to antimicrobial compounds. To predict the putative function of the ORFs from the antimicrobial genetic clusters, the sequences were analyzed against the NCBI database using the BLAST online platform (https://www.ncbi.nlm.nih.gov/)[38]. The core gene of each genetic cluster was aligned with its closest relative using Clustal Omega[56]. The predicted protein and peptides had their molecular mass, isoelectric point (pI), grand average of hydropathicity (GRAVY), and aliphatic index (AI) determined using the online ProtParam (https://web.expasy.org/protparam/)[57]. The putative cellular localization of the identified protein and peptide was predicted using the online platform PSORTb v3.0.3[58]. Genomic data is available at GenBank/EMBL under accession no. CP157073-CP157081.

Bacteriocin purification

One litre of *Leuconostoc lactis* APC 3969 culture was grown overnight at 30°C in MRS broth. The culture was then centrifuged at 8,280 *g* for 20 minutes at 10°C, and cells were separated from the supernatant. The cells were mixed with 250 ml 70% IPA and stirred at room temperature for 3-4 hours with the objective to extract the bacteriocin associated with the cell mass. This cell suspension was then centrifuged, and the supernatant was retained. A rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) was used to remove all the IPA from the supernatant before applying it to a 2 g, 12 ml C18 solid phase extraction column pre-equilibrated with methanol and water. The column was washed with 20 ml 25% ethanol and 20 ml 70% IPA. The IPA was removed from an aliquot of the C18 IPA eluent and applied to an analytical Jupiter Proteo (C12, 4.6 x 250 mm, 4 μ, 90 Å) high-pressure liquid chromatography column (Phenomenex, Cheshire, UK) running a 30-60% gradient at 1 ml/min where mobile phase A is H_2O + 0.1% TFA and mobile phase B is 100% acetonitrile + 0.1% TFA. The HPLC eluent (2.5 ml/min) was monitored at 214 nm, and fractions were collected at 1-minute intervals. Fractions were assayed on *L. innocua* DPC3572 using well diffusion assays[46], as previously described, and active fractions were evaluated for the antimicrobial mass of interest using MALDI-TOF mass spectrometry.

Minimal inhibitory concentration of leucocyclicin C against *Clostridium perfringens* EM124

The MIC of the pure leucocyclicin C against approximately 1×10^5 CFU/ml of the target strain *C. perfringens* EM124 was assayed in a 96-well microtiter plates (Sarstedt, Co. Wexford, Ireland) using a Stratus Microplate Reader (Cerillo, Virginia, USA) to measure the optical density at 600 nm ($O.D._{600}$) as previously described[48]. Initially, 4 times the test concentration (13.155 µM) solution of the peptide was resuspended in 70% propan-2-ol. Then, 100 µL of CRM (Clostridial Reinforced Medium) was added to all wells, followed by 100 µL of the peptide solution to the first well. Afterwards, a two-fold serial dilution was carried out from the first well until the twelfth well. Optical densities at 600nm were collected every hour for 23 h at 37°C in an anaerobic environment. The MIC was determined as the lowest concentration necessary to inhibit the target strain completely. The assay was done in triplicate.

Data availability

The accession number of the *L. lactis* APC 3969 complete genome is CP157073-CP157081. All other data generated or analyzed in this project are included in this published article. Any additional information or data can be addressed to the corresponding author upon reasonable request.

Received: 4 June 2024; Accepted: 5 February 2025
Published online: 21 February 2025

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Acknowledgements

We would like to thank Jim Flynn (Teagasc) for providing the bovine milk collected in bulk samples for this project.

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Conceptualization, F.M.F., M.C.S., R.P.R., C.H. and C.S.; Analysis collection, F.M.F., P.M.O., X.H., C.B., E.K., D.H. and M.C.S.; Data analysis, F.M.F., P.M.O. and C.B.; Supervision, R.P.R., C.H., C.S., L.D., J.W., A.D. and O.F.; Project administration, R.P.R., C.H., C.S. and L.D.; Funding acquisition, R.P.R., C.H. and C.S.; Writing, F.M.F. and X.H.; Review and editing, R.P.R., C.H., C.S., P.M.O., C.B., L.D., J.W., A.D. and O.F. All authors have read and agreed to the published version of the manuscript.

Funding

Co-funded by a research grant from Kraft-Heinz Company, Science Foundation Ireland (SFI) under Grant Number SFI/ 12/RC/2273_P2 and the European Union (ERC, BACtheWINNER, 101054719). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them. This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under the INSPIRE COFUND Marie Skłodowska-Curie grant agreement No. 101034270.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-89450-x.

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