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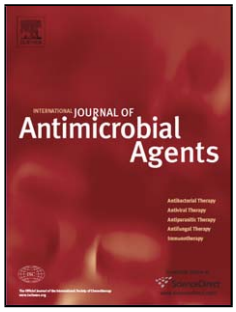

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Purification, characterisation and identification of acidocin LCHV, an antimicrobial peptide produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine

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ABSTRACT

In the last two decades, antimicrobial peptides (AMPs) have been gaining attention as antimicrobial alternatives to chemical food preservatives and commonly used antibiotics. *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine produces a small AMP with a molecular weight of 1.1 kDa, designated acidocin LCHV. In this study, the AMP was extremely heat stable (90 min at 130 °C), was active over a wide pH range and was found to be sensitive to proteolytic enzymes (trypsin, pepsin and proteinase K). Acidocin LCHV has a broad spectrum of activity both against Gram-positive and Gram-negative pathogens, including several that are classified as Especially Dangerous Infections by the World Health Organization as well as meticillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) was used to determine the molecular mass and sequence of the purified peptide. Complete killing with immediate impact on cells was observed within a very short period of time (10 min).

1. Introduction

There has been a rapid worldwide increase in pathogenic bacteria that are resistant to multiple antibiotics. The alarming increase and spread among pathogens of bacterial resistance to all clinically useful antibacterial agents has been one of the most serious public health problems of the past decade. In the majority of cases, infections caused by multidrug-resistant (MDR) strains cannot be treated by currently available antibiotics. There is therefore a pressing need to develop new antibiotics and novel antimicrobial agents.

Lactic acid bacteria (LAB) have long been used as supplements to restore intestinal balance disorders by altering the gut flora as well as by their immunomodulatory effects and production of antibacterial substances. A probiotic is a 'live microbial food supplement that beneficially affects the host animal by improving the intestinal microbial balance' [1]. To be considered a probiotic, a bacterial strain must be of human origin and safe for human use. Probiotics have preventative as well as a curative effect on several types of diarrhoea of different aetiologies.

Many probiotic strains, for example *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Escherichia coli* and certain strains of bifidobacteria and enterococci have been studied for use in the treatment and prevention of a wide range of diseases [2–4]. Probiotic microorganisms are thought to counteract disturbances in the normal microflora and thereby reduce the risk of colonisation by pathogenic bacteria [5,6].

Several theories have been proposed to explain the antimicrobial and beneficial effects of probiotics, including their capacity to compete for nutrients and their ability to secrete antimicrobial substances such as organic acids, bacteriocins and peptides [7].

Lactobacillus acidophilus n.v. Er 317/402 strain Narine was isolated from newborn baby faeces by Dr L.A. Yerzinkyan in 1963 and named 'Narine' after his granddaughter [8]. The Narine strain was studied in Armenia and neighbouring countries for many decades where the academic achievements were not made public to Western countries as it was a military secret and regulated by the KGB. It was not possible to disclose information on the strain at the time as a product obtained from its fermentation was used in the former USSR as a supplement for astronauts [2].

In this study, for the first time we have explored the isolation, identification and characterisation of a novel antimicrobial peptide (AMP) produced by *L. acidophilus* n.v. Er 317/402 strain Narine (given the trivial name acidocin LCHV), which has activity against a range of Gram-positive and Gram-negative pathogens.

2. Materials and methods

2.1. Bacterial strains and media

Lactobacillus acidophilus n.v. Er 317/402 strain Narine (also known as a *L. acidophilus* 1991) was kindly provided by Dr Rosa Madoyan (Nor-Narine Ltd., Yerevan, Armenia). Other LAB used as reference strains were provided by Dr Marina Karakhanyan (Armenian State Agrarian University). Further reference strains

[obtained from American Type Culture Collection (ATCC), Rockville, MD) used in this study are listed in Table 1. The cultures used in this study were maintained as frozen stocks at –80 °C and were transferred twice into the appropriate medium before use and incubated according to the respective growth condition of each strain.

Lactobacillus acidophilus n.v. Er 317/402 strain Narine was grown in Man–Rogosa–Sharpe (MRS) medium containing Tween 80 and milk whey (MW) nutrient medium [milk whey (350 mL), potato extract (4.5 mL) and/or potato extract powder (2.5 mg), manganese sulphate (8.0 g) and sodium acetate (0.3 g)]. The volume of the mixture was made up to 1 L with water whilst maintaining the pH in the range 6.0–6.5. Media were sterilised at 121 °C for 15 min and then cooled to 37 °C and inoculated with 5% (v/v) fresh fermentation of *L. acidophilus* n.v. Er 317/402 strain Narine. The culture was incubated at 37 °C for 64 h in anaerobic conditions.

2.2. Determination of in vitro antimicrobial activity

The inhibitory activity against reference strains was determined by the spot-on lawn method [9]. Reference strain lawns were prepared by pouring 0.125 μL of diluted overnight cultures [5×10^5 colony-forming units (CFU)/mL] over the surfaces of pre-poured ISO Sensitivity Test Agar plates. A 10 μL aliquot was used to determine antimicrobial activity of the peptide acidocin LCHV. Activity was expressed as arbitrary units (AU)/mL and was defined as the reciprocal of the highest dilution showing a clear zone of inhibition. One AU of acidocin LCHV was defined as 10 μL of the highest dilution of the AMP solution that yielded a definite zone of inhibition on a lawn of indicator strains [10]. The titre of the AMP produced was calculated as

(1000/*d*) × *D*, where *D* is the dilution factor and *d* is the amount of AMP on each spot [11].

2.3. Sensitivity of acidocin LCHV to heat, pH and proteolytic enzymes

To determine the effect of temperature on the activity of the AMP, acidocin LCHV was exposed to various heat treatments of 60, 70, 80, 90, 121 and 130 °C for 90 min. Peptide activity was then determined by the agar disk diffusion method as described by Andrews [12]. Aliquots of 500 μL of AMP samples were treated with trypsin in 0.05 M Tris hydrochloride (pH 8.0), pepsin in 0.2 M citrate (pH 6.0), and proteinase K in 1 N NaOH (pH 6.5) to determine sensitivity to proteases (all obtained from Sigma Chemical Co., St Louis, MO). The sample was also treated with catalase and untreated samples were used as controls [13].

The determine the effect of pH, AMP samples were adjusted to pH 3, 4, 5, 6, 7 and 8 with hydrochloric acid (HCl) and sodium hydroxide (NaOH) [14].

2.4. Purification and characterisation of the antimicrobial peptide produced by Lactobacillus acidophilus n.v. Er 317/402 strain Narine

2.4.1. Ammonium sulphate precipitation

Following cultivation of *L. acidophilus* n.v. Er 317/402 strain Narine, cells were removed by centrifugation (5000 × *g*) and 40%, 60%, 70% and 80% saturation was reached by slow addition of solid ammonium sulphate (Sigma Chemical Co.) and held overnight at 4 °C with stirring. Samples were pelleted by centrifugation (12 000 × *g* for 30 min). The collected fractions were then dissolved in 0.01 M K-Na

phosphate buffer (pH 6.5) and dialysed using a 1 KDa cut-off membrane against the same buffer at 4 °C overnight. Antimicrobial activity was found in the supernatant but not in the re-dissolved precipitate.

A 1 mL aliquot of supernatant was applied to a Sephadex G-15 column (3 cm × 20 cm) equilibrated with 0.01 M K-Na phosphate buffer (pH 6.5) at a flow rate of 1 mL/min, run on an ÄKTA protein purification platform (Amersham Biosciences, Uppsala, Sweden). The column was washed with the same buffer until no absorbance was detected at 280 nm. Fractions of 5 mL were collected and antimicrobial activity was determined.

The active fractions were pooled and further purified by reverse-phase high-performance liquid chromatography (HPLC) on a C_{18} column (11 mm × 300 mm) (PerkinElmer, Shelton, CT). Fractions of 20 μL were eluted with the following mobile phase at a flow rate of 0.5 mL/min using solvent A (60% methanol) and solvent B (40% water) for 25 min.

Fractions were monitored at 220–284 nm and collected manually. Antimicrobial activity was assayed for all fractions with measurable absorbance at 220 nm.

2.4.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Schägger and von Jagow [15] was used for SDS-PAGE. The gel was composed of 4% acrylamide and 0.5% bisacrylamide in the stacking gel and 16.5% acrylamide and 0.5% bisacrylamide in the separating gel. A 15 μL aliquot of AMP was mixed with 15 μL of a two-fold concentrated sample buffer and heated for 15

min at 70 °C as described by Deraz et al. [16]. Molecular mass markers were from Bio-Rad (Hercules, CA) (1423—26 625 Da). Electrophoresis was run at a constant voltage (150 V) and the gel was stained with silver stain [17].

2.4.3. Mass spectrometry analysis

Active peptide fractions from HPLC were analysed by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) (Applied Biosystems, Foster City, CA). Ten microlitres of the antimicrobial sample was mixed with an equal volume of matrix solution (α-cyano-hydroxycinnamic acid) and 1 μL of the prepared solution was spotted onto a 10 × 10 MALDI plate and left to dry at room temperature for 10 min.

Prior to sequence analysis, the sample was desalted using a C18 ZipTip® (ZTC18S; Millipore, Billerica, MA). The sample was prepared using 10 μL of 50% acetonitrile containing 0.1% (w/v) trifluoroacetic acid and was applied to a nanospray capillary (disposable borosilicate tips) and scanned between m/z 1000 and 4500 in TOF-TOF mode. For MS/MS, a range of m/z 100–2000 was chosen [18]. The obtained amino acid sequence was compared with deposited sequences using the NCBI BLAST program (http://www.expasy.org/tools/blast).

2.5. Killing kinetics

Staphylococcus aureus and *Salmonella enterica* serovar Typhimurium strains were selected and grown overnight in Mueller–Hinton (MH) broth (Oxoid, Basingstoke, UK) and diluted in fresh medium to an optical density of 0.1 absorbance units. Then, 50 μL of the AMP was added to 200 μL of *S. aureus* culture (56×10^8 CFU/mL) and

200 μL of *S.* Typhimurium (49 × 10^8 CFU/mL). The viable count was monitored up to 120 min. Aliquots were taken at defined intervals and were plated in triplicate on MH agar. Plates were incubated at 37 °C and CFU were counted after 24 h [19].

3. Results

3.1. Antimicrobial peptide production

To study acidocin LCHV production by *L. acidophilus* n.v. Er 317/402 strain Narine, a liquid nutrient medium was developed that facilitated optimum growth of the bacteria and the production of larger amounts of the antimicrobial agent than using traditional methods.

The presence of potato extract, which contains starch, monosaccharides and disaccharides that stimulate growth of the strain, increased production of the AMP. During prolonged incubation of up to 64 h, the titre of cells reached 5.0–6.0 × 10^9 CFU and the highest antimicrobial activity was detected at later stages.

The initial activity of 12 800 AU/mL was detected after culture growth in MW medium, whereas an activity of only 1600 AU/mL inhibitory activity was detected using the conventional MRS medium. It has previously been reported [20,21] that the detergent Tween 80 affects the solubility and activity of hydrophobic antimicrobial agents owing to its emulsifying properties. Production of acidocin LCHV both in MRS and MW medium started at very late exponential growth phase. During cultivation, the culture antimicrobial activity in MRS medium was very low compared with the high activity in MW medium. Similar problems have been observed for acidocin D20079 [16]. In MW medium, maximum antimicrobial activity

was detected after 54–58 h and it remained at this high level until the end of cultivation. The effects of Tween 80 present in MRS on reducing the antimicrobial activity level of bacteriocins have been reported by other authors [21–23].

3.2. Spectrum of inhibitory activity

AMP solution was prepared by ammonium sulphate precipitation of cell-free supernatant from *L. acidophilus* n.v. Er 317/402 strain Narine. A 10 μL portion from the 1:256 dilution was the highest dilution of active fraction that gave a definite zone of inhibition (expressed in mm) on a lawn of indicator strains (Table 1). The arbitrary unit was calculated as 25 600 AU/mL (1000/10 × 256) [10].

Acidocin LCHV had a broad spectrum of activity against a wide range of pathogenic microorganisms, including several bacteria classified as Especially Dangerous Infections (EDI) by World Health Organization (WHO) (Table 1).

It appears that acidocin LCHV was more active against Gram-positive bacteria than against Gram-negative bacteria and fungal pathogens (inhibition zone measurement) when the same concentrations (10 μL) were used. This may be due to the inherent physiological differences between indicator strains used. The novel antimicrobial agent was not active against several related bacteria, suggesting that it has a novel mode of action.

3.3. Stability of acidocin LCHV produced by Lactobacillus acidophilus n.v. Er 317/402 strain Narine

The effects of heat, proteolytic enzymes and pH were determined. Acidocin LCHV was considered to be extremely heat stable as antibacterial activity was not altered by heat treatment after 90 min at 130 °C (Fig. 1).

The pH stability of acidocin LCHV was studied in the range of pH 3–8. It was observed that acidocin LCHV was active at pH values from 3–6.5 but reduced at higher pH levels. pH adjustment to 6.5 was chosen to eliminate the possible effect of organic acids.

The activity of acidocin LCHV was lost after treatment with proteolytic enzymes, whereas treatment with catalase did not affect the activity, excluding an inhibitory activity due to the presence of hydrogen peroxide. These data clearly show that the antimicrobial substance was of a proteinaceous nature. Storage of the active compound at 4 °C for >2 years and in a frozen state over 10 months did not affect the antibacterial activity.

3.4. Purification and identification of acidocin LCHV produced by Lactobacillus acidophilus n.v. Er 317/402 strain Narine

During the purification protocol, the activity of the antimicrobial substance was concentrated by ammonium sulphate purification. Recovery was 40%. The antimicrobial activity was found in the supernatants but not in the precipitates. The active fraction of supernatant was subjected to gel filtration on a Sephadex G-15 column. At this stage of the purification the recovery increased up to 80% (Table 2).

The purification factor reached 25 600 AU/mL and the recovery was 8% after separation of the active fraction on a HPLC C_{18} column.

3.5. SDS-PAGE and mass spectrometry

No protein bands were detected by SDS-PAGE analysis. These results suggested that the concentration of the active peptide was low and/or the molecular weight of the peptide was too small and was rapidly eluted from the gel.

To determine accurately the molecular mass of the AMP, MS analysis was carried out. The purified sample was analysed by MALDI-TOF/TOF, which revealed a major peak at 1.1 kDa (1151.704 Da). This peak was exposed to MS/MS fragmentation, which yielded the sequence of 11 residues Asn-Val-Gly-Val-Leu-X-Pro-Pro-X-Leu-Val.

3.6. Killing kinetics

Experiments were carried out to study the immediate impact of the peptide on cells. The mode of action of the peptide showed single-hit kinetics when exponentially growing cells were treated with the AMP for 120 min (Fig. 2). Within 10 min, complete killing was observed both with *S. aureus* and *S.* Typhimurium. Survival of subpopulations and subsequent re-growth was not observed.

4. Discussion

Intestinal LAB in humans are closely associated with the host's health because they are an important biodefence factor in preventing colonisation and subsequent

proliferation of pathogenic bacteria in the intestine [24,25]. A role for lactobacilli in suppressing undesirable intestinal microflora was first proposed by Eli Metchnikoff, who suggested that it would be possible to modify the gut microflora and replace harmful microbes by useful ones [3]. The use of probiotics to maintain health must be considered promising, although much remains to be elucidated. A number of clinical studies have been performed on the ability of probiotic strains to prevent or treat gastrointestinal infections. Despite major research efforts to find effective treatments, infectious diseases remain a global problem claiming millions of lives every year [26].

A probiotic food supplement derived from *L. acidophilus* n.v. Er 317/402 is widely used in Armenia and neighbouring countries as a breast milk substitute (added as a supplement to dairy milk) and to treat gastrointestinal problems including various forms of dysbacteriosis. In addition, this supplement is used to alleviate symptoms of inflammatory bowel diseases and acute infections, during and especially after taking antibiotics, as an immune stimulator in virus and somatic diseases, and in *Helicobacter pylori*-associated pathogenic chronic gastritis of B-type and peptic ulcers. Clinical trials of this probiotic food supplement have been undertaken in Armenia, Russia, Ukraine and the Baltic countries [2,27,28]. However, the nature of this inhibitory substance has never before been characterised.

MS analysis showed that this factor was peptidic in nature and had a molecular weight of 1151.704 Da. MS/MS analysis identified the sequence as Asn^{1}-Val^{2}-Gly^{3}-Val^{4}-Leu^{5}-X^{6}-Pro^{7}-Pro^{8}-X^{9}-Leu^{10}-Val^{11}, where the Xs represented residues that we have identified as Leu^{6} or Asn^{6} and Met^{9} or Pro^{9}. Unfiltered BLAST searches using this sequence have not revealed any sequences with homology in the SWISS-

PROT/TrEMBL database. Similar BLAST sequences have also not identified any homology in the genome sequences of the two sequenced strains of *L. acidophilus*.

The AMP was heat stable (90 min at 130 °C), active at pH values from 3–6.5 and had broad-spectrum activity against a range of Gram-positive and Gram-negative pathogens, including several classified as EDI by the WHO. Furthermore, acidocin LCHV was active against meticillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*, which are prominent hospital pathogens that are difficult to treat and are a major cause of hospital morbidity and mortality worldwide. Interestingly, the novel antimicrobial agent was not active against several related bacteria, including *Lactobacillus plantarum*, *Leuconostoc mesenteroides* ssp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Streptococcus salivarius* ssp. *thermophilus*, suggesting that it may have a novel mode of action.

Other natural bacterially derived AMPs have not been exploited previously in this manner. The most well know natural AMP in commercial use is nisin. This natural peptide was derived from *L. lactis* and was approved for use in food by the Joint FAO/WHO Expert Committee on Food Additives in 1969. Nisin is now used in over 50 countries as a food preservative in a broad range of foods ranging from dairy products and meat to beer. Interestingly, nisin has not been used to treat bacterial infections. A number of other bacterially derived AMPs are also used as food preservatives [29–33]. Lu et al. [3] isolated and characterised seven small peptides from *Lactobacillus* GG, two of which were NPSRQERR and PDENK, that retained the antimicrobial activity detected with LGG conditional media. The antimicrobial activity of these peptides was observed both against Gram-negative (*E. coli* EAEC

042 and *S.* Typhimurium) and with less potency against Gram-positive bacteria (*S. aureus*).

Heat stability of low molecular weight peptides, which would be advantageous for transport and usage as a drug [34], has been suggested [20,35] to arise from complex patterns of disulphide intramolecular bonds that stabilise secondary structures by reducing the number of possible unfolded structures. We will undertake further characterisation of acidocin LCHV to reveal its full structure. It has been reported by several authors that lactobacilli are able to produce antimicrobial substances when grown in specific media [36,37]. In 1959, Vincent et al. [38] described what was called lactocidin, a substance obtained from solid agar medium seeded with *L. acidophilus*. This substance was more active against Gram-negative than Gram-positive bacteria.

During purification, optimal recovery was achieved by including ammonium sulphate precipitation, followed by gel filtration and HPLC reverse-phase chromatography (Table 2). This resulted in an increase of specific activity and an 8% recovery, which is similar to results obtained for the antimicrobial agents and bacteriocins produced by other *Lactobacillus* spp. [39]. Treatment of *S.* Typhimurium and *S. aureus* to study the mode of action of acidocin LCHV resulted in rapid killing with immediate impact on the cells. Killing kinetics was considerably faster and complete killing of the cells was observed within a very short period of time both on Gram-positive and Gram-negative strains.

Several theories have been proposed to explain the exact mechanism by which AMPs kill bacteria. The antimicrobial activity of these peptides has been described

as a ‘carpet-like’ mechanism, where the AMPs are proposed to bind to the surface of the membrane and cover it in ‘’carpet-like’ manner and disturb its barrier function [18,40].

MRSA and other multiresistant bacteria have emerged as serious pathogens over the past decade and, despite major research efforts aimed at finding an effective drug, increasing resistance has compromised therapy [41]. Use of small peptides as anti-infective drugs is a novel approach and would offer several advantages over current treatments. Here we have demonstrated that a new AMP has a broad spectrum of activity against a wide range of pathogens, including EDI and MDR pathogenic microorganisms. These compounds could be synthesised readily and the costs for these using a solid-phase approach would be inexpensive. This would offer opportunities for a developing country to organise the production of this peptide.

It is accepted that the use of AMPs would be safe for the treatment of infectious diseases owing to the fact that they are elaborated by probiotics [3]. However, more studies are necessary to investigate the possibility of using this novel antimicrobial agent as an anti-infective agent and to progress it through pre-clinical studies in preparation for entry into clinical trials.

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Competing interests

None declared.

Ethical approval

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Fig. 1. Effect of heat treatment on acidocin LCHV produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine demonstrated on the indicator strain *Bacillus cereus*: 1, control; 2, 60 °C; 3, 70 °C; 4, 80 °C; 5, 90 °C; 6, 121 °C; and 7, 130 °C.

Fig. 2. Bactericidal effect of acidocin LCHV produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine on *Salmonella enterica* serovar Typhimurium (●) and *Staphylococcus aureus* (■). ▲, control, without acidocin LCHV treatment. CFU, colony-forming units.

Table 1

Sensitivity (inhibition zone measurement) of pathogenic microorganisms to acidocin LCHV produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine

Indicator strain	Inhibition zone (mm) [a]
Gram positive bacteria	
Bacillus cereus ATCC 12432	35 ± 0.3
Bacillus subtilis ATCC 12434	34 ± 0.5
Clostridium difficile ATCC 9689	29 ± 0.4
MRSA ATCC-BAA 1720	30 ± 0.6
Klebsiella pneumoniae ATCC 13883	35 ± 0.3
Pseudomonas aeruginosa ATCC 10145	36 ± 0.5
Rhodococcus fascians ATCC 12975	35 ± 0.4
Serratia marcescens ATCC 14227	34 ± 0.3
Staphylococcus aureus ATCC 10390	37 ± 0.4
Staphylococcus epidermidis ATCC 12228	35 ± 0.3
Listeria monocytogenes ATCC 23073	37 ± 0.3
Gram-negative bacteria	
Brucella abortus 544	28 ± 0.5
Escherichia coli VTEC KCTC 12900	33 ± 0.5
E. coli NCF BS 55	33 ± 0.4
Francisella tularensis	25 ± 0.6
Salmonella enterica serovar Stanley	35 ± 0.5
Salmonella enterica serovar Typhimurium ATCC 14028 NA	35 ± 0.4
Shigella flexneri ATCC 11836	34 ± 0.4
Vibrio cholerae 2590	25 ± 0.5
Yersinia pestis 3344	26 ± 0.4
Y. pestis 2835	26 ± 0.5
Yersinia enterocolitica 2840	32 ± 0.4
Y. enterocolitica	29 ± 0.3
Fungal pathogens	
Alternaria solani MYA-995	25 ± 0.4
Fusarium moniliforme ATCC 10052	15 ± 0.6
Fusarium oxysporum ATCC 12581	13 ± 0.3

Helminthosporium sativum ATCC 11404	24 ± 0.4
Lactic acid bacteria (*n*)	
Lactobacillus plantarum (2)	–
Leuconostoc mesenteroides ssp. *lactis* (3)	–
Lactococcus lactis (2)	–
L. lactis subsp. *cremoris* (2)	–
L. lactis subsp. *lactis* biovar *diacetylactis* (1)	–
Streptococcus salivarius ssp. *thermophilus* (2)	–

MRSA, meticillin-resistant *Staphylococcus aureus;* –, no inhibition.

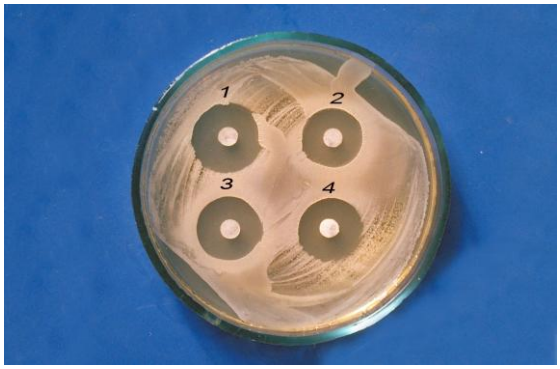
[a] Average value of three replicates.

Table 2

Purification of acidocin LCHV by precipitation with ammonium sulphate, gel filtration and high-performance liquid chromatography (HPLC) on a C_{18} column

Purification stage	Volume (mL)	Activity (AU/mL)	Total activity (AU)	Recovery (%)
Supernatant	100	12 800	1 280 000	100
Ammonium sulphate precipitation	10	51 200	512 000	40
Gel filtration	40	25 600	1 024 000	80
HPLC	4	25 600	102 400	8

AU, arbitrary units.



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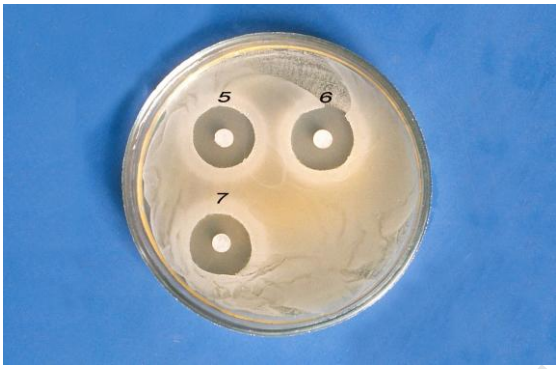

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