Isolation, Characterization, and Applicability of Helveticin 34.9, A Class Iii Bacteriocin Produced By Lactobacillus Helveticus 34.9

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Abstract

Traditionally fermented foods and beverages are still produced and consumed at a large scale in Romania. They are rich sources for novel lactic acid bacteria with functional properties and with potential application in food industry or health. *Lactobacillus helveticus* 34.9, isolated from a home-made fermented milk is able to inhibit the growth of other bacteria, such as other lactic acid bacteria, but also strains of *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Halobacillus hunanensis*, a halobacterium isolated from the degraded wall of a Romanian monastery. *L. helveticus* 34.9 produces a large bacteriocin (35 KDa), active in a wide pH range, but inactivated by heat and proteinase K treatment. Bacteriocin production was enhanced under stress conditions, especially when combined stresses were applied. Its mode of action and degree of inhibition depended on the indicator strain and on the concentration of bacteriocin that was used. *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T cells from a suspension were killed within 8 h, but the viability of *H. hunanensis* 5Hum cells was only reduced to 60%. However, the bacteriocin was able to prevent the bacterial growth of both indicator strains when added to the medium prior inoculation. Scanning electron microscopy images revealed morphological changes induced by the bacteriocin treatment in both sensitive strains, but more severe in the case of *L. delbrueckii* subsp. *bulgaricus*. This class III bacteriocin, with inhibitory activity against various bacterial species, may find application in food and non-food related fields, including in the restoration of historical buildings.

Introduction

Lactic acid bacteria (LAB) are well known for their capacity to produce various antimicrobial substances, including: organic acids, ethanol, hydrogen peroxide, bacteriocins, and antibiotic-like peptides (Reis et al. 2012). Based on this property, LAB have major potential to be used in biopreservation, which is a novel, natural, and more desirable way of preservation, involving the use of nonpathogenic microorganisms and/or their products for extending the shelf life of foods (De Martinis et al. 2001).

Among the antimicrobial compounds, bacteriocins have been intensively studied and are of very high perspective because they have a great potential to be utilized not only in food preservation, but also in other fields, such as: human and animal health, agriculture, and environmental protection (Juturu and Wu 2018). Bacteriocins may be used as natural antibiotics, in human or animal healthcare products, where undesired and potentially resistant microorganisms must be controlled (Cotter et al. 2013), but also in packaging and nanotechnology (de Melo et al. 2013; Bali et al. 2014). Some bacteriocins have been shown to have antiviral or antifungal activity (Todorov et al. 2010; Yasmin et al. 2015), while some LAB strains and their produced bacteriocins are able to control the oral microbiota, preventing halitosis or oral diseases (Burton et al. 2010).

Bacteriocins are defined as “ribosomally synthesized antimicrobial peptides produced by one bacterium, that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum)” (Cotter et al. 2005). Bacteriocins are classified according to their properties (chemical

structure, molecular mass, enzymatic susceptibility, genetics, mechanism of action, thermostability, etc.) into three main groups: (i) class I, constituted by posttranslationally modified, heat-stable peptides, with a low molecular mass, below 5 kDa; (ii) class II, unmodified and heat-stable peptides, with a molecular mass below 10 kDa; and (iii) class III, heat-labile large proteins, above 30 kDa (Nowakowski et al. 2018; Kumariya et al. 2019).

LAB belonging to most genera are able to produce bacteriocins, mainly targeted against several important foodborne pathogens such as *Listeria monocytogenes*, *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., and, in some cases, against *Bacillus* spp. (Kaškonienė et al. 2017). Bacteriocins typically have a broad mechanism of action, quick killing effect and low tendency to induce resistance (Chikindas et al. 2018).

Since the first discovery of nisin, many bacteriocins with unique structures and different modes of activity have been described, and the genes coding for the production, secretion and immunity of most have been reported. As microbial diversity is very wide, the search for new bacteriocins with a broad inhibitory spectrum has led to an extensive research in this field during past decades. There is an increasing interest in antibacterial compounds with great stability in different media, tolerance to heat and pH variation, and resistance to proteolytic enzymes (Kaškonienė et al. 2017).

Important sources for LAB strains producing such novel bacteriocins are traditionally fermented foods, in which no starter strains are used, and fermentation is carried out by microbiota naturally present in the raw material or in the environment. In this context, Romanian home-made fermented foods (dairy products, fermented vegetables, *bors*, *braga*) have been shown to contain LAB strains with antibacterial activity against spoilage bacteria and some foodborne pathogens (Grosu-Tudor and Zamfir 2013; Grosu-Tudor et al. 2014; Angelescu et al. 2019).

Lactobacillus helveticus 34.9 had been isolated from a sample of Romanian home-made fermented milk (Zamfir et al. 2006), and it had been shown to exhibit antibacterial activity against *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T (Stefan et al. 2018). The objectives of this study were to identify the nature of inhibition, to isolate the inhibitory compound, and to characterize it, in terms of production under different environmental conditions, stability, mode of action, and potential applications.

Materials And Methods

Bacterial Strains and Growth Conditions

Lactobacillus helveticus 34.9 was used as the producer of antibacterial activity, while *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T was used as the indicator strain. Both the producer and the indicator strain were maintained at -80°C in MRS broth (de Man et al. 1960), in the presence of 25% (v/v) of glycerol as a cryoprotectant. Before use, they were propagated twice, for 24h, in MRS broth, at 37°C.

Antibacterial activity assessment

Cell free culture supernatant (CFCS) was obtained from fresh *L. helveticus* 34.9 culture, by centrifugation (13000 x g, 10 min, 4 °C). Antibacterial activity was assayed by both agar spot (De Vuyst et al. 1996), and agar well diffusion (Balouiri et al. 2018) methods. Briefly, ten ml of the CFCS were spotted onto fresh lawns (soft agar media, 0.7 % (w/v) agar) containing 100 ml of the indicator culture. Alternatively, 50 ml of the CFCS were added to wells cutted into agar media previously inoculated with the indicator. Plates were then incubated for 24h to allow the growth of the indicator strain. The presence of clear inhibition zones around the spots/wells was recorded as a positive result.

Evidence of bacteriocin production

In order to establish the nature of the inhibitory compound, several additional tests were done. Firstly, to remove the antibacterial activity of organic acids, the pH of CFCS was adjusted to 6.5-7.0, before testing. Secondly, the influence of several proteolytic enzymes, such as: trypsin (Sigma-Aldrich Chemie GmbH, Germany), pronase E (Merck KGaA, Darmstadt, Germany), proteinase K (Carl Roth, Karlsruhe, Germany), and pepsin (Sigma-Aldrich), on the antibacterial activity was investigated. Enzymes were dissolved in 0.2 M sodium phosphate buffer (pH 7.5), except pepsin, which was dissolved in 0.2 M HCl/KCl buffer (pH 2.0) and were added to the CFCS, at a final concentration of 1 mg/ml. Supernatants were incubated with proteinase K for 2h at 55°C, and with other enzymes for 2h at 37°C, before being tested. The CFCS diluted with the above mentioned buffers, and enzymes solutions without CFCS were used as positive and negative controls, respectively.

Quantitative assay of bacteriocin activity

Serial twofold dilutions in water, of the bacteriocins samples, were spotted (10 µl) onto fresh lawns of the indicator strain, or added (50 µl) to wells cutted in the inoculated agar medium (as described above). The activity was defined as the reciprocal of the highest dilution for which a clear inhibition zone could be still observed, and was expressed in activity units (AU) per millilitre of sample.

Antibacterial Activity under Stress Conditions

Previous results showed an increase of the antibacterial activity of *L. helveticus* 34.9 when grown under different stress conditions, such as an initial pH of 4.5, or in the presence of low concentrations of NaCl or bile salts (BS) in the growth medium (Stefan et al. 2018). In the present study, the bacteriocin producing strain was cultivated under more stressful conditions, such as higher concentrations of bile salts (Sigma-Aldrich, up to 2%), or under combined stresses, as they are presented in Table 1. The antibacterial activity was quantitatively assayed, using *L. delbrueckii* subsp. *bulgaricus* 6901^T as indicator strain.

Bacteriocin isolation and characterization

The bacteriocin produced by *L. helveticus* 34.9 was concentrated and extracted as described before (Zamfir et al. 1999). Briefly, the strain was inoculated (2% inoculum) in 500 ml MRS broth supplemented

with 2% NaCl and with an initial pH of 4.5, and incubated overnight at 37°C. CFCS was neutralized with 10N NaOH and treated with 40% ammonium sulphate, with gentle stirring, to precipitate the proteins. Afterwards, the mixture was centrifuged (13,000 x g, 10 min, 4 °C) and the pellicle was resuspended in 5 mM potassium phosphate buffer, pH 6.5 (crude bacteriocin). The bacteriocin was finally extracted with 15 volumes of chloroform: methanol (2:1, v/v). After 2h of incubation at 4 °C, the suspension was centrifuged (13,000 x g, 15 min, 4°C) and the precipitate was resuspended in ultrapure water.

Characterization was further done using the crude bacteriocin sample.

Antibacterial inhibition spectrum

To determine the activity spectrum of *L. helveticus* 34.9 bacteriocin, several bacterial strains were used as indicator, including other LAB, some bacterial pathogens, and a strain of *Halobacillus hunanensis*, namely 5Hum, previously isolated from the degraded wall of a Romanian monastery (Cojoc et al. 2019). Table 2 shows a list of the indicator strains and the growth conditions used for each of them. The antibacterial activity of the crude bacteriocin was assayed using the well diffusion method. The diameters of the inhibition zones around the wells were measured, as an indication of the strength of the antibacterial activity.

Heat sensitivity and pH stability of the bacteriocin

Crude bacteriocin was subjected to different pH and thermal treatments. Untreated samples were used as controls and the antimicrobial activities were quantitatively determined against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T by agar spot method.

The pH stability was tested by adjusting the pH of the crude bacteriocin from 2 to 10 (using sterile 1M HCl or 1M NaOH) with increments of two units, and allowing the samples to stand for one hour at room temperature before testing the inhibitory activity.

To determine the heat stability of the antibacterial activity, independent incubations of the crude bacteriocin were carried on at 60°C, and 100°C, and the activity was tested after 15, 30, and 60 min of treatment.

Molecular Mass Determination

Tricine sodium dodecylsulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE, Schägger and von Jagow 1987) was carried on in a Biometra Minigel Twin (Biometra, Germany) to estimate the molecular mass of the produced bacteriocin. CFCS, crude bacteriocin, and bacteriocin extracted with chloroform/methanol mixture, were used for the run. Electrophoresis was conducted at a constant voltage of 30 V in the stacking gel, and 90 V in the separating gel. For the bioassay, the gel was washed during 5 h with sterile ultrapure water and overlaid with a top layer of soft MRS agar (0.7% agar) inoculated with the indicator strain, *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T. The broad range

molecular weight markers (10-225 KDa, Promega, USA) were stained with Coomassie Brilliant Blue (Carl Roth GmbH, Germany).

Mode of action

Bactericidal effect of the bacteriocin

Cells from 24h cultures of the sensitive strains, *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T and *H. hunanensis* 5Hum, were washed with sterile potassium phosphate buffer (pH 6.5) and suspended in the same buffer to get about 7-8 log CFU/ml. In case of 5Hum strain, the buffer was supplemented with 10% NaCl, to minimize the osmotic stress. Crude bacteriocin was filter sterilized and serially diluted before being added to the cells suspensions. Final activities of about 6400, 3200, and 1600 AU/ml (determined against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T) were used. Samples were maintained at 37°C. At various time intervals, viable cell counts (CFU/ml) were determined. All experiments were done in triplicate. Untreated cells served as controls.

Bacterial survival rate (%) was calculated according to the formula

Survival rate (%) = $(N/N_0) \times 100$, where

N represents $\log_{10}$ colony forming units per ml suspension,

N_0 represents $\log_{10}$ colony forming units per ml at time zero.

Growth inhibition of the sensitive cells

Crude bacteriocin was filter-sterilized and added to MRS and MH broth, respectively (final activities between 1600 and 6400 AU/ml). Each medium was further inoculated (5%) with the corresponding sensitive strain. The growth was monitored by hourly measurements of the OD at 600nm, using a FLUOstar Omega microplate reader (BMG-Labtech GmbH, Germany). Cultures without bacteriocins served as controls.

Scanning electron microscopy

The impact of the bacteriocin produced by *L. helveticus* 34.9 on the sensitive cells' morphology was observed by scanning electron microscopy (SEM), using a previously described protocol (Zamfir et al. 2016). Cells of *L. delbrueckii* subsp. *bulgaricus* LMG6901^T and *H. hunanensis* 5Hum were collected by centrifugation from the exponentially growing cultures, washed and treated for 24h with the bacteriocin (final concentration of 6400 AU/ml). Cellular suspensions without bacteriocin served as controls. After centrifugation (10,000 x g, 10 min, 4°C), cells were fixed for 2h at 4°C with 4% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.4). Water was then extracted from the samples using a graded series of ethanol: 50-75-100%, for 15 min in each bath. The last baths were in ethanol/acetone (1/1) and

acetone 100%. A drop of each sample was spotted on a microscopic blade, air-dried and coated with gold. Cells were visualized with a JSM-6610LV scanning electron microscope (Jeol).

Statistical Analyses

All the experiments were done in triplicate or quadruplicate. The results were processed using Microsoft Excel software (Microsoft Corp.) and are expressed as mean value $\pm$ SD (standard deviation).

Results

Evidence of bacteriocin production

CFCS obtained from *L. helveticus* 34.9 cultures developed at 37°C, showed an antibacterial activity of 200 AU/ml against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T (Table 1). The pH adjustment to 6.5-7.0, and treatment with tripsin, pepsin, and pronase E did not affect the activity (results not shown). On the contrary, no activity was detected after treatment with proteinase K. Enzymes alone did not show activity, while CFCS treated with buffers only, had unaltered activity (results not shown).

Antibacterial Activity under Stress Conditions

As observed in Table 1, growth and bacteriocin production were improved when *L. helveticus* 34.9 was cultivated at 37°C as compared with results at 42°C, both in the presence or absence of other stress factors. When cultivated under optimal growth conditions (MRS medium, 37°C), the antibacterial activity was 200 AU/ml. A low initial pH (4.5) of the growth medium and the presence of small concentrations of BS (0.1%) inhibited the bacterial growth, but did not affect the final bacteriocin activity. A further increase of the BS concentration to 0.2%, resulted in a dramatic decrease of the growth, but stimulated the bacteriocin production. Higher concentration of BS (2%) resulted in a decrease of both growth and inhibitory activity. Some other stress conditions resulted in an increase of the antibacterial effect, although the bacterial growth was limited. The highest bacteriocin activities were recorded for the cultures obtained in MRS with initial pH of 4.5, concomitantly supplemented with 2% NaCl, and in MRS supplemented with both 2% NaCl and 0.1% BS (3,200 AU/ml).

At 42°C, in the absence of other stress factors, the activity was 100 AU/ml. Most of the singular stresses resulted in a significantly slower growth, but had little or no effect on the antibacterial activity, as compared with the control. Unlike at 37°C, the presence of NaCl in the medium did not improve the activity. However, the combined stresses led to a significant increase of the antibacterial activity. The highest activities were recorded, as for the growth at 37°C, in the presence of both NaCl and BS, and in the presence of NaCl in a medium with low pH. In these two media, the activity increased 16 to 32 times compared with the control, both when the strain was grown at 37°C and at 42°C (Table 1).

Bacteriocin isolation and characterization

The bacteriocin activity increased from 3,200 to 25,600 AU/ml after ammonium sulphate precipitation of the CFCS. A further chloroform/methanol extraction step resulted in a decrease of the inhibitory activity down to 6,400 AU/ml (Table 3). Since the highest activity was reached after ammonium sulphate precipitation, this sample was further used for characterization.

Antibacterial inhibition spectrum

Crude bacteriocin of *L. helveticus* 34.9 was screened for inhibitory activity against a large variety of bacteria including some pathogenic strains (Table 2). It was able to inhibit the growth of strains belonging to the following species: *L. delbrueckii*, *L. helveticus*, *L. sakei*, *Enterococcus faecium*, *Bacillus subtilis*, *B. cereus*, and *Staphylococcus aureus*. High activities (diameters of the inhibition zones larger than 10 mm) were detected against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T, *L. helveticus* 102, and *L. sakei* 13558. Moreover, the bacteriocins showed a significant antagonistic effect against *Halobacillus hunanensis* 5Hum (Table 2, Fig. 1).

Heat sensitivity and pH stability

Crude bacteriocin, with an inhibitory activity of 25,600 AU/ml, was used to test the heat sensitivity and the stability at different pH values. The bacteriocin maintained its activity in a wide pH range, between 2.0 and 10.0, but with significantly lower values at pH 2.0 and above 8.0 (Table 3). On the other hand, bacteriocin showed a high sensitivity to heat, its activity dramatically decreasing after 15 min of incubation at 60°C, and being completely inactivated by a 15 min incubation at 100°C.

Molecular mass determination

A clear inhibition zone was observed in the indicator lawn covering the gel, for all three samples analyzed by Tricine-SDS-PAGE, namely CFCS, crude bacteriocin, and chloroform/methanol extract (Fig. 2). The bands corresponded to a molecular mass of about 35 KDa.

Mode of action

Bactericidal effect of the bacteriocin

The bactericidal effect against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T was clearly demonstrated, as shown in Fig. 3a. The viability of the sensitive strain started to decrease within the first two hours of incubation in the presence of bacteriocin, and it reached 0% after 8 - 12 h of incubation, depending on the bacteriocin concentration. A sharp decrease of the viability down to 80% was observed for *H. hunanensis* 5Hum cells, within the first two hours of treatment with bacteriocins (Fig. 3b), but further incubation did not result in a significant change. More important, the sensitive cells kept a high viability percentage, of 60% to 70%, depending on the bacteriocin concentration, even after 48 h of treatment.

Growth inhibition of the sensitive cells

The inhibitory effect of the crude bacteriocin added before inoculation of the sensitive strain was directly related to its concentration (Fig. 4). At maximum bacteriocin concentration, no bacterial growth was detected within 24 h of incubation, for any of the indicators. Addition of intermediate concentrations, of 3200 AU/ml, resulted in a slower growth, compared with the control, while the lowest concentrations used in the study (1600 AU/ml) did not show a significant inhibitory effect against the sensitive strains.

Scanning electron microscopy

Untreated cells of *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T have a typical rod shape, with smooth edges, and about 5-10 mm length (Fig. 5a). Bacteriocin treatment induced significant morphological changes of the sensitive cells. As shown in Fig. 5b-d, we could detect the presence of transmembrane pores on the cell surface, but also many ghost cells emptied by their content.

On the other hand, *H. hunanensis* 5Hum control cells are small rods, of about 1-2 mm length, also with smooth edges (Fig. 6a). No transmembrane pores were detected in the treated cells, and the morphological changes were less severe in this case. We observed a surface roughening and a shrinkage of the cells after incubation with the crude bacteriocin (Fig. 6b-c). Few elongated cells were also found in the treated samples (Fig. 6c).

Discussion

Bacteriocins represents one of many natural defense mechanisms bacteria use to compete against microorganisms in the same environment. The primary application of LAB bacteriocins has always been in food preservation (Field et al. 2015). However, considering the discovery of new bacteriocins with novel properties, their application spectrum can be enlarged in other fields such as horticulture, biomedical, personal care industries, environmental protection, etc. (Juturu and Wu 2018). The present study describes the properties of a bacteriocin produced by *L. helveticus* 34.9, further named **helveticin 34.9**, including its mode of action and inhibition spectrum. Based on the results, different fields of application have been identified for this compound, including non-food applications.

Helveticin 34.9 was produced both in optimal growth conditions, and in stressful environments. The influence of acidic and osmotic stress, but also the presence of bile salts had been investigated in our study, since these stress conditions can be encountered during food processing and during the passage through the gastrointestinal tract. Growth of *L. helveticus* 34.9 at the optimal temperature (37 °C) resulted in a higher antibacterial activity, as compared with 42 °C. Helveticin production was also stimulated when the producing strain was cultivated under some stress conditions, such as the presence of NaCl or moderate concentrations of BS in the growth medium. A combination of two different stress factors, such as a low initial pH and addition of NaCl, or addition of both NaCl and BS, seemed to be very efficient for a significant increase of the inhibitory activity, although correlated with a much slower bacterial growth.

Despite bacteriocins are primary metabolites, being directly correlated with bacterial growth, it was shown that a good growth is not enough for a good bacteriocin production and that some bacteriocins are

overproduced under some stress environmental conditions (Verluyten et al. 2004). The pH, water activity, and nutrients concentration of the complex environments, but also the incubation temperature will affect both the bacterial growth and bacteriocin production (Dicks et al. 2018). It has been previously shown that production of bacteriocins and immune proteins is often mediated by quorum-sensing mechanisms, and that some environmental stressors may induce the production (Nes and Eijsink 1999).

Interestingly, the active compound produced by *L. helveticus* 34.9 was able to maintain its effect in a wide pH range (2.0-10.0), although with lower activities at pH 2.0 and over 8.0. Many bacteriocins are stable and active in a much narrow pH range and render inactive at pH higher than 8.0 (Meng et al. 2021). Also, helveticin 34.9 was only inactivated by proteinase K, but not by trypsin or pepsin.

These results suggest that *L. helveticus* 34.9 and its bacteriocin may find applications in the probiotic sector, being able to maintain the inhibitory activity in conditions specific for the digestive tract, such as low pH, and the presence of bile salts or digestive enzymes. Moreover, the good production of helveticin 34.9 in the presence of NaCl, but also its broad pH-adaptability, makes the producing strain suitable to be used in certain foods such as fermented meat, dairy products, or fermented vegetables.

Helveticin 34.9 was extracted and partially purified by ammonium sulphate precipitation. A further chloroform/methanol extraction resulted in a decrease of the final activity. A possible explanation could be a low hydrophobicity of this bacteriocin. It has been pointed out by several authors that extraction and purification technique differ from one class of bacteriocins to another and depends on the protein nature (Kaškonienė et al. 2017).

Crude bacteriocin could be detected by Tricine-SDS-PAGE in a band corresponding to a molecular mass of about 35 KDa. Moreover, its activity was significantly reduced by heat treatment such as 15 min incubation at 60 °C. Based on all its properties, helveticin 34.9 can be assigned to class III of bacteriocins, comprising large, heat sensitive proteins.

Although most bacteriocins belonging to class III have a narrow inhibitory spectrum, recent studies described proteins of this class able to inhibit both Gram-positive and Gram-negative foodborne pathogens, including *Salmonella*, *Yersinia*, *Listeria*, *Enterobacter*, *Shigella*, etc., acting by weakening their cell wall, and increasing the permeability of the membrane (Meng et al. 2021). Helveticin 34.9 was active against a broad spectrum of bacteria, inhibiting some related species, such as other LAB strains, but also two *Bacillus* strains, and one strain of *S. aureus*, confirming its applicability in food industry. Interestingly, helveticin 34.9 was able to inhibit the growth of a strain of *Halobacillus hunanensis*, an antibiotic resistant halobacterium isolated from the mural painting of Humor Monastery (Suceava county, Romania), where it acts as a biodeteriogen, causing an undesirable pink staining of the mural paintings (Cojoc et al. 2019). Finding new directions in restoration of cultural heritage is an actual topic. Specific-acting antimicrobial compounds, such as helveticin 34.9, could be included in products used in the restoration process, such as “prevention” mortars.

Regarding their mode of action, bacteriocins compete other microorganisms by pore-formation, with a consecutive leakage of intracellular substrates, disruption of proton motive force, inhibition of synthesis or degradation of the cell-wall, inhibition of protein synthesis and disruption of DNA replication, etc. (García-Bayona and Comstock 2018; Kumariya et al. 2019). Class III bacteriocins are less studied from this point of view, but their main target is not the cell membrane, like for the other classes, but the cell wall, and their mode of action seems to be either bacteriolytic or non-lytic (Alvarez-Sieiro et al. 2016). Helveticin M has been recently characterized and shown to disrupt the cell wall of Gram-positive bacteria and outer membrane of Gram-negative bacteria, being thus effective against both Gram-positive and Gram-negative bacteria (Sun et al. 2018).

Our results showed that helveticin 34.9 has a dual mode of action, depending on the indicator strain. For instance, the bacteriocin was not able to kill the sensitive cells of *H. hunanensis* from a suspension, but it could repress the growth of this pathogen, when added in a sufficient amount, prior inoculation. In contrast, *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T, completely lost its viability in suspension after 8-12 h of treatment with 6400-1600 AU/ml of helveticin.

SEM results confirmed the difference between the mode of inhibition against the two indicator strains. In the treated culture of *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T, accumulation of cell debris outside the cell walls, and the presence of many ghost cells may suggest that helveticin acts in this case by pore formation. On the other hand, in *H. hunanensis* 5Hum culture, no such modifications were detected after the treatment with helveticin. In this case, the bacteriocin seems to affect the cell wall, leading to a surface roughening and cell shrinking, but it also seems to disrupt the cell division, resulting in long, unseparated cells.

Some bacteriocins have been shown to kill sensitive cells by interfering with the cell division process, as well as with other biological functions. The process occurs at different bacteriocin concentrations and is directly related to the target microorganisms. Interference with bacterial septum formation may also result in bacteriostatic action for some bacteriocins (Asaduzzaman et al. 2009). This might be the case also for helveticin 34.9. As observed by SEM, the morphological changes in *H. hunanensis* 5Hum cells were not so dramatic as compared with the other indicator. The cells did not loose their content and, most probably, they were not killed by the treatment. Therefore, when inoculated in a medium without bacteriocin, they were able to recover and divide normally, as seen in the killing assay.

In conclusion, the present study opens new perspectives for the applicability of LAB bacteriocins, including in the non-food area. Results are of great importance given that class III bacteriocins is much less studied compared with the other classes, due to the narrow inhibitory spectrum and limited application. However, this class of antibacterial compounds might still find interesting, specific applications in various fields, such as the one we report here. Even if helveticin 34.9 was not able to kill *H. hunanensis* 5Hum cells in suspension, it may be successfully used as a prevention method, since it was able to repress the growth of the sensitive cells, when present in the medium before inoculation. This is the first report of an LAB bacteriocin with inhibitory activity against a halobacterium, with potential

application in restoration of historical buildings. We consider that such reports, on very specific applications, may be a good starting point for deeper studies leading to new inventions and new applications of LAB bacteriocins.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: Data sharing not applicable to this article as no datasets were generated or analysed during the current study

Conflicts of interest/Competing interests: No conflict of interest

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Authors' contributions: MZ designed the research, conducted experiments and wrote the manuscript. SSGT and IRA conducted experiments and analyzed data. RLC provided the strain of *H. hunanensis* and conducted the experiments with this strain. GM conducted SEM experiments. All authors read and approved manuscript.

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Authors' contributions: MZ designed the research, conducted experiments and wrote the manuscript. SSGT and IRA conducted experiments and analyzed data. LRC provided the strain of *H. hunanensis* and conducted experiments with this strain. GMM conducted SEM experiments. All authors read and approved manuscript.

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Tables

Table 1. Antibacterial activity of *L. helveticus* 34.9 cultivated in different stress conditions against *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T

Growth conditions	37°C		42°C	
	OD _{600nm}	Inhibitory activity (AU/ml)	OD _{600nm}	Inhibitory activity (AU/ml)
Control	3.8	200	2.3	100
Initial pH 4.5	1.5	200	0.7	<100
2% NaCl	3.4	800	2.8	100
0.1% BS	0.9	200	0.4	200
0.2% BS	0.3	800	0.2	<100
2% BS	0.2	100	0.2	<100
pH 4.5 + 2% NaCl	0.9	3,200	0.3	1,600
pH 4.5 + 0.2% BS	0.1	800	0.1	400
2% NaCl + 0.2% BS	0.4	3,200	0.2	3,200

Table 2. Bacterial strains used as indicators, corresponding growth media and temperatures of incubation, and sensitivity to the crude bacteriocin produced by *L. helveticus* 34.9, as determined by well diffusion method

Indicator strain	Growth medium	Growth temperature	Inhibition zone* (mm)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> LMG 6901 ^T	MRS broth	37 °C	11.8 ± 0.1
<i>Lactobacillus helveticus</i> 102	MRS broth	37 °C	10.4 ± 0.2
<i>Lactobacillus helveticus</i> 122	MRS broth	37 °C	n.i.
<i>Lactiplantibacillus plantarum</i> P26	MRS broth	37 °C	n.i.
<i>Enterococcus faecalis</i> LMG13566	MRS broth	37 °C	n.i.
<i>Enterococcus faecalis</i> LMG16216	MRS broth	37 °C	n.i.
<i>Enterococcus faecium</i> LMG14203	MRS broth	37 °C	8.5 ± 0.1
<i>Leuconostoc mesenteroides</i> LMG13562	MRS broth	28 °C	n.i.
<i>Latilactobacillus sakei</i> LMG13558	MRS broth	37 °C	10.8 ± 0.2
<i>Pediococcus pentosaceus</i> LMG13561	MRS broth	30 °C	n.i.
<i>Pediococcus pentosaceus</i> LMG13561	BHI	37 °C	n.i.
<i>Salmonella enterica</i> ATCC 14028	BHI	30 °C	8 ± 0.2
<i>Bacillus subtilis</i> B17	BHI	30 °C	7 ± 0.2
<i>Bacillus cereus</i> CBAB	BHI	37 °C	n.i.
<i>Escherichia coli</i> ATCC 25922	BHI	37 °C	8.4 ± 0.2
<i>Staphylococcus aureus</i> ATCC 25923	BHI	37 °C	n.i.
<i>Listeria monocytogenes</i> ATCC 1911	MH (10% NaCl)	30 °C	12.6 ± 0.3
<i>Halobacillus hunanensis</i> 5Hum			

* Values indicate the mean (of three values) diameter of the inhibition zone ± standard deviation (SD); n.i. = no inhibition

Table 3. Bacteriocin activity determined by agar spot method, after various treatments; *L. delbrueckii* subsp. *bulgaricus* LMG 6901^T was used as indicator strain

Treatment	Bacteriocin activity (AU/ml)
CFCS*	3,200
Ammonium sulphate precipitation (Crude bacteriocin – Control)	25,600
pH 2.0	6,400
pH 4.0	25,600
pH 6.0	25,600
pH 8.0	6,400
pH 10.0	3,200
60°C 15 min	400
30 min	200
60 min	100
100°C 15 min	0
30 min	0
60 min	0
Chloroform/methanol extraction	6,400

* CFCS was obtained from cultures developed at 37 °C in MRS medium supplemented with 2% NaCl and with an initial pH of 4.5

Figures

Figure 1

Antagonistic effect of the bacteriocin produced by *L. helveticus* 34.9 against *H. hunanensis* 5Hum

Figure 2

Tricine-SDS-PAGE of the bacteriocins isolated from *L. helveticus* 34.9 (a-c): the gel was overlaid with the indicator lawn, *L. delbrueckii* subsp. *bulgaricus* LMG 6901T. (a) CFCS; (b) crude bacteriocins; (c) bacteriocins extracted with chloroform/methanol (2/1, v/v); (d) – molecular weight markers stained with Coomassie Brilliant Blue (see Materials and Methods)

Figure 3

Decrease of the survival rate of *L. delbrueckii* subsp. *bulgaricus* LMG6901T (a) and *H. hunanensis* 5Hum (b) in the presence of variable concentrations of bacteriocins produced by *L. helveticus* 34.9

Figure 4

Growth of *L. delbrueckii* subsp. *bulgaricus* LMG6901T (a) and *H. hunanensis* 5Hum (b) in the presence of variable concentrations of bacteriocins produced by *L. helveticus* 34.9

Figure 5

Scanning electron microscopy of *L. delbrueckii* subsp. *bulgaricus* LMG6901T: control cells (a); morphological changes of the sensitive cells induced by bacteriocin treatment (b-d)

Figure 6

Scanning electron microscopy of *Halobacillus hunanensis* 5Hum: control cells (a); morphological changes of the sensitive cells induced by bacteriocin treatment (b-c)