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The partial characterization of the antibacterial peptide bacteriocin G₂ produced by the probiotic bacteria *Lactobacillus plantarum* G₂

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Abstract: The aim of this study was the partial characterization of the antimicrobial peptide bacteriocin G₂ produced by probiotic bacteria *Lactobacillus plantarum* G₂, which was isolated from a clinical sample of a healthy person. Antimicrobial substance was secreted in the supernatant of an *L. plantarum* G₂ culture, and showed a diverse spectrum of antimicrobial activity of all the tested strains of the genera *Lactobacillus* and the pathogenic bacteria *Staphylococcus aureus* and *Salmonella abony*. Isoelectric focusing revealed that bacteriocin G₂ is a cationic peptide (pI about 10) with a molecular mass of 2.2 kDa according to tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SDS-PAGE. The antimicrobial activity of bacteriocin G₂ was diminished by the proteolytic action of trypsin and proteinase K. Bacteriocin G₂ preserved its biological activity in the temperature range 40–60 °C (15 min), which was lost at 80 °C. Bacteriocin G₂ was stable in the pH range 2–9, while treatment with 1 % Tween 80 and 1 % urea resulted in increased antimicrobial activity. The probiotic strain *L. plantarum* G₂ produces the antimicrobial substance proteinaceous in nature with bacteriocin characteristics. Bacteriocin production is one of the key properties of probiotic bacteria with clinical potential as anti-infective agents, which will increase the likelihood of its *in vivo* efficacy.

Keywords: *Lactobacillus plantarum*; probiotic; bacteriocin.

INTRODUCTION

Bacteria employed in probiotic applications help to maintain or restore the natural microbial flora of a host. The ability of probiotic bacteria to successfully outcompete undesired species is often due to, or enhanced by, the production of

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potent antimicrobial toxins.¹ Some antimicrobial substances are non-specific, such as short-chain fatty acids or hydrogen peroxide, while others are specific with a very narrow killing range, such as bacteriocins, bacteriocin-like inhibitory substances (BLIS), and bacteriophages.^{2–4} Bacteriocins have been designated as bacterial substances with the capacity to inhibit, even in low concentrations, the multiplication of other taxonomically similar bacteria.⁵ They comprise a large and functionally diverse family of toxins found in all major lineages of Bacteria and Archaea, but there are certain features that unite them as a family; they are all ribosomally synthesized proteinaceous compounds and are active against bacteria closely related to the producing bacteria.⁶ In an attempt to organise this diverse family, bacteriocins are roughly classified into two main groups, the toxins produced by Gram-negative bacteria and those produced by Gram-positive bacteria.⁷

Bacteriocins from Gram-positive bacteria are generally cationic, amphiphilic, membrane-permeabilizing peptides, approximately 2–6 kDa in size.⁸ They are particularly attractive when the goal of probiotic application is to supplement, rather than dramatically alter, the natural bacterial flora of a host.¹ Bacteriocin nisin was found to be safe for human consumption by the Food and Drug Administration and has thus gained popularity in probiotic research.¹

Numerous strains of bacteriocin producing *Lactobacillus plantarum* have been isolated in the last two decades. Several of these plantaricins were characterized and their amino acid sequence determined.⁹

The aim of this work was to test bacteria with probiotic characteristics, *L. plantarum* G₂, for antimicrobial peptide production. The antimicrobial spectrum and some properties of the bacteriocin G₂ are described herein for the first time.

EXPERIMENTAL

Bacterial strains and growth media

The bacteriocin G₂ producing strain used in this study was isolated from a clinical sample of a healthy person, and according to its phenotypic and genotypic characteristics, it was classified as *L. plantarum* G₂. The strain was stored at –20 °C in MRS (de Man, Rogosa and Sharpe, Merck) medium containing 15 % glycerol. MRS broth (Merck) was used for cell propagation.

Indicator bacteria strains

The bacteria used as indicator strains were: *L. acidophilus* ATCC 314, *L. rhamnosus* ATCC 7469, *L. leishmany* ATCC 7830, *L. plantarum* G₁, *L. casei* G₃, *Enterococcus faecalis* ATCC 29219, *Staphylococcus aureus* ATCC 6538–P, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Klebsiella* sp. ATCC 10031, *Bacillus subtilis* ATCC 6633, *B. cereus* ATCC 11178, *Micrococcus luteus* ATCC 93419, *Salmonella abony* ATCC 6017.

L. leishmany ATCC 7830 was used as the indicator bacterium for the estimation of antimicrobial activity.

Production of bacteriocin G₂

For bacteriocin production, *L. plantarum* G₂ cells were grown anaerobically (in 1000 ml Erlenmeyer flasks) in MRS broth (pH 6.4), at 37 °C and 58 rpm for 24 h, in rotary shaker incubator (Adolf Kühner, Switzerland). The culture was centrifuged at 4000 × g, for 20 min at 4 °C. The pH of the supernatant was adjusted to 6.5–7.0 with 1.0 M NaOH, to exclude the antimicrobial effect of organic acids, followed by filtration of the supernatant through a 0.2 µm pore-size cellulose acetate filter (Sigma–Aldrich). The supernatant S was used for screening the antimicrobial activity.

Detection of antimicrobial activity

The antimicrobial activity of the bacteriocin produced by *L. plantarum* G₂ was screened using the agar well diffusion (AWD) assay.¹⁰ Pre-poured MRS agar plates were overlain with 0.1 mL suspension of the indicator strain (containing 10⁶ CFU mL⁻¹). Wells of 6 mm in diameter were cut into the agar plate using a cork borer and 100 µL of the supernatant S was placed into each well. After 18 h of incubation at 37 °C, the zone of inhibition (diameter) was measured.

Bacteriocin assay

The critical dilution assay described by Mayer-Hartings *et al.*¹¹ was used to quantify the inhibitory activity of the bacteriocin against the respective sensitive indicator strain *L. leishmany*. A serial two-fold dilution of the supernatant was made in 0.1 M Tris-HCl buffer, pH 7. The activity of each dilution was determined by the AWD assay. The antimicrobial activity is expressed in arbitrary units (AU mL⁻¹). One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

Sensitivity to enzyme activity of bacteriocin G₂

To investigate the sensitivity of the bacteriocin to different enzymes (all obtained from Sigma), neutralized supernatant S samples of the tested strain were mixed with enzyme solutions of catalase, pronase E, proteinase K, trypsin, chymotrypsin, lipase, lysozyme and α-amylase (final enzyme concentration was 1 mg mL⁻¹), incubated for 1 h at the optimum temperature for each of the enzymes and the residual activities were measured using the AWD assay. Two controls, one with sterile MRS and the respective enzyme (1 mg mL⁻¹), and the other one with untreated bacteriocin were included.

Heat resistance and pH stability of bacteriocin G₂

To determine the heat stability of bacteriocin G₂, aliquots of the neutralized cell-free supernatant of tested strain were heated at 40, 60 and 80 °C for 15, 30, 60 and 90 min, and immediately cooled in an ice water bath. The heat resistance was also checked after autoclaving the bacteriocin at 121 °C for 15 min. The residual bacteriocin activity was determined by the AWD assay.

To test the stability at different pH, aliquots of neutralized supernatant were adjusted to pH values from 2–12 using 4 M HCl and 4 M NaOH, respectively and subsequently incubated for 1 h at 37 °C. The residual activities were measured after neutralizing the aliquots to pH 7.

Sensitivity of bacteriocin G₂ to surfactants

To examine sensitivity of bacteriocin G₂ to surfactants, SDS, Tween 80, Triton X-100 and urea were used. Neutralized cell-free supernatant samples of tested strain were mixed with surfactants at a final concentration of 1 % (w/v) and incubated for 2 h at 37 °C. Residual

bacteriocin activities were measured using the AWD assay. The surfactants at a concentration of 1 % in neutralized MRS broth were used as controls.

Preparation of crude supernatant

Crude supernatant was prepared from 500 mL of an *L. plantarum* G₂ culture. The cells were grown to the stationary phase in MRS broth at 37 °C. The cell-free supernatant of the culture was collected by centrifugation at 4000 × *g* for 20 min at 4 °C. To concentrate the produced bacteriocin G₂ 10-fold, the supernatant was filter sterilized (0.22 µm), and ultra-filtrated on 5 kDa cut-off membrane (Millipore). The obtained solution, designated crude supernatant fluid, was used for bacteriocin characterization by electrophoretic methods, *i.e.*, sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SDS-PAGE, and isoelectric focusing.

Reducing and non-reducing tricine SDS PAGE

The molecular mass of bacteriocin G₂ was estimated in a tricine–SDS-PAGE, as described by Schagger and Von Jagow.¹² To test for the presence of interchain disulphide bonds between the bacteriocin subunits or intrachain disulphide bonds essential for its activity, reducing and non-reducing sample buffers for the tricine–SDS-PAGE were used.

Electrophoresis was performed in vertical gels (16.5 % acrylamide) at 30 V for 1 h, and 90 V for 5 h. To determine the apparent molecular mass of the bacteriocin after tricine–SDS-PAGE, the gel was cut into two slices. One half was fixed and stained with Coomassie Brilliant Blue. The other slice was assayed for antimicrobial activity according to Bhunia *et al.* (1987), with a slight modification.¹³ The gel prepared for growth inhibition was fixed in 20 % 2-propanol and 10 % acetic acid for 5 min and washed in deionised water for 24 h. Afterwards, the gel was placed on a MRS prepared agar plate and overlaid with 20 mL of soft MRS agar seeded with *L. leishmany* (10⁶ CFU mL⁻¹). After incubation of the plate for 24 h at 37 °C, the location of the zone of growth inhibition was identified and photographed. For molecular mass determination, Amersham Low-Range Rainbow Molecular Weight Markers (3500–40000 Da) were used. The protein standards molecular masses (Da) and colours were as follows: 38000, blue; 31000, orange; 24000, green; 17000, blue; 12000, red; 8500, yellow and 3500, blue.

Isoelectric focusing

Isoelectric focusing, IEF, was performed at 10 °C in a Multiphor II electrophoresis unit according to the manufacturer's instructions (Pharmacia). The isoelectric point was determined by using a broad *pI* calibration kit (*pI* 3.5–10, Pharmacia). After IEF, the gel was washed with distilled water for 30 s and cut into two slices. One slice was fixed and stained with Coomassie Brilliant Blue and the other one was assayed for antimicrobial activity by the agar overlay method described above.

RESULTS AND DISCUSSION

Spectrum of antimicrobial activity of bacteriocin G₂

To examine the effect of the bacteriocin from *L. plantarum* G₂ on other microorganisms, neutralized cell-free supernatant was tested for antimicrobial activity against Gram-positive and Gram-negative bacteria by the well diffusion agar test. As shown in Table I, the bacteriocin was active against all the tested strains of the genera *Lactobacillus*: *L. acidophilus*, *L. rhamnosus*, *L. leishmany*, *L. plan-*

tarum G₁ and *L. casei* G₃. The bacteriocin G₂ also inhibited the growth of the pathogenic bacteria *S. aureus* and *S. abony*. Bacteriocin G₂ displayed a relatively broad spectrum of antimicrobial activity as it inhibited 7 of the 14 indicator strains.

TABLE I. Inhibitory activity of neutralized cell-free supernatant of *L. plantarum* G₂ against various indicator strains, which is expressed as the diameter of the zone of inhibition

Indicator organism	Antimicrobial activity of bacteriocin G ₂ ^a
<i>L. acidophilus</i> ATCC 314	+
<i>L. rhamnosus</i> ATCC 7469	+
<i>L. leishmany</i> ATCC 7830	++
<i>L. plantarum</i> G ₁	+
<i>L. casei</i> G ₃	+
<i>E. faecalis</i> ATCC 29219	-
<i>S. aureus</i> ATCC 6538-P	+
<i>P. aeruginosa</i> ATCC 9027	-
<i>E. coli</i> ATCC 8739	-
<i>Klebsiella</i> sp. ATCC 10031	-
<i>B. subtilis</i> ATCC 6633	-
<i>B. cereus</i> ATCC 11178	-
<i>M. luteus</i> ATCC 93419	-
<i>S. abony</i> ATCC 6017	+

^a -: No inhibition zone; +: 5 mm < zone < 10 mm; ++: 10 mm < zone < 15 mm

The inhibitory spectrum of bacteriocins produced by different species of lactobacilli varies greatly.¹⁴ Most bacteriocins inhibit only lactobacilli or closely related Gram-positive bacteria, whereas others are active against a broad spectrum of Gram-positive and Gram-negative bacteria.¹⁵⁻¹⁹

Effect of enzymes, heat and pH on the antimicrobial activity of bacteriocin G₂

Bacteriocin G₂ was tested for its sensitivity to various enzymes. The antibacterial activity was retained after catalase treatment, indicating that antibacterial activity was not due to H₂O₂. As shown in Table II, trypsin and proteinase K were the only proteases that partially or completely inhibited the antimicrobial activity. The fact that the bacteriocin was inactivated by trypsin and proteinase K indicates its proteinaceous nature, however, despite this, the substance was rather persistent to proteolytic cleavage of pronase E and chymotrypsin. Most bacteriocins are resistant to all proteolytic enzymes, but a few of them, including nisin, plantaricin C, plantaricin D, are sensitive to the actions of some proteases.²⁰⁻²⁴ The antimicrobial activity of bacteriocin G₂ was not affected by lipase, lysozyme or α -amylase, suggesting that its biological activity was not dependent on the presence of a lipid or sugar moiety.

Incubation of the neutralized supernatant containing bacteriocin G₂ at different temperatures for 15, 30 and 90 min showed that it was completely stable up

to 40 °C (Table III). No loss of activity was detected after 15 min at 60 °C, but inactivation occurred when the incubation was continued for a longer period. A total loss of activity was observed after incubation at 80 °C. Heat-stability is a major feature of low-molecular-weight bacteriocins; however, some bacteriocins produced by *Lactobacillus* strains were inactivated by 10- to 15-min treatment at 60–100 °C.^{25,26}

TABLE II. Antimicrobial activity of bacteriocin G₂ detected after enzyme treatment, expressed as residual bacteriocin activity

Surfactant	Residual bacteriocin activity ^a , %
Catalase	100
Proteinase K	40
Pronase E	100
Chymotrypsin	100
Trypsin	0
Lysozyme	100
Lipase	100
α-Amylase	100

^aThe percentage of the initial activity

The activity of bacteriocin G₂ was stable throughout the pH range 3–7 (Fig. 1). Above pH 7, the activity decreased and was completely lost at pH 10. Bacteriocins differ greatly with respect to their sensitivity to pH. Many are considerably more tolerant to acid than to alkaline pH values.²⁷ As most bacteriocins and bacteriocin-like substances, bacteriocin G₂ was also stable in acidic and neutral pH values, indicating that the substances are well adapted to the environment of the bacteria that produce them.²⁸

TABLE III. Antimicrobial activity of bacteriocin G₂ detected after thermal treatment, expressed as residual bacteriocin activity

Treatment	Residual bacteriocin activity, %
40 °C, 15 min	100
40 °C, 30 min	100
40 °C, 60 min	100
40 °C, 90 min	100
60 °C, 15 min	100
60 °C, 30 min	0
60 °C, 60 min	0
60 °C, 90 min	0
80 °C, 15 min	0
80 °C, 30 min	0
80 °C, 60min	0
80 °C, 90 min	0
Autoclaving 121 °C, 15 min	0

To examine the hydrophobic nature of bacteriocin G₂, the neutralized supernatant was treated with surfactants at a final concentration of 1 % (w/v). The sensitivity to the surfactants is given in Table IV. Treatment with Tween 80 and urea resulted in increased antimicrobial activity. SDS and Triton X-100 had no effect on the bacteriocin activity. Many bacteriocins contain hydrophobic domains and tend to form large aggregates. The large macromolecules can be disaggregated by the use of surface-active compounds. Desegregation can result in a significant increase in bacteriocin activity.²⁹

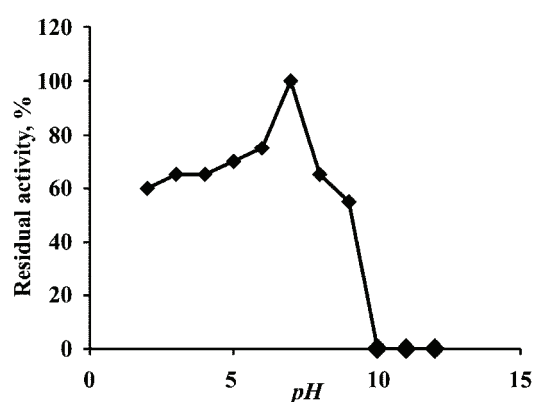


Fig. 1 Effect of pH treatment on the activity of bacteriocin G₂.

TABLE IV. Effect of surfactants on bacteriocin G₂ activity, expressed as residual bacteriocin activity

Surfactant	Residual bacteriocin activity, %	
	Treated supernatant	Surfactant control
None (control)	100	–
SDS	167	150
Tween 80	133	0
Triton X-100	117	108
Urea	117	0

Molecular mass determination

Electrophoretic analysis of the concentrated supernatant of *L. plantarum* G₂ prepared using a sample buffer containing the reducing agent 2-mercaptoethanol showed a wide protein band occupying most of the gel. The gel slice overlaid with the indicator strain revealed a single clear inhibition band, corresponding to a molecular mass of 2.2 kDa. Similar molecular masses have been reported for a few other bacteriocins, including plantaricin A.⁹ A tendency of bacteriocins produced by other lactic acid bacteria to aggregate was reported, which might have contributed to the reason why the bacteriocins could not pass through a 5 kDa cut-off membrane.^{30,31} Bacteriocin G₂ retained antimicrobial activity after treatment with reducing agents indicating that it does not have intramolecular disul-

phide bonds that are essential for its activity. Bacteriocin G₂ resolved in non-reducing tricine–SDS-PAGE, and subsequently overlaid with indicator strain showed a single clear band with the same molecular mass as found with the reducing gel.

pI determination

To determine the isoelectric point of bacteriocin G₂, isoelectric focusing was performed. A single clear inhibition band was detected on the part of the gel overlaid with the indicator strain, corresponding to a *pI* value of about 10. Bacteriocin G₂ is a cationic protein, as are most bacteriocins.¹⁰ The high isoelectric point allows bacteriocins to interact at physiological pH with the anionic surface of bacterial membranes.

CONCLUSIONS

Based on the presented results, it may be concluded that the antimicrobial substance from the culture supernatant of *L. plantarum* G₂ is a peptide with a diverse spectrum of antimicrobial activity. Stability in acidic and neutral pH range (up to pH 9), resistance to proteolytic cleavage but sensitivity to trypsin and heat stability at physiological temperatures allow bacteriocin G₂ to be a competitive advantage of *L. plantarum* G₂ probiotic preparations.

ИЗВОД

ПАРЦИЈАЛНА КАРАКТЕРИЗАЦИЈА АНТИБАКТЕРИЈСКОГ ПЕПТИДА КОЈИ ПРОИЗВОДИ ПРОБИОТСКА БАКТЕРИЈА *Lactobacillus plantarum* G₂

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Циљ овог истраживања је била парцијална карактеризација антимикурног пептида из пробиотске бактерија *Lactobacillus plantarum* G₂ изоловане из клиничког узорка здраве особе. Антимикурно једињење из *L. plantarum* G₂, означено као бактериоцин G₂, добијено из супернатанта бактеријске културе, показало је широк спектар антимикурне активности, инхибирајући раст свих испитиванх врста рода *Lactobacillus*, као и патогених бактерија *Staphylococcus aureus* и *Salmonella abony*. Бактериоцин G₂ је осетљив на протеолитичко дејство трипсина и протеиназе К. Антимикурна активност је стабилна у опсегу 40–60 °С (15 мин), али се губи на температури од 80 °С. Установљено је да је бактериоцин стабилан на рН вредностима између 2 и 9. Дејство Tween-а 80 и урее је довело до повећане инхибиторне активности. Према IEF, бактериоцин G₂ је катјонски протеин, са *pI* вредношћу око 10, а молекуласка маса одређена на основу трицин–SDS-PAGE је 2,2 kDa. Пробиотски сој *L. plantarum* G₂ продукује антимикурно једињење протеинске структуре са карактеристикама бактериоцина. Синтеза бактериоцина је једна од кључних особина пробиотских бактерија које имају клинички потенцијал као антиинфективни агенси, јер значајно повећава вероватноћу њихове *in vivo* ефикасности.

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