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Physical and biochemical characterisation of bacteriocins produced by three *Lactobacillus* species

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Abstract

The bacteriocins produced by Lactic acid bacteria (LAB) species (*L. plantarum* L2, *L. paraplantarum* L5 and *L. pentosus* L11) were characterized with respect to their sensitivity to heat, pH, cations, surfactants, inhibitors and papain. The activity of bacteriocin produced by *L. plantarum* L2 and *L. pentosus* L11 was stable at 100°C for 20 minutes while 87.5% of the activity of *L. paraplantarum* L5 bacteriocin was retained. The bacteriocin activity of *L. pentosus* L11 and *L. plantarum* L2 was unaltered after 15 minutes autoclaving while there was a reduction in that of *L. paraplantarum* L5 (6.25%). The bacteriocin activity of the LAB strains at pH 3 to pH 5 was higher than the values of the control and pH 3 supported the highest activity in all the isolates. Tween 80 boosted all bacteriocin activity while 5 mM-10 mM of benzoic acid augmented bacteriocin activity of *L. plantarum* L2. The activity of *L. pentosus* L11 bacteriocin was unaffected at all concentrations of ammonium ion (NH_4^+) used. At 5 mM concentrations of Ca^{2+}, the activity of the three *Lactobacillus* bacteriocins were slightly increased (10%). Crude papain and three fold aqueous dilutions of crude papain completely inhibited bacteriocin activity. The properties exhibited by these bacteriocins are an indication that they and their producing strains may find applications in industrial processes.

Keywords: Lactic acid bacteria; bacteriocin activity; antimicrobial compound characterisation.

Introduction

Among many gram positive bacteria, genera belonging to the lactic acid bacteria (LAB) produce a diversity of antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bactericidal proteins (commonly referred to as bacteriocins) during their growth and fermentation processes [1]. Bacteriocin synthesis is ribosome-directed with activity against closely or distantly-related strains [2, 3]. These potential functions as well as other technical ones make bacteriocins interesting in many industries (especially, food) and research fields [4] since LAB is credited with a 'GRAS' (generally recognized as safe) status and is a component of almost all human diets [5]. While a variety of microorganisms exist in raw milk, only LAB produce lactic acid from milk sugars through fermentation. The presence of LAB in dairy products causes aromatic and physicochemical transformations [6] and produce bacteriocins capable of inhibiting spoilage and pathogenic microorganisms [7]. In order to ascertain safety and control food pathogens, [8], bacteriocins are gaining important industrial applications especially as starter cultures [9], therefore, the properties of three bacteriocins produced from *Lactobacillus* species isolated from milk were investigated in this work. The effects of pH, temperature increases, surfactants, inducers and cations on the activities of the bacteriocins produced by these bacteria were studied.

Materials and methods

Microorganisms and bacteriocin production

Bacteriocin-producing *Lactobacillus plantarum* L2, *Lactobacillus paraplantarum* L5 and *Lactobacillus pentosus* L11 with antibacterial activity as previously reported [10] with bacteriocin activity units of 7670, 5330 and 6330 AU/ml respectively were obtained from the culture collection of our previous work in the

Department of Microbiology and used. Bacteriocin was produced as cell-free supernatant broths from 3 day old LAB cultures cultivated in modified MRS broth at 25°C, treated with 5mg/ml catalase and adjusted to pH 7.0 [10].

Bacteriocin characterization

The bacteriocins were characterised with respect to their sensitivity to pH, heat, cations, surfactants, inhibitors and enzyme denaturation. In each case, 30 µl of bacteriocin was put in 5 mm agar wells bored in MRS agar plates seeded with the indicator *L. brevis* strain. Triplicate experiments were set up, bacteriocin activity was scored as a percentage of the average of recorded zones of inhibition of test bacteriocin divided by that of control (the activity of the untreated bacteriocin).

Effect of pH on bacteriocin activity: The sensitivity of the bacteriocin samples to various pH values (pH 3 to 9) was carried out by a modification of the method of Guatam and Sharma [11]. Equal volumes of the bacteriocin samples and 10 mM phosphate buffer (adjusted with 0.01 M NaOH where necessary) were incubated for 1 hour and then assayed for bacteriocin activity.

Effect of temperature on bacteriocin activity: Bacteriocin samples were incubated at 100ºC over 5, 10, 15 and 20 minutes and at 121ºC for 15 minutes respectively. The residual bacteriocin activity was used to determine bacteriocin responses to temperature treatments [12].

Effect of surfactants on bacteriocin activity: Equal volumes of the bacteriocin were mixed with 0.5%, 1.0%, 1.5% and 2.0% concentrations of Tween 80, Triton X-100 and sodium dodecyl sulfate (SDS) using a modification of the method of Ogunbanwo *et al* [13] for 1hour and after which assaying for bacteriocin activity was carried out.

Effect of inducers/inhibitors on bacteriocin activity: The responses of bacteriocins to 5 mM, 10 mM, 15 mM and 20 mM concentrations of urea, ethylene diaminetetraacetic acid (EDTA) and benzoic acid was investigated by incubating a mixture of equal volumes of the compounds and bacteriocin samples together for 1 hr and then assayed for bacteriocin activity [14]. Effect of cationson bacteriocin activity: Varying molarities (5 mM-20 mM) of chloride salts of Ca^{2+}, Mg^{2+}, Na^{+} and NH_4^{+} were incubated with equal volumes of the bacteriocin samples for one hour after which the bacteriocin activity was determined [15].

Proteolytic activity of papain on bacteriocin activity: The sensitivity of the bacteriocin samples to enzyme using crude papain was done by the modification of the method of Kormin *et al* [16]. A mature but unripe pawpaw was given a 5-10 mm deep incision to allow for release of sap. Equal volume of this sap was used in its concentrated (10^0) and aqueous diluted forms (10^{-1} to 10^{-3}), mixed with bacteriocin samples and incubated for 1 hour. The residual bacteriocin activity was then determined.

Bacteriocin activity: This was scored as percentage inhibition of indicator organism (*Lactobacillus brevis*) from a 5 mmagar-well diffusion assay in which the bacteriocin was used against the seeded susceptible LAB strain to determine bacteriocin activity [16]. The activity was determined by calculating the percentage ratio of the diameter of the inhibition zone of each test against that of the control [10].

Statistical analysis

All values used were the average of triplicate readings of each experiment.

Results and discussion

The bacteriocin produced by the three LAB species (*Lactobacillus plantarum* L2, *L. paraplantarum* L5 and *L. pentosus* L11), was active at all pH ranges (3-8), but highest between pH 3-5 (Figure 1a). The lower the pH, the higher the percentage zone of inhibition recorded. The highest activity (177%) was recorded from *L. paraplantarum* L5 bacteriocin at pH 3 and followed closely at the same pH by activity of *L. plantarum* L2 bacteriocin which recorded an activity increase equivalent to 71.43% above the control value. At pH 8, 15%, 16% and 25% reduction respectively, was recorded in *L. plantarum* L2, *L. paraplantarum* L5 and *L. pentosus* L11. Ogunbanwo *et al* [13], Gautam and Sharma [11] and Aslam *et al* [14], reported an acidic range (between pH 2 and 6) for maximum bacteriocin activity. Lee *et al* [17] also reported a plantaricin C7 stable between pH 2 and 8. Joshi *et al* [12] reported a bacteriocin by *Lactobacillus* isolate CA44 with >61% activity over a wide pH range of 3-8 against a *Staphylococcus aureus* strain. Another bacteriocin, produced by *Lactococcuslactis* D53, recorded its highest activity at a pH range between 3-5 [8].

At 100°C the bacteriocin activity of *L. plantarum* L2 and *L. pentosus* L11 was higher than the control between 5 to 15 minutes (7%-25% increase) and at 20 minutes the activity was the same value as the control (Figure 1b). After autoclaving at 121°C for 15 minutes the bacteriocin activity of *L. plantarum* L2 remained unchanged (100%) while the bacteriocin activity of *L. paraplantarum* L5 was reduced by 6.25%. The activity of the bacteriocin from *L. pentosus* L11 was however increased by 23.08%. Sarika *et al* [18] reported that the bacteriocin GP1 produced by

Lactobacillus rhamnosus had a remarkable stability after heat treatment at 121°C for 20 minutes. Sifour *et al* [19] also reported that the activity of bacteriocin produced by *L. plantarum* F12 remained constant after heating at 100°C for 30 minutes followed by subsequent decline after 60 minutes. In contrast, Todorov *et al* [20], reported that lacticin NK24 produced by *Lactococcus lactis* NK24, lost 87.5% of its activity after 30 min at 100°C and was completely inactivated after 15min at 121°C. Guatam and Sharma [11] also reported that bacteriocin activity was retained after heat treatment at 100°C for 20 minutes and 121°C for 10 minutes. Joshi *et al* [12] however, reported a bacteriocin which possessed only 28% of activity after 20 minutes at 100°C and completely lost its activity after incubation for 15 minutes at 121°C.

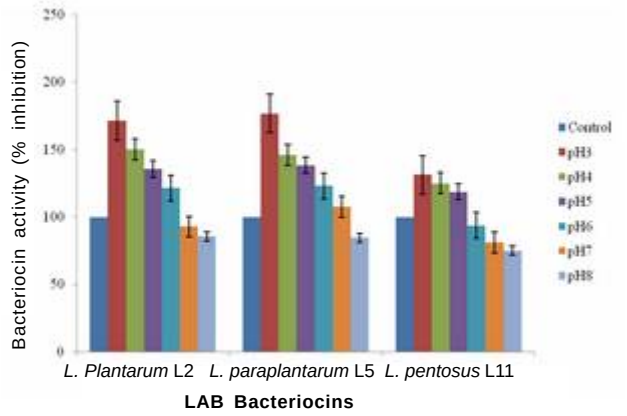

Figure 1a. Effect of pH on bacteriocin activity produced by *L. plantarum* L2, *L. paraplantarum* L5 and *L. pentosus* L11.

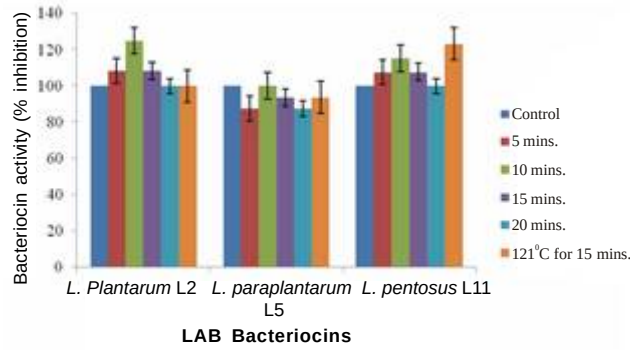

Figure 1b. Effect of temperature increase with time (100°C at 5-20 minutes and autoclaving) on bacteriocin activity of the LAB isolates.

Bacteriocin activity at different concentrations of benzoic acid, EDTA and Urea (Figure 2a, 2b, 2c) ranged from 86% to 120%; 87% to 107% and 83% to 114% respectively. Lower concentrations of Benzoic acid at 5 mM and 10 mM enhanced the bacteriocin activity of *L. plantarum* L2 (20% and 6.7%, respectively). Bacteriocin activity of *L. paraplantarum* L5 was however increased with 15 mM and 20 mM benzoic acid (7% and 14% respectively). 5 mM concentration of EDTA reduced bacteriocin activity but higher concentrations increased the activity of the bacteriocin of *L. plantarum* L2. The addition of EDTA reduced the activity of bacteriocin of *L. paraplantarum* L5 and *L. pentosus* L11 at all concentration used. All concentrations of urea reduced bacteriocin activity of *L. plantarum* L2 while it had no effect on bacteriocin activity of *L. paraplantarum* L5 at all concentrations. The activity of the bacteriocin of *L. pentosus* L11 was increased with 5 and 10 mM urea additions but was reduced gradually as the concentration of urea was increased (15 and 20 mM). Kaktcham *et al* [15] observed that while EDTA enhanced the activity of *L. rhamnosus* 1K bacteriocin, additions of urea had no effect on its bacteriocin activity. Ali and Musleh, [21] reported a plantaricin whose activity was not affected by treatments with urea and EDTA.

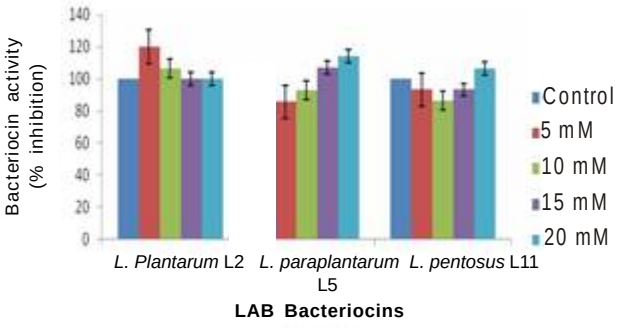

Figure 2a. Effect of increasing concentrations of benzoic acid on bacteriocin activity.

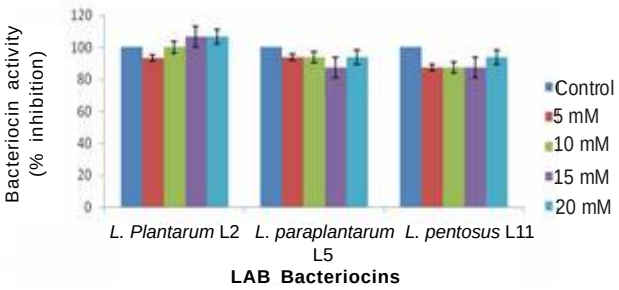

Figure 2b. Effect of increasing concentrations of EDTA on bacteriocin activity.

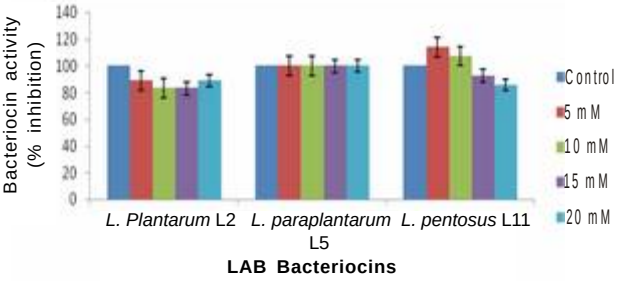

Figure 2c. Effect of increasing concentrations of Urea on bacteriocin activity.

In Figure 3a, additions of Triton X-100 to LAB bacteriocins slightly reduced activity of *L. pentosus* L11 at all concentrations resulting in a least activity of 86% at 1.5% Triton-X additions. Triton X-100 however, had no effect on the activity of the bacteriocin produced by *L. paraplantarum* L5 at all concentrations used. Increasing concentration of Tween 80 boosted the activity of all the bacteriocins (Figure 3b), showing the least effect on the activity of the bacteriocin from *L. pentosus* L11. In Figure 3c, SDS totally destroyed the activity of bacteriocin from *L. pentosus* L11. The highest activity (15% increase) after SDS additions were recorded at 5 mM and 15 mM in *L. paraplantarum* L5 and *L. plantarum* L2, respectively. These findings were similar to that of Ali and Musleh [21].

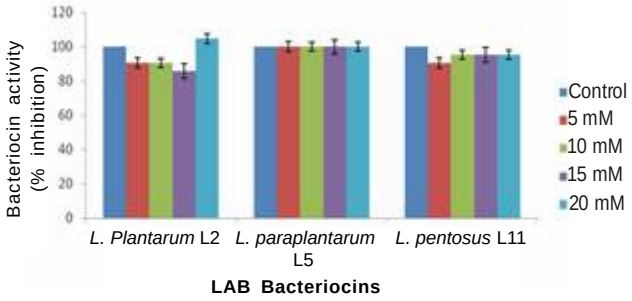

Figure 3a. Effect of increasing concentrations of Triton X-100 on bacteriocin activity.

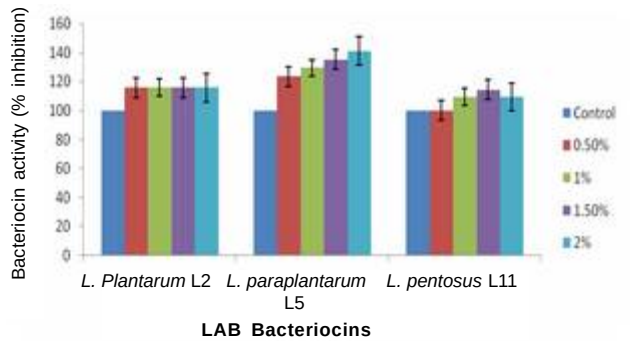

Figure 3b. Effect of increasing concentrations of Tween 80 on bacteriocin activity.

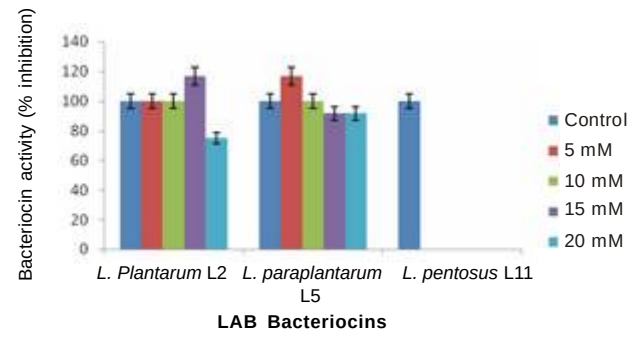

Figure 3c. Effect of increasing concentrations of SDS on bacteriocin activity.

Additions of lower concentrations of Na^+ slightly reduced the activity of *L. plantarum* L2 bacteriocin but at 20 mM it resulted in a 5% increase (Figure 4a). At 5-15 mM additions of Na^+, the activity of *L. pentosus* L11 bacteriocin was unaffected, however, it reduced at 20 mM (90%). Katcham *et al* [15] reported a *L. rhamnosus* 1K bacteriocin which was not sensitive to Na^+. 10 mM and 15 mM additions of NH_4^+ resulted in a 5% and 11% increase in the activities of *L. paraplantarum* L5 and *L. plantarum* L2 respectively (Figure 4b). While 20 mM NH_4^+ additions resulted in an overall activity of 94% in *L. plantarum* L2 bacteriocin, all other additions of NH_4^+ did not alter bacteriocin activities.

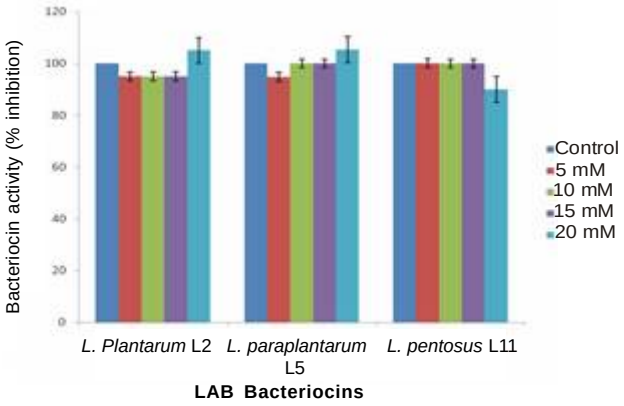

Figure 4a. Effect of increasing concentrations of Naz on bacteriocin activity.

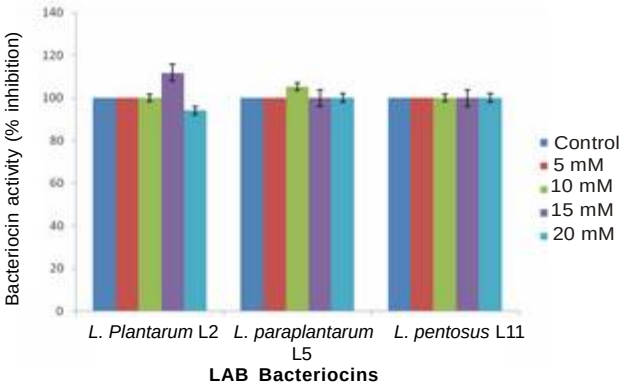

Figure 4b. Effect of increasing concentrations of NH„ z on bacteriocin activity.

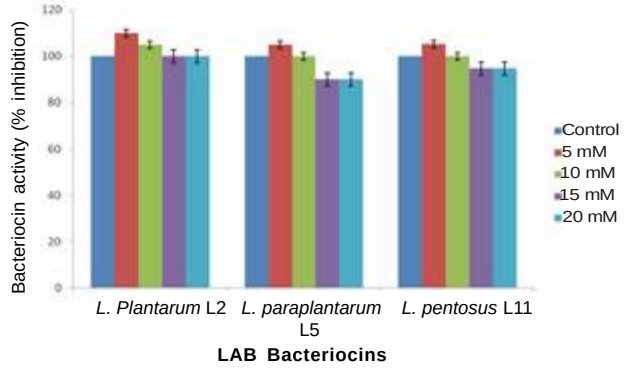

Figure 4c. Effect of increasing concentrations of Ca²z on bacteriocin activity.

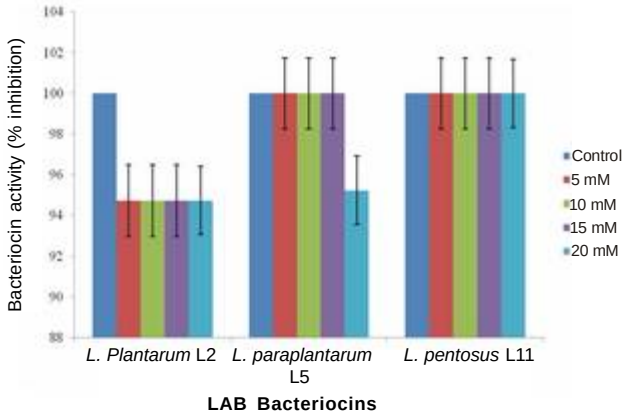

Figure 4d. Effect of increasing concentrations of $Mg^{2}z$ on bacteriocin activity.

At lower concentrations of Ca^{2+} the activity of the bacteriocin from *L. plantarum* L2 and *L. pentosus* L11 respectively was boosted, yielding a maximum activity of 110% and 105% at 5 mM (Figure 4c). Although 5 mM concentration of Ca^{2+} increased the bacteriocin activity of *L. paraplantarum* L5, the activity was unchanged at 10 mM and reduced at 15 and 20 mM concentrations. The activity of *L. pentosus* L11 bacteriocin was increased at 5 mM, unaffected at 10 mM concentration and reduced to 94% at 15 mM and 20 mM concentrations. In Figure 4d, increasing concentration of Mg^{2+} had no effect on the bacteriocin activity of *L. pentosus* L11 but reduced that of *L. plantarum* L2 to 95%. Mg^{2+} at 5 mM, 10 mM and 15 mM concentration had no effect on bacteriocin activity of *L. paraplantarum* L5 but at 20 mM bacteriocin activity was reduced to 95%. Shayesteh *et al* [22] also reported a bacteriocin from a *Bacillus* strain which was totally unaffected by the addition of different heavy metals.

Crude papain (a natural proteolytic extract from unripe pawpaw) completely inhibited bacteriocin activity. Papaya latex proteases are known to be composed of four cysteine proteases [23]. These proteolytic enzymes in their crude form were found to totally destroy bacteriocin activity even at 10^{-3} dilution, confirming the LAB bacteriocins to be protein in origin. Kormin *et al* [16] reported complete inactivation of the bacteriocin produced by *L. plantarum* BS2 upon papain additions. Joshi *et al.*[12] also reported a total inactivity of the bacteriocin of a *Lactobacillus* isolate CA44 with papain.

Conclusions

The activities of the bacteriocins produced by the lactic acid bacteria possessed a wide range of pH tolerance, considerable stability to heat (even at high autoclaving temperatures), surfactants, inhibitors/inducers and metal ions. The characteristics displayed by these bacteriocins make them potential candidates in industrial processes especially food processes involving low pH or extreme temperatures.

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