

Bioprotective Potential of Bacteriocinogenic *Enterococcus gallinarum* Strains Isolated from Some Nigerian Fermented Foods, and of Their Bacteriocins

IYABO C. OLADIPO^{1,2*}, ABIODUN I. SANNI³, WRITACHIT CHAKRABORTY⁴, SOMNATH CHAKRAVORTY⁴, SAYANTAN JANA¹, DEEP S. RUDRA¹, RATAN GACCHUI⁴ and SNEHASIKTA SWARNAKAR¹

¹Department of Physiology, Drug Development Diagnostics and Biotechnology Division,
Indian Institute of Chemical Biology, Jadavpur University, Kolkata, India

²Department of Science Laboratory Technology, Ladoke Akintola University of Technology,
Ogbomoso, Oyo State, Nigeria

³Department of Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria

⁴Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India

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Abstract

Enterococcus gallinarum strains isolated from some Nigerian fermented foods were found to produce bacteriocins. The bacteriocins had a broad spectrum of activity against both Gram-positive and negative bacteria. The effects of the bacteriocins and bacteriocinogenic organisms on *Staphylococcus aureus* infections in rats were evaluated. *Sprague-Dawley* rats were infected with *S. aureus* MTCC 737 and treated with *E. gallinarum* T71 and different concentrations of the bacteriocins from *E. gallinarum* W211 and T71. *Staphylococcus aureus* infection caused significant upregulation of aspartate aminotransferase and alanine aminotransferase levels in sera of the infected rats. Moreover, gelatin zymography revealed that infected gastric tissues showed elevated matrix metalloproteinase-9 activity. Bacteriocin treatments reduced the MMP-9 activity and inhibited the expressions of both Tumour Necrosis Factor Alpha (TNF- α) and Interleukin-1 Beta (IL-1 β) dose dependently, pointing to a potential role of the bacteriocins in attenuating inflammatory responses to *Staphylococcus aureus* infection. Gastric and GIT damage caused by staphylococcal infection were reduced in the *Enterococcus gallinarum* T71 and bacteriocin-treated groups also dose dependently. We conclude that these bacteriocins may have useful biomedical applications.

Key words: *Staphylococcus aureus*, inflammation, Interleukin-1 Beta and matrix metalloproteinase-9

Introduction

Enterococci are components of the normal flora of man and animals. They are usually thought to be harmless commensals and preparations containing *Enterococci* (especially *E. faecium*) are sold in health food stores in Europe and elsewhere as “probiotics” (NFID, 1998). Most strains of *E. faecium* appear to be beneficial to the health; they can be found naturally in raw materials and are used as starter cultures for fermented foods. Other strains are feared as opportunistic pathogens, causing serious illnesses in hospitals all over the world (Aakra *et al.*, 2007). Furthermore, the ability of *Enterococci* to produce antimicrobial peptides, known as bacteriocins (enterocins), which can be used as food biopreservatives is also remarkable. Bacteriocins are microbially produced membrane-active peptides with antimicrobial activities usually against closely related strains (Klaenhammer, 1993). Bacteriocin production

has been reported in both Gram-positive and negative bacteria (Jack *et al.*, 1995). Certain bacteriocins produced by lactic acid bacteria inhibit a variety of food-borne pathogens including *Bacillus cereus*, *Clostridium perfringens*, *Listeria* species and *S. aureus* (Jeevanram *et al.*, 2008). Most bacteriocin-producing lactic acid bacteria have been isolated from foods, and very few bacteriocin producers have been isolated from humans (Flynn *et al.*, 2002). It has been proposed that bacteriocins might be used not only in food preservation, but also selective antimicrobials to inhibit pathogens without affecting the normal flora (Gillor *et al.*, 2008). Other proposed biomedical applications include treatment of mastitis infections in cows (Sears *et al.*, 1995), and gingivitis and gastrointestinal infections such as peptic ulcers caused by *Helicobacter pylori* (Blackburn and Projan, 1994).

There is evidence that nisin modulates the immune system of mice when included in the diet (De Pablo

* Corresponding author: I.C. Oladipo, Science Laboratory Technology Department, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria; e-mail: xtiocoker@gmail.com

et al. 1999) and the immune system of turbot after intra-peritoneal injection (Villamil *et al.*, 2002). Enterocins are of importance due to their potential applications in food preservation since one of the distinctive characteristics of some enterocins and class IIa pediocin-like bacteriocins in particular, is strong listericidal effects (Cleverland *et al.*, 2001).

Although, the effectiveness of enterocins against food spoilage and pathogenic bacteria in various food systems is well established (Aymerich *et al.*, 2000), little information is available on their possible antibacterial and anti-inflammatory activities. Therefore, the objective of this study was to determine the effect of bacteriocins produced by *Enterococcus gallinarum* strains in controlling *Staphylococcus aureus* infections *in vivo*.

Experimental

Materials and Methods

Chemicals. Slanetz and Bartley medium and Brain Heart Infusion agar were obtained from Oxoid chemicals, Canada. Gelatin from porcine skin, trichloroacetic acid (TCA), tritonX-100 (TX) and EDTA-free protease inhibitor cocktail were obtained from Sigma (St. Louis, USA). MMP-9 and MMP-2 as standard were purchased from Chemicon (Hampshire, UK) and anti-TNF α , anti-IL1 β , anti- β -tubulin antibodies from Santa Cruz Biotechnology, USA.

Sample Collection. Samples of traditionally fermented vegetable condiments which included ugba, ogiri, okpehe and West African soft cheese (*wara*) were purchased from local markets in Nigeria. The samples were packaged separately in polythene bags and transported aseptically to the laboratory for analysis.

Bacterial strains and culture conditions. To isolate presumptive *Enterococci*, 10 g of each sample were homogenized in 90 ml peptone water. Serial 10-fold dilutions were made and aliquots plated on Slanetz and Bartley Medium. After 48hrs at 37°C, typical small pinkish colonies of presumptive *Enterococci* were picked at random from the plates and subcultured in Brain Heart Infusion broth (Oxoid, Canada) at 37°C for 18hrs. The cultures were kept frozen at -20°C in BHI broth containing glycerol (50%).

Identification of Strains. Strains were identified by their physiological and biochemical characteristics, as described by Schleifer and Kilpper-Balz (1984). To confirm the identities of the isolates, total genomic DNA was extracted as described by Sambrook *et al.* (1989) and the 16S rRNA genes sequenced using primers FD1 (5'-AGAGTT TGATCCTGGCTCAG-3') forward and RD1 (5'-AAGGAGGTGATCCAGCC-3') reverse (Weisburg *et al.*, 1991).

Determination of bacteriocin production and antimicrobial spectra. The bacteriocinogenic strains T71 and W211 were grown in brain heart infusion (BHI) broth at 37°C. Bacteriocin was extracted by centrifuging at 6708 g for 20 minutes, 4°C. The supernatants from the early stationary growth phase were neutralized with 1 M NaOH and filter sterilized through 0.22 mm filters. The antimicrobial activity of the supernatants was determined by well diffusion assay, 10 μ L aliquots of supernatants were placed in wells (3-mm diameter) cut in cooled soft BHI agar plates previously seeded with indicator microorganisms. After 2 hours at 4°C, the plates were incubated at 30°C or 37°C for growth of the target organism; after 24 hours, the plates were examined for growth inhibition zones (Ogunbanwo *et al.*, 2003).

Purification of bacteriocins. Crude bacteriocin supernatant was precipitated with 70% saturation ammonium sulphate with constant stirring at 4°C for 3 hours, then centrifuged at 6708 g for 15 minutes 4°C, after centrifugation the precipitate were resuspended in 0.05 M potassium phosphate buffer (pH 6.5) followed by dialysis against the same buffer at 4°C. The dialyzed protein was applied to a DEAE-cellulose A-50 column pre-equilibrated with 0.05 M potassium phosphate buffer (pH 6.5). The columns were washed with 3 volumes of equilibration buffer and bound proteins were eluted with phosphate buffers of increasing molarity and decreasing pH. Fractions with high bacteriocin activity were pooled and concentrated in a lyophilizer (Rajaram, 2010). Protein was measured by the method of Lowry (Lowry *et al.*, 1951).

In-vivo evaluation of the effect of the bacteriocinogenic strains and their bacteriocin – Ethics statement. Sprague Dawley rats (150 g) bred in-house with free access to food and water were used for all experiments. The experiments were designed to minimize animal suffering and to use the minimum number of rats needed for valid statistical evaluation. This study was carried out in strict accordance with the guidelines of the Council of Scientific and Industrial Research, Government of India. The protocol was approved by the Animal Ethics Committee of the Indian Institute of Chemical Biology (Permit Number: 147/1999 CPCSEA) affiliated to the Indian Institute of Chemical Biology (a unit of the Council of Scientific and Industrial Research), Kolkata. All experiments were performed under standard controlled conditions. Control and experimental animals were fasted for 12 hours with free access to water before each experiment (Swarnakar *et al.*, 2005).

Infection and treatment. *Staphylococcus aureus* (MTCC 737) was obtained from the Culture Collection of the Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India and sub-cultured

on LB agar. Prior to infection the weights of all the animals were recorded and all rats were confirmed to be healthy. The animals were randomly divided into 8 groups of 10 animals each. The rats in Group 1, the control were not infected but received normal saline (0.4 µl) while the other seven groups were infected by oral administration with 0.1 ml of *S. aureus* (3.0×10^7 CFU/ml). Group 2 animals were given 427 AU/ml of ENT71, Group 3 1280 AU/ml of ENT71, Group 4 was infected but received no bacteriocin, Group 5 was given 640 AU/ml of ENT71, Group 6 640 AU/ml of ENTW21, Group 7 3.8×10^7 CFU/ml of T71 culture and Group 8 received 640 AU/ml of the combination of both ENT71 and T71 live culture (3.8×10^7 CFU/ml). This treatment was administered twice in a week for 3 weeks (Hena and Sudha, 2010).

Clinical examination. A modification of the method of Hena and Sudha (2010) was used for clinical examination of the experimental animals. The animals were weighed on a daily basis; total feed and fluid consumption were measured daily. Blood samples were drawn from all the animals and the blood was allowed to clot at room temperature and then centrifuged at 310 g for 12 minutes. The serum was collected and used for analysis, which included total protein, blood glucose, albumin, globulin, and enzymes activities (AST and ALT).

Serum biochemical parameters analysis. Glucose analysis: The quantitative determination of serum glucose was carried out using commercially available diagnostic experimental kits purchased from Diagnosticum Limited (Budapest, Hungary). **Albumin assay:** Quantitative colorimetric albumin determination was carried out by using Albumin Assay kit which was purchased from BioSino Biotechnology & Science Inc. (China). Serum total protein was carried out according to Lowry's method. Total protein minus albumin equals globulin. **Transaminases assay:** The determination of AST and ALT was based on the fact that phenyl hydrazone which was produced after incubating the substrate with the enzyme was measured spectrophotometrically. The amount of phenyl hydrazone formed was directly proportional to the enzyme quantity. Stanadard kits for the determination of AST and ALT were obtained from Span diagnostics, Surat (Gujurat). Colorimetric procedure in which the oxaloacetate and/or pyruvate formed in either the AST or ALT reaction is combined with 2, 4-dinitrophenylhydrazine to yield a brown-coloured hydrazone which is measured at 505 nm.

Harvesting of visceral organs. The animals were anesthetized by ketamine (12 mg kg^{-1} body weight) followed by cervical dislocation for killing, dissected and the visceral organs (liver, spleen, kidney and stomach) removed, weighed and preserved in 10% formalin (Biswas *et al.*, 2010).

Tissue extraction. The fundic portion of the gastric mucosa was suspended in phosphate-buffered saline (PBS) containing protease, minced, incubated for 10 minutes at 4°C and centrifuged at 9660 g for 15 minutes. The supernatant were saved as a PBS extract. The pellet was extracted in lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 plus protease inhibitors) and centrifuged at 12000 rpm for 15 minutes to obtain Triton-X (TX) extracts, which were preserved at -70°C (Swarnakar *et al.*, 2005).

Evaluation of MMP-2 and -9 activities. To assay MMP-2 and -9 activities, the mucosal extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels containing 1 mg/mL gelatin under non-reducing conditions. The gels were washed in 2.5% Triton X-100 and incubated in CAB buffer (40 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM CaCl_2) for 18 h at 37°C and stained with 0.1% Coomassie blue followed by destaining. Zones of gelatinolytic activity failed to stain and the zymograms were quantified by densitometry (Image QUANT, Amersham Biosciences, the Netherlands) linked to appropriate software (Swarnakar *et al.*, 2005).

Evaluation of TNF-α and IL-1β activities. Tissue extracts (100 µg/lane) were resolved by 10% reducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 2 hours at room temperature (~25°C) in 3% BSA solution in 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 0.02% Tween 20 (TBST), followed by overnight incubation at 4°C in 1:200 dilutions of the respective primary antibodies (TNF-α and IL-1β) in TBST containing 0.2% BSA. The membranes were washed five times with TBST and incubated with horse radish peroxidase-conjugated secondary antibody (1:20000) for 1.5 hour. Bands were visualized by enhanced chemiluminescence using luminol peroxide substrate solution. The blots were stripped and reused for β-tubulin (SC-9935), after blocking with 3%BSA (Swarnakar *et al.*, 2005; Paul *et al.*, 2008).

Histological assessment of the gastric tissues and GIT. For histological studies, stomachs and small intestines were fixed in 10% formalin and embedded in paraffin. The sections (5 µm) were cut with a microtome, stained with hematoxylin and eosin, and assessed under an Olympus microscope (Olympus Optical Co., GMBH, Hamburg, Germany). Images were captured using Camedia software (E20P 5.0 Megapixel; Hamburg, Germany) at 20X magnification (Swarnakar *et al.*, 2005).

Statistical analysis. Statistical analysis of was carried out using GraphPad Instat 3 software. Comparison between groups was made using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

Results and Discussion

Identification of bacterial strains. Isolates were differentiated on the basis of their morphological appearance, and physiological and biochemical tests. Presumptive *Enterococcus* strains were Gram-positive, catalase and oxidase negative, non-spore forming cocci occurring in singles and pairs. They were able to grow in the presence of 6.5% NaCl, at pH 9.6 and at 10°C and 45°C. They were able to hydrolyze esculin, pyrrolidonyl- β -naphthylamide, arginine and unable to hydrolyze starch. The *Enterococcus* strains were also differentiated based on their sugar fermentation pattern. The strains were able to ferment glucose, mannitol, arabinose, raffinose, sucrose, and unable to ferment sorbose, sorbitol and pyruvate. Furthermore, ammonia was produced from the hydrolysis of arginine, and H₂S was not produced.

The results of the biochemical test were confirmed at the gene level by performing PCR in a PCR SPRINT Thermal cycler (Thermo Electron Corporation, Japan). PCR conditions were standardized, with a set of primers to amplify the 16S rRNA gene. Molecular sequencing of the five positive strains using both forward and reverse primers yielded DNA sequences of about 1500 bp. The sequences were aligned with their closest matches from a BLAST search, and analysis of the data revealed that the sequences had their best match with *E. gallinarum*. The sequences of the five strains were $\geq 99.0\%$ similar to those of *Enterococcus gallinarum* in Genbank. *Enterococcus gallinarum* W211 (isolated from wara), *E. gallinarum* U82 (from ugba), *E. gallinarum* T71 (from okpehe), *E. gallinarum* W184 (from wara) and *E. gallinarum* C103 (from ogiri) were given accession numbers JF915769, JF774412, JF774411, JN020631 and JF774410, respectively. The various methods used above permitted the positive identification of isolates. The five strains that were presumed to be *Enterococcus gallinarum* from their phenotypes were confirmed by genomic methods as described by Oladipo *et al.* (2013).

Determination of bacteriocin production and antimicrobial spectra. The antimicrobial activities of the bacteriocins produced by the strains were determined against selected indicator organisms, and it was observed that the bacteriocins from *E. gallinarum* C103 and U82 had no antimicrobial activity against any of the pathogens tested, while the bacteriocin from *E. gallinarum* W184 had antimicrobial against *Serratia marcescens* only. The bacteriocin produced by *Enterococcus gallinarum* T71 had antimicrobial activity against *Micrococcus luteus*, *Proteus mirabilis*, *Proteus vulgaris*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Salmonella typhimurium*, and *Bacillus pumilus*. Finally, the bacteriocin from *E. gallinarum* W211 had antimicrobial

activity against *Proteus vulgaris*, *Bacillus cereus*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Salmonella typhimurium*.

Of the five strains identified, two strains had a wide spectrum of activity against the indicator organisms used. Interest in the *Enterococci* has increased in recent decades and most of the *in vitro* studies have dealt with the ability of *Enterococci* to produce bacteriocins (spectrum of inhibitory activity, physicochemical properties of the bacteriocins, applications in food products, etc.). We found that the bacteriocins from *Enterococcus gallinarum* T71 and W211 had inhibitory activity against both Gram positive and negative bacteria even though Gram negative bacteria are usually considered resistant to many of the bacteriocins produced by *Enterococci* species. Some reports support our findings in that certain bacteriocins of lactic acid bacteria, notably the class 2 bacteriocin, pediocin, inhibit a limited number of Gram negative bacteria (Cintas *et al.*, 2001; Moreno *et al.*, 2006). A brief search of the literature revealed that only a small percentage of the isolated bacteriocins have antibacterial activity against more phylogenetically distant Gram-positive bacteria and, under certain conditions, Gram-negative bacteria (Line *et al.*, 2008). De Kwaadsteniet *et al.* (2005) described the characterization of a 3,944-Da bacteriocin produced by *Enterococcus mundtii* with activity against Gram-positive and negative bacteria. Line *et al.* (2008) reported an unusual broad-spectrum antimicrobial activity of Enterocin E-760 (E-760), including activity against Gram-negative organisms.

In-vivo evaluation of the effects of the bacteriocinogenic strains and their bacteriocins. None of the rats died during the experimental period. The weight of all the groups increased between the first and third week except for Group 4, which was infected with *S. aureus* and not treated (Fig. 1a). Food intake was reduced, while water intake increased in Group 4 compared to the others (Fig. 1b). The weights of the visceral organs (liver, spleen, kidney and stomach) excised from the experimental rats are shown in Table I. *Staphylococcus aureus* infection caused increases in size of the visceral organs (liver, spleen, kidney and stomach) (Group 4). This may have been related to the lack of increase in body weight, as the animals consumed less food and a high fluid intake was observed.

Biochemical evaluation of sera. For biochemical analysis, sera were collected before the animals were killed. It was observed that serum total protein and the globulin fraction increased in the infected and untreated rats compared to the uninfected control, whereas serum albumin decreased. Treatment with the bacteriocins from *E. gallinarum* W211 and T71 normalized serum protein levels. An increase in blood glucose was

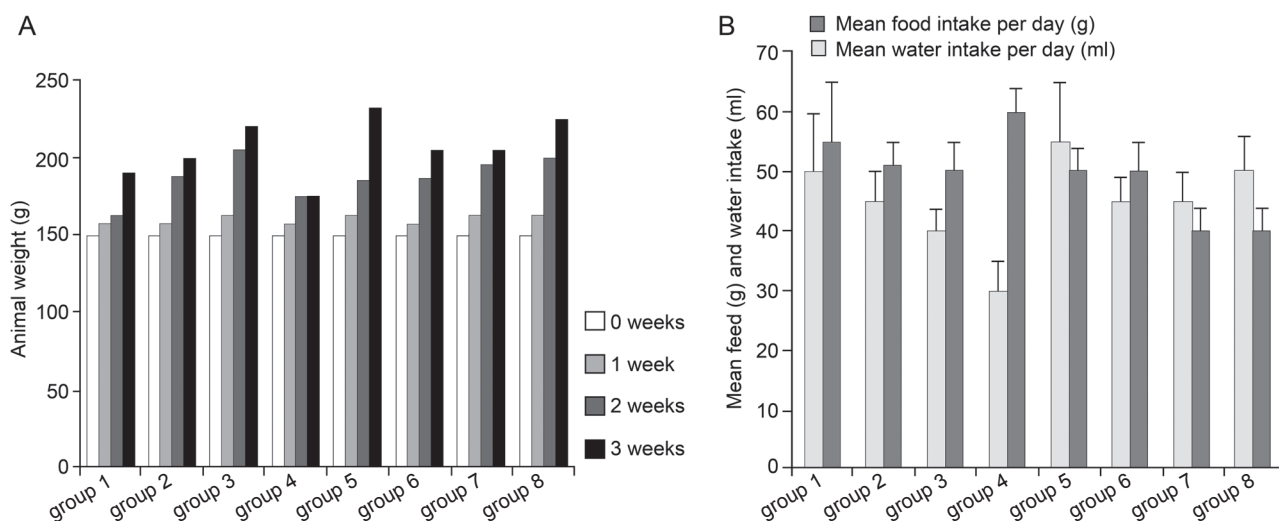


Fig. 1. Physiological and behavioural changes in experimental rats. Bar diagram represents mean weight of experimental rats before and during treatment (a) mean feed and water intake of experimental rats before and during treatment (b).

observed in the *S. aureus*-infected rats that were bacteriocin-treated. Serum enzymatic evaluation showed that AST and ALT levels increased in the *S. aureus*-infected rats (group 4), whereas the bacteriocin-treated

groups had lower activity than group 4. This confirms the development of pathological changes in liver function during prolonged *S. aureus* infection. There was also a decrease in AST level in Group 8 (Table II). Treatment with the bacteriocins from *E. gallinarum* W211 and T71 also reversed enzyme levels, supporting their bioprotective activity against *S. aureus* infection.

Evaluation of MMP-2, MMP-2-9, TNF- α and IL-1 β activities. *Staphylococcal* infection caused stomach inflammation by increasing both secreted and total MMP-9 activity (Fig. 2). Treatment with bacteriocins from *E. gallinarum* T71 and *E. gallinarum* W211 decreased the increase by up to ~3 fold, dose dependently. Moreover, treatment with *E. gallinarum* T71 and the bacteriocin from *E. gallinarum* T71 also reduced pro-MMP-9 activity. Elevated expression of MMP-9 plays an important role in gastric inflammation and ulceration by remodeling tissues and degrading extracellular matrix in disease (Swarnakar *et al.*, 2005). In the present study, prolonged *S. aureus* infection increased

Table I
Average weight of the visceral organs of experimental rats

Group	Liver	Spleen	Kidney		Stomach
			Right	Left	
Group 1	7.20 \pm 0.17	0.60 \pm 0.01	0.80 \pm 0.01	0.76 \pm 0.04	2.36 \pm 0.03
Group 2	7.08 \pm 0.17	0.61 \pm 0.01	0.79 \pm 0.01	0.73 \pm 0.03	2.33 \pm 0.03
Group 3	7.23 \pm 0.03	0.60 \pm 0.01	0.80 \pm 0.01	0.74 \pm 0.04	2.36 \pm 0.01
Group 4	7.40 \pm 0.02	0.64 \pm 0.02	0.89 \pm 0.02	0.79 \pm 0.01	2.43 \pm 0.01
Group 5	7.21 \pm 0.02	0.60 \pm 0.01	0.76 \pm 0.05	0.73 \pm 0.03	2.35 \pm 0.01
Group 6	7.19 \pm 0.01	0.59 \pm 0.01	0.78 \pm 0.02	0.74 \pm 0.04	2.34 \pm 0.02
Group 7	7.10 \pm 0.02	0.60 \pm 0.01	0.80 \pm 0.01	0.76 \pm 0.02	2.35 \pm 0.02
Group 8	7.11 \pm 0.01	0.59 \pm 0.01	0.80 \pm 0.01	0.75 \pm 0.05	2.36 \pm 0.02

Values are mean \pm SD, where SD = Standard Deviation

Table II
Biochemical analysis of the blood serum of experimental rats.

Group	Total protein g/L	Albumin μ mol/L	Globulin g/L	Blood glucose mmol/L	AST μ kat/L	ALT μ kat/L
Group 1	73.10 \pm 0.01	6.18 \pm 0.02	30.50 \pm 0.01	1.60 \pm 0.02	2.38 \pm 0.01	1.22 \pm 0.01
Group 2	72.70 \pm 0.02	6.12 \pm 0.03	30.50 \pm 0.03	1.59 \pm 0.02	2.11 \pm 0.01	1.19 \pm 0.02
Group 3	72.00 \pm 0.02	6.16 \pm 0.02	29.50 \pm 0.01	1.59 \pm 0.01	2.26 \pm 0.03	1.19 \pm 0.02
Group 4	76.00 \pm 0.01	5.70 \pm 0.01	36.70 \pm 0.01	2.30 \pm 0.02	2.46 \pm 0.01	1.34 \pm 0.02
Group 5	72.60 \pm 0.01	6.18 \pm 0.02	30.00 \pm 0.03	1.61 \pm 0.02	2.23 \pm 0.02	1.20 \pm 0.01
Group 6	73.00 \pm 0.01	6.18 \pm 0.03	30.40 \pm 0.02	1.54 \pm 0.01	2.29 \pm 0.02	1.22 \pm 0.02
Group 7	72.90 \pm 0.01	6.15 \pm 0.02	30.50 \pm 0.02	1.59 \pm 0.02	2.23 \pm 0.01	1.13 \pm 0.02
Group 8	72.10 \pm 0.03	6.16 \pm 0.02	29.60 \pm 0.01	1.54 \pm 0.03	0.48 \pm 0.01	1.19 \pm 0.03

Values are mean \pm SD, where SD = Standard Deviation

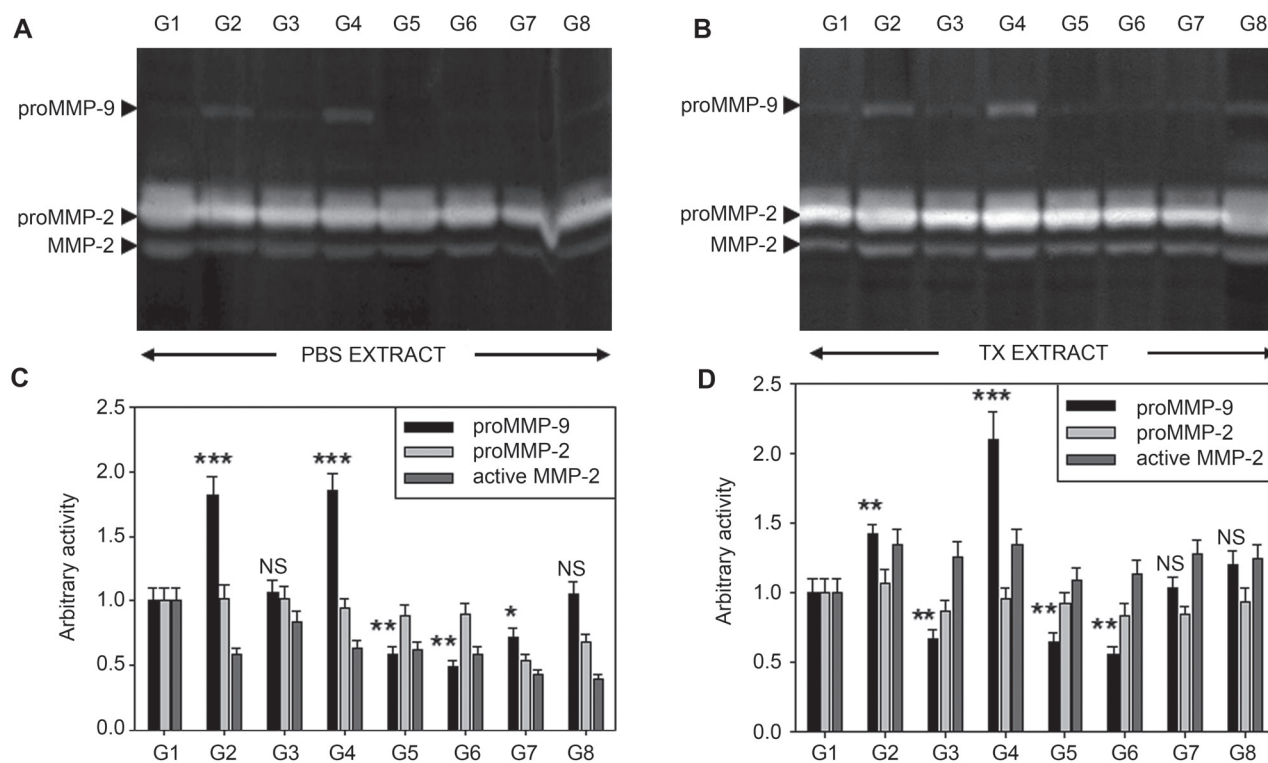


Fig. 2. Effect of varying doses of bacteriocins on staphylococcal infection and associated proMMP-9 activities. Gelatin zymography showing proMMP-9 activity in each PBS (a) and Tx (b) extracts from tissues. Histogramic representation of rat groups *versus* arbitrary activity of MMP-9 and MMP-2 activity for PBS (c) and Tx (d) extracts respectively as measured by densitometric analysis using Lab image software. Values are \pm SD. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ and NS, non-significant versus the appropriate control using ANOVA followed by Student-Newman-Keuls test. G1- G8 = Group1 – Group 8.

inflammation and mucosal damage as a result of increased MMP-9 activities. Moreover, the increased expression of TNF- α and IL-1 β due to infection was reversed dose-dependently by bacteriocin treatment. Furthermore, the levels were also reduced in group 8, consisting of rats treated with cells of *E. gallinarum* T71 together with *E. gallinarum* T71 bacteriocin (Fig. 3). The elevation of inflammatory cytokines (TNF- α)

during *S. aureus* infection further intensified the gastric damage. Furthermore, TNF- α also induces other inflammatory cytokines like IL-1 β , thereby augmenting inflammatory responses. We found above that the bacteriocins from *E. gallinarum* W211 and T71 were able to reduce the expression of inflammatory cytokines and down-regulate MMP-9 activities and related mucosal damage, supporting the bioprotective nature of the

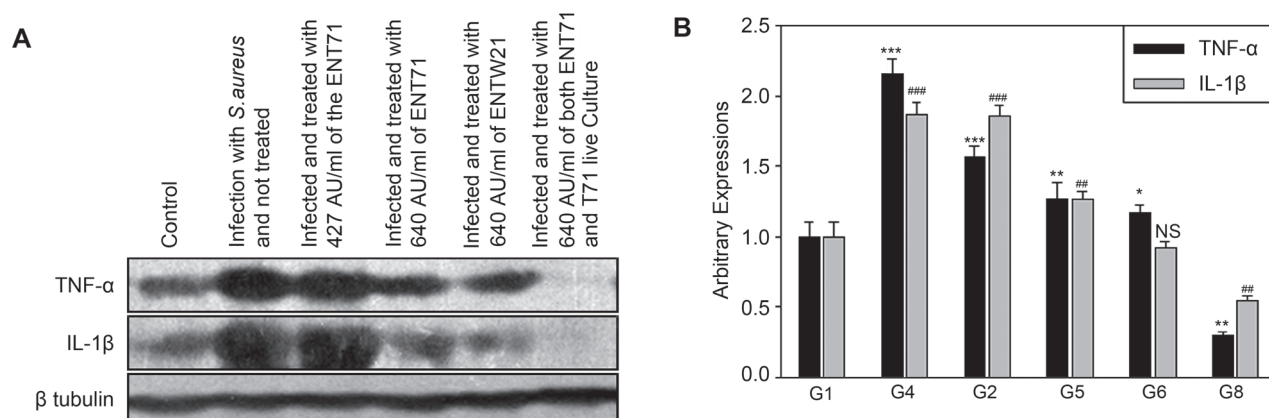


Fig. 3. Effect of varying doses of bacteriocins on staphylococcal infection and associated TNF- α and IL-1 β expressions. Western blot showing each Tx extract from tissue probed with respective primary antibodies of TNF- α , IL-1 β and β tubulin (a) an histogramic representation (b) Protein band intensities were quantified by densitometric analysis using Lab image software from three blots from independent experiments in each case. Values are \pm SD. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ and NS, nonsignificant versus the appropriate control using ANOVA followed by Student-Newman-Keuls test. G1-G8 = Group1-Group 8.

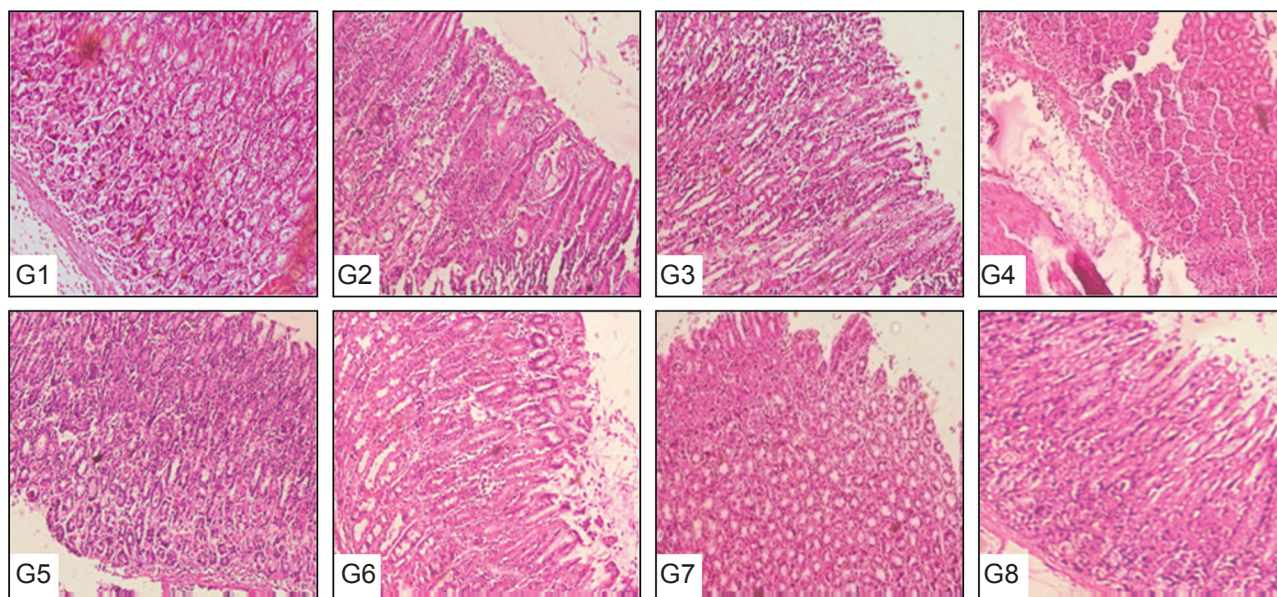


Fig. 4. Histology of rat stomach after infection with *Staphylococcus aureus* and treatment with bacteriocin. G1-G8=Group1 – Group 8

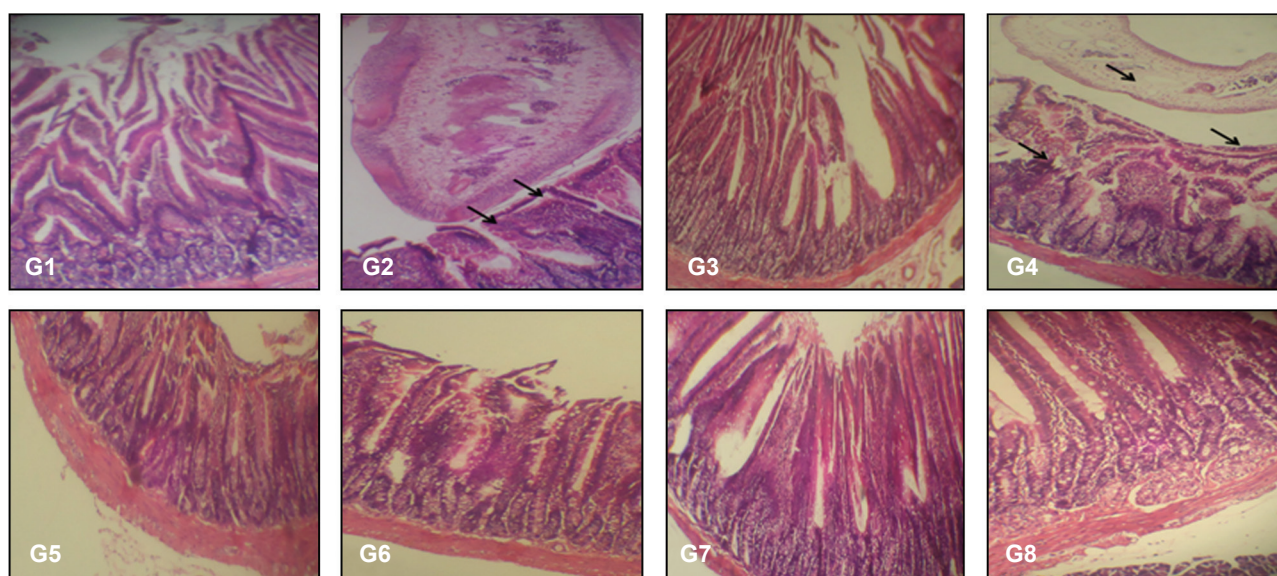


Fig. 5. Histology of the small intestine of experimental rats after infection with *Staphylococcus aureus* and treatment with bacteriocin. G1-G8=Group 1 – Group 8

bacteriocins. Also, the combination of a live culture of *E. gallinarum* T71 and *E. gallinarum* T71 bacteriocin suppressed the expression of TNF- α and IL-1 β , confirming their probiotic and bioprotective activities.

Histological assessment of gastric tissues and GIT

The histology of the stomachs of *S. aureus*-infected and untreated rats (Group 4) revealed ulceration of the mucosa as well as inflammation of the cells of the epithelia lining the glands of the mucosa. These effects were greatly reduced in the rats treated with bacteriocin-producing organisms and various doses of bacteriocin (Fig. 4). The histology of the small intestine of the untreated group (Group 4) revealed blunted and disintegrated villi tips, large numbers of bacteria along

with a small amount of mucosal debris in the intestinal lumen. In addition the thickness of the serosa was gradually eroded. These abnormalities were less marked in the rats treated with 50 μ L of the bacteriocin from *E. gallinarum* T71; no visible lesions were seen, but the villi were broad at their base and tips, and there were large numbers of bacterial sections in the lumen. No lesion was evident in the other treated groups (Fig. 5). Histological analysis revealed direct evidence supporting the probiotic nature of the strains and the usefulness of bacteriocins as bioprotective agents, as reported by Dodd and Gasson (1994).

The *Enterococcus gallinarum* T71 and W211 bacteriocins had broad spectrum inhibitory activity against

both Gram positive and Gram negative indicator bacteria, and a unique ability to attenuate gastric inflammation and GIT damage caused by *S. aureus* infection. At the levels tested, bacteriocin dose of 640 AU/ml from both *E. gallinarum* T71 and W211 caused a reduction greater than 60% in MMP-9 activities; lower dosages also affected the MMP-9 activities, although this was not as dramatic. The combination of the bacteriocinogenic *E. gallinarum* T71 and the bacteriocin produced by this strain reduced TNF- α and IL-1 β expressions as well as gastric and GIT damage profoundly. Therefore, the special characteristics of these *E. gallinarum* strains can positively contribute to their use as probiotics. The selective use of these bacteriocinogenic strains and their bacteriocins may be beneficial for biomedical purposes.

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