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(RESEARCH ARTICLE)

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Study of anti-bacterial activity of bacteriocins isolated from lactic acid bacteria grown on millet media

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Abstract

Millets are grains that have high nutritive values. The current study aimed at studying the effect of prebiotics on the production of bacteriocins and their antibacterial activity. Bacteriocins were extracted from probiotic species of *Lactobacillus plantarum* and *Lactocaseibacillus rhamnosus* grown in millet modified medium and MRS medium. Results of anti-microbial assays of bacteriocin indicate enhanced activity in presence of proso millet. Bacteriocin isolated from *L. rhamnosus* growing in proso modified media had a zone of inhibition 15 ± 0.35mm against *B. cereus* and 16.8 ± 0.19mm against *E. cloacae*. Molecular weight was determined by SDS-PAGE and was found to be ~ 48 kDa. Thus, it could be concluded that proso millet acts as an efficient prebiotic. Crude, unprocessed extracts of millets were used in this study and significant results were obtained, suggesting that including them in everyday diet could render a source of natural prebiotic that is affordable and available commonly across the globe.

Keywords: Prebiotic; Proso Millet; Lactobacillus; Bacteriocin; Pathogen

1. Introduction

Bacteriocins are small size, heat-stable peptides synthesised by bacteria that are active against other organisms and against which the producer has a definite immune mechanism [1]. They are produced by a variety of microorganisms including, Gram-positive and Gram-negative bacteria along with some archaea [2]. Bacteriocins synthesised by Gram-positive bacteria, commonly lactic acid bacteria (LAB), are divided into two categories namely: Class I - lanthionine-containing bacteriocins/lantibiotics and Class II - non-lanthionine containing bacteriocins/lantibiotics[3]. According to the statistics provided by the World Health Organization, nearly 4.2 million individuals are subjected to death each year as a result of ingesting pathogenic bacteria-infected food[4]. The majority of the reported foodborne disorder outbreaks are known to be caused by common pathogens such as *Salmonella*, *Campylobacter*, *Listeria monocytogenes*, Norovirus, and *Shiga* toxin-producing *Escherichia coli*. Other pathogens that are reported to occasionally contribute to illnesses are *Clostridium sp.*, *Staphylococcus aureus*, *Bacillus cereus*, *Yersinia enterocolitica*, parasites, and some others [5].

The mechanism by which bacteriocins kill deadly pathogens, the variables that determine the efficiency of bacteriocins in foods and the physical chemistry of the bacteriocin/pathogen interaction and are less known. Such understanding is mandatory for bacteriocins to be used more commonly and for their potency to be increased through means of genetic engineering [6]. Since many bacteriocin producers in this bacterial group are probiotics, bacteriocins produced by LAB have sparked a lot of research, particularly in recent years. Since these micro-organisms are widely present in our food, particularly fermented foods, they are typically considered as safe for human ingestion [7]. The gastrointestinal tract is another typical habitat for LAB, where they generate complicated molecular crosstalk with the host and the other microbes [8]. Bacteriocins are frequently viewed as weapons, with a wide range of inhibitory spectra to compete with other bacteria in the same niche. The majority of bacteriocins target species or genera that are closely related to the producers; however, some can have a significantly greater spectral range [9].

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A few studies have shown the presence of prebiotic oligosaccharides in grains that are rich in dietary fibres, including rice, buckwheat and wheat [10, 11, 12]. Considering the fact that millets are easily available, stress and heat-resistant crops, rich in dietary fibres, they could be potentially considered as prebiotics that enhance growth and development of probiotics that can neutralize pathogens through bacteriocins in the gastro-intestinal tract

2. Material and methods

2.1. Sample collection

Millets (namely barnyard, browntop, foxtail, finger, kodo, little, pearl, proso, and sorghum) were procured from the local market in Bengaluru. Cultures of *Lactobacillus plantarum* (MTCC 1407), *Lactocaseibacillus rhamnosus* (MTCC 1408), *Bacillus cereus* (MTCC 1307) and *Enterobacter cloacae* (MTCC 509) were obtained from MTCC, Chandigarh, India.

2.2. Preparation of modified MRS media

Lactobacillus strains were grown on standard MRS media and modified MRS media namely proso mMRS (PmMRS) and foxtail mMRS (FmMRS) containing respective millet extracts as the source of carbohydrate replacing glucose in MRS media.

Composition of modified MRS (mMRS) (g/L): Peptone – 10g; Beef extract – 10g; Yeast extract – 8g; Extract from 20g millets; Sodium acetate trihydrate – 5g; Polysorbate – 1g; Tri-ammonium citrate – 2g; Magnesium sulphate – 0.2g; Manganese sulphate – 0.05g; Agar – 15g.

The components were dissolved in distilled water (1L) and pH was adjusted to 6.2 and sterilized by autoclaving at 121°C for 15 minutes.

2.3. Bacteriocin extraction

Bacteriocin produced by the LAB was extracted following the protocol given by Zhao [2]. About 50 ml of 18hr cultures of *Lactobacillus plantarum* and *Lactocaseibacillus rhamnosus* in MRS, PmMRS and FmMRS broth were centrifuge at 8000 rpm for 10 minutes at 4°C. The pellet was suspended in 50 mL of respective broths. The suspension was sonicated for 20 minutes and centrifuged at 8000 rpm for 10 minutes at 4°C. The cell free extract was centrifuged. The supernatant was collected, and equal volume of chloroform was added. The mixture was vigorously shaken and incubated overnight at 4°C. The interfacial layer was collected and centrifuged at 12,000 rpm for 10 minutes at 4°. The pellet was resuspended in 1mL of phosphate buffer (0.05M; pH-7.0). The resulting suspension was stored at 4°C for further use.

2.4. Antimicrobial assay of crude bacteriocin extract.

The anti-microbial assay was done on Muller Hinton (MH) agar. *B. cereus* and *E. cloacae* were the pathogens used. The cultures were swabbed on MH agar and wells were made using a 6mm corkborer. Tetracycline (10µg) was used as a positive control, and phosphate buffer was taken used as a negative control. Bacteriocin extracts from MRS, PmMRS and FmMRS were loaded, and plates were incubated at 37°C for 24 hrs and the zones of inhibition were observed.

2.5. Determination of molecular weight by SDS – PAGE

Molecular weights of the crude bacteriocin extracts were determined [2]. To achieve proper resolution separation of complex mixture of proteins SDS-PAGE is used. The standard protocol by Laemmli [13] was followed. Two gels were made, one with 5% stacking gel and the other with 12% separating gel. 25 µl aliquot of extracted bacteriocin was mixed with 25 µl of loading dye and boiled at 100°C for 10 minutes. 40 µl of this mixture was loaded in the wells along with a ladder. Electrophoresis was carried out at a constant voltage of 50 millivolts.

2.6. Analysis of the effect of pathogens on the prebiotic strains through SEM analysis

The microbial cultures of *L. plantarum* and *L. rhamnosus* were inoculated in MRS and PmMRS broths (5% inoculum each). After 24hrs, pathogens were inoculated into probiotic cultures in equal proportions and incubated at 37°C on a shaker incubator at 100 rpm for 24hrs. One drop of the culture was suspended on a glass slide and was subjected for drying in a desiccator for 48hrs. The preparations were then analysed by scanning electron microscopy at IISc., Bengaluru.

3. Results and discussion

3.1. Antimicrobial assay of bacteriocin

Antimicrobial assay of bacteriocin extracts from *L. plantarum* in the different modified and standard media showed no zone of inhibition. Only positive control had a consistent zone of inhibition 23 ± 2 mm as seen in Plate 1. Bacteriocin extracts from *L. rhamnosus* grown in PmMRS media had a significant zone of inhibition 15 ± 5mm against *B. cereus* (Plate 2 (a)) and 16.8 ± 1 mm against *E. cloacae* (Fig 2 (b)). The antimicrobial assay was conducted in replicates of 5.

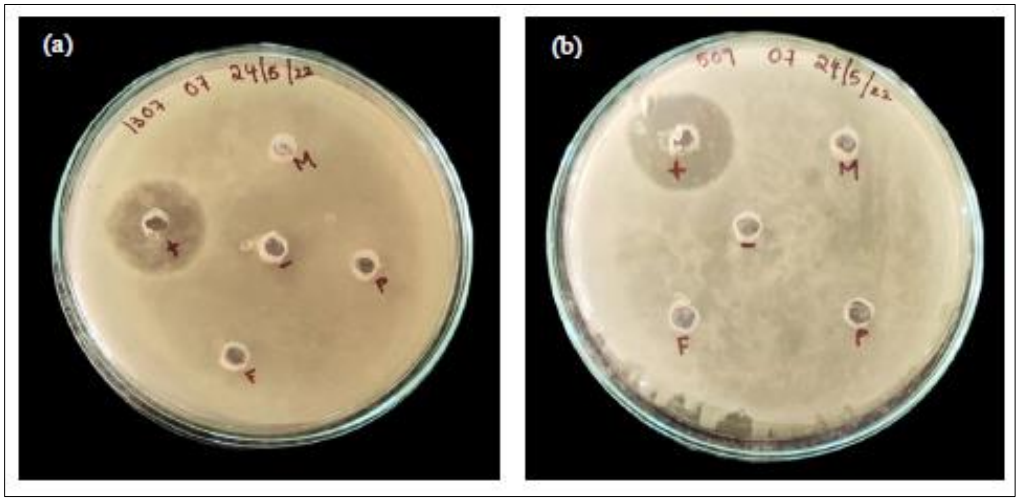

Figure 1 Anti-microbial assay of *L. plantarum* bacteriocin extract against (a) *B. cereus* and (b) *E. cloacae*

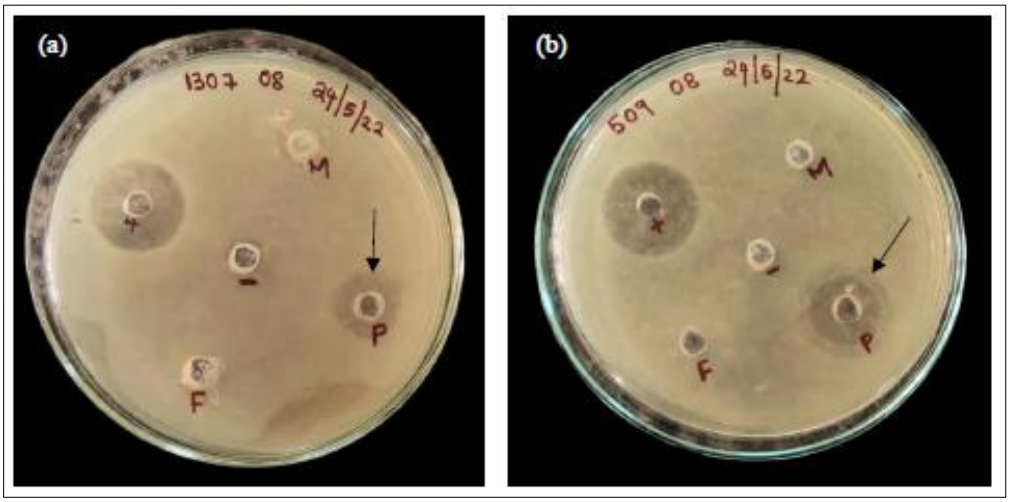

Figure 2 Zone of inhibition for bacteriocin extract produced by *L. rhamnosus* grown in PmMRS against (a) *B. cereus* and (b) *E. cloacae*

In a study conducted on *L. bulgaricus* from yoghurt, significant inhibiting potential has been observed against *S. typhi* (16.5 mm), *E. coli* (15.25 mm), and *B. subtilis* (15.32 mm), with a maximum of 18.32 mm against *V. cholerae* [14]. *Lactobacillus* also showed very strong activity against *Bacillus mycoides* with the zone of inhibition of 15-18mm in diameter [15]. Similar values in the range of 15-16mm has been obtained in the present experiment as well.

3.2. Determination of molecular weight by SDS – PAGE

Two prominent bands for P07 (*L. plantarum* on PmMRS) and P08 (*L. rhamnosus* on PmMRS) were obtained and the molecular weight determined was ~ 48 kDa (Fig 3). Proso millet samples showed the presence of bacteriocin. No bands were obtained from other samples suggesting further purification of crude extracts.

Bacteriocins obtained from *L. plantarum* isolated from sourdough have found to be around 10 kDa as according to Zangeneh [16]. The values of ~ 48 kDa obtained in the current study is well in the range of 2.5 to 100 kDa described for bacteriocins of *Lactobacillus* species [17].

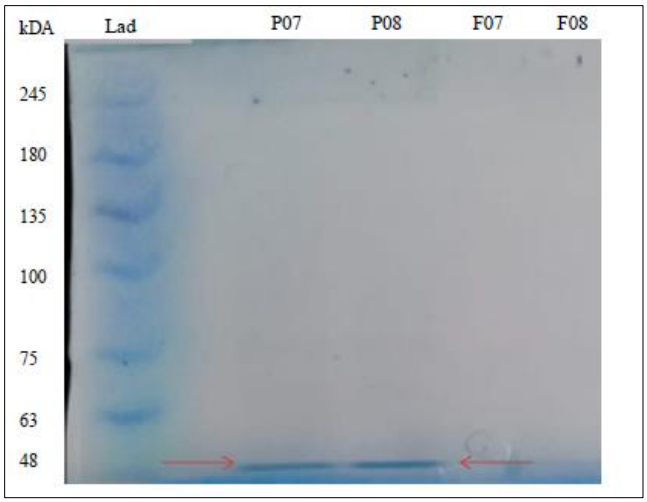

Figure 3 Bands obtained on SDS-PAGE gel with extracted bacteriocin (indicated by arrows)

3.3. Analysis of probiotic pathogen interaction through SEM analysis

Figure 4a and 4b shows the SEM images for probiotic bacteria in PmMRS with B. cereus at two different magnifications. It is evident from the micrograph, rupturing of pathogenic cells could be seen on interaction with the probiotic spp. Some extracellular polymeric substances (EPS) are clearly seen in Fig 5a and 5b. These may be bacteriocin secreted by probiotic bacteria in response to the pathogens growing in the medium. This shows that, the probiotic help in suppression of bacteria, more efficiently in presence of prebiotics such as millet. A similar SEM image showing the enhanced production of EPS by probiotic strains in the presence of pathogens was reported by Mathivanan [18], in their investigation.

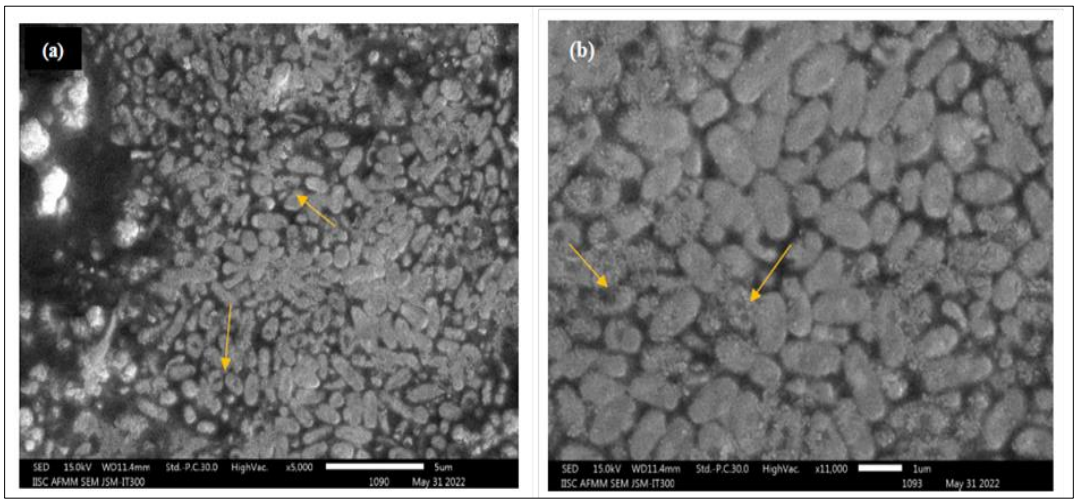

Figure 4 (a) and (b): SEM images showing ruptured cell walls of *B. cereus* in the presence of probiotic sp

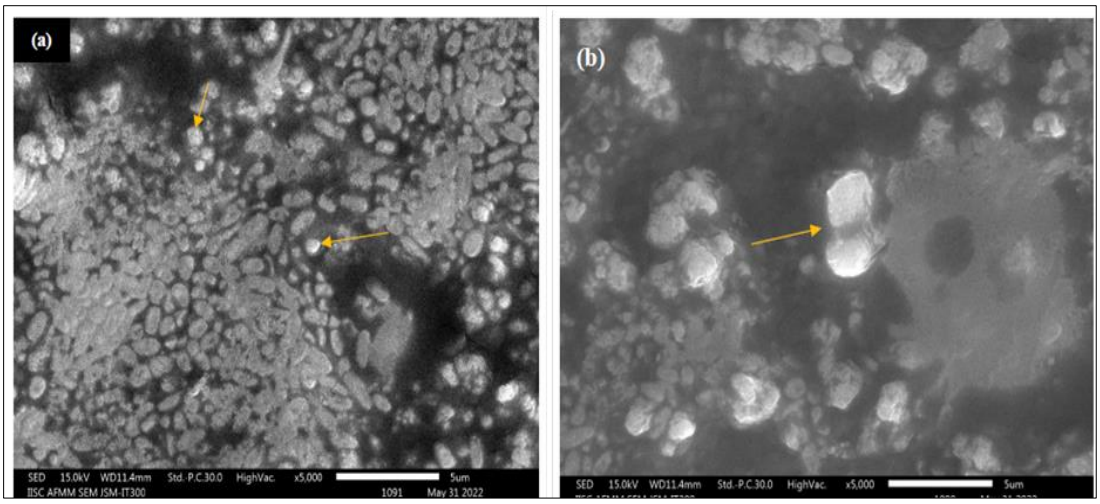

Figure 5 (a) and (b): SEM image showing EPS secreted by pathogen in response to probiotic bacteriocin

4. Conclusion

Antagonistic activity of the crude bacteriocin extracts against the selected pathogens suggests that the activity intensified in the presence of millets. The molecular weights of the extracted bacteriocin were found to be ~ 48kDa. Amongst the two millets, proso millet showed better prebiotic properties compared to foxtail millets, thus proving as a superior prebiotic grain. Results obtained in this *in vitro* study hints that gut microbiota could be modulated to through interventions like including natural source of prebiotics in diet. They act as substrates for probiotics and lead to exploitative competition and directly influence the production of bacteriocins that can harm the gut pathogens.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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