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Biopreservation potential of antimicrobial protein producing *Pediococcus* spp. towards selected food samples in comparison with chemical preservatives

Sinosh Skariyachan*, Sanjana Govindarajan

R & D Centre, Department of Biotechnology, Dayananda Sagar College of Engineering, Bangalore-560 078, Karnataka, India

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ABSTRACT

The present study elucidates biopreservation potential of an antimicrobial protein; bacteriocin, producing *Pediococcus* spp. isolated from dairy sample and enhancement of their shelf life in comparison with two chemical preservatives. The antimicrobial protein producing *Pediococcus* spp. was isolated from selected diary samples and characterised by standard microbiology and molecular biology protocols. The cell free supernatant of *Pediococcus* spp. was applied on the selected food samples and monitored on daily basis. Antimicrobial potential of the partially purified protein from this bacterium was tested against clinical isolates by well diffusion assay. The preservation efficiency of bacteriocin producing isolate at various concentrations was tested against selected food samples and compared with two chemical preservatives such as sodium sulphite and sodium benzoate. The bacteriocin was partially purified and the microbiological qualities of the biopreservative treated food samples were assessed. The present study suggested that 100 µg/l of bacteriocin extract demonstrated antimicrobial potential against *E. coli* and *Shigella* spp. The treatment with the *Pediococcus* spp. showed enhanced preservation at 15 mL/kg of selected samples for a period of 15 days in comparison with sodium sulphite and sodium benzoate. The microbiological quality of food samples treated with biopreservative showed lesser total bacterial count (CFU/g) in comparison with the food samples applied with chemicals ($p \leq 0.05$). Thus, the present study suggests that bacteriocin producing *Pediococcus* probably provides enhanced shelf life to the selected food samples and can be used as biopreservatives.

1. Introduction

Food spoilage is one of the major concerns in the society (Soomro et al., 2002). The use of synthetic chemical preservatives have increased over the past few decades and also considerable scientific data have emerged on the intolerance of food additives with various health issues (Kregiel, 2015; Sweis and Cressey, 2018). Hyperactivity and other neurophysiological issues have been reported in children as regards/ due to the consumption of chemical preservatives (Trasande et al., 2018). Some of these preservatives are carcinogenic while others are known to cause various side effects, which included breathing difficulties, heart damage and other health implications (Tuormaa, 1994).

The adverse effect of commonly used preservatives can be surmounted by the application of biopreservatives. This approach involves the use of natural microbiota or their antimicrobial products for extending the shelf life and enhancing the food safety. The common microorganisms used for biopreservation of food products include lactic acid bacteria (LAB) due to their abundance, probiotic nature and generally recognised as safe (GRAS) status (Mokoena et al., 2016; Perez et al., 2014). There are many proteins produced by genera of lactic acid bacteria which are responsible for antimicrobial activities (Barbosa et al., 2017; Elayaraja et al., 2014; Li et al., 2015). Most of them are bacteriocins and they are categorized in to three groups namely class I, class II and class III (Chanos and Mygind, 2016; de Oliveira Jr. et al., 2015). *Pediococcus* spp. is one of the most important LAB that are considered as probiotics. They are Gram-positive, non-motile, non-spore forming, catalase-negative, facultative anaerobic cocci arranged in tetrads (Porto et al., 2017). There are various strains of *Pediococcus* that produces protein known as pediocin, which is considered as effective antimicrobial bacteriocin (Papagianni and Anastasiadou, 2009; Porto et al., 2017).

The applications of antimicrobial proteins extracted from various bacteria have been studied for their biopreservation potential towards various food samples (Ishibashi et al., 2015; Mader et al., 2015;

* Corresponding author.
E-mail addresses: sinoshskariya@gmail.com, sinosh-bt@dayanandasagar.edu (S. Skariyachan).

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Mahmood et al., 2015). Nisin extracted from *Lactococcus* spp. was licensed for various industrial applications. Pediocin exhibits imperative technological properties such as thermo stability and activity at wide range of pH, making them attractive classes of biopreservative (Maldonado-Barragán et al., 2013; Mehta et al., 2013). The application of bacteriocin producing LAB serve as promising alternative to traditional chemical based preservation in food industry. LAB can produce other antimicrobial compounds other than bacteriocin which can also aid in food preservation (Barros et al., 2001; Hernández-Saldaña et al., 2016). Hence, this study tried to elucidate the biopreservation potential of bacteriocin producing *Pediococcus* spp. isolated from curd sample towards selected food products in comparison with two chemical preservatives.

2. Materials and methods

2.1. Description of samples

Eight samples were identified for the isolation of *Pediococcus* spp. based on their availability and day-to-day applications in food industry. The samples identified in the current study included three types of milk such as pasteurized milk (supplemented with probiotics) (S1), house hold milk obtained from goat (*Capra aegagrus hircus*) (S2) and buffalo (*Bubalus bubalis*) (S5), dairy products such as fermented home-made curd from cow, *Bos taurus* (S3), fermented batter of rice grain (S4), fresh cream (S6), paneer (S7) and butter (S8). Curd, butter and fresh cream were prepared from cow milk. The samples were collected in sterile containers and were aseptically transported to the laboratory. All the samples were processed within 4 h of collection after refrigeration at 4 °C.

All the media components used were supplied by Hi-media, Mumbai India. Low molecular weight markers with a range of 2.7–18 kDa (SDS7B2) were supplied by Sigma Aldrich, India. All chemicals used were of analytical grade and standard quality.

2.2. Isolation and enumeration of Pediococcus spp. from the selected samples

One ml of collected milk and other samples (S1–S8) were serially diluted (1: 10) and plated on *Pediococcus* selective medium (PSM) with ampicillin (Simpson et al., 2006) by pour plate method (Fankhauser, 1989). After 48 h at 30 °C, the plates showed bacterial colonies were enumerated (CFU/g) by digital colony counter and the pure cultures of the selected bacteria were prepared by inoculating the colonies on nutrient agar slants and nutrient broth.

2.3. Microbiological characterisation

The isolated colonies were streaked on nutrient agar by standard technique (Pelczar and Reid, 1958). Gram staining and motility test were performed to study morphological characteristics of the bacteria. In addition to PSM medium, the phenotypic characteristics of the isolates were studied by plating the isolates on various selective media such mannitol salt agar (Kateete et al., 2010) and blood agar (Buxton, 2003). The bacteria that demonstrated Gram positive, non-motile cocci in tetrad and other the standard phenotypical characteristics of *Pediococcus* spp. were selected for further biochemical analysis as per Bergey's manual (Bergey et al., 1974).

2.4. 16S rRNA gene sequencing

The pure isolate that showed the morphological and phenotypic characteristics of *Pediococcus* spp. and demonstrated predominant growth in PSM and mannitol salt agar were selected for 16S rDNA characterisation. The amplification of 16S rRNA gene from the pure isolate was carried out by polymerase chain reaction using the primers 16SF: AGAGTTTGATCCTGGCTCAG and 16SR: ACGGCTACCTTGTTACGACTT by standard protocol (Turner et al., 1999). The amplified 16S rDNA gene was subjected to agarose gel electrophoresis (1.8% agarose) and visualized by ethidium bromide staining. Forward and reverse DNA sequencing of the amplicon was carried out on ABI 3730xl Genetic Analyzer (Applied Biosystem). The 16S rDNA sequence was subjected to similarity searching by BLAST search (www.ncbi.nlm. nih.gov/BLAST) to identify the best homologous sequences. The evolutionary relatedness of these sequences was performed by constructing a phylogram by neighbour joining approach with bootstrapping and the phylogram was visualized by Tree View software. The 16S rRNA sequence was deposited to GenBank (www.ncbi.nlm.nih.gov/genbank) and assigned an accession number KY673793.

2.5. Extraction and partial purification of the protein from Pediococcus spp.

Pediococcus spp. were inoculated in tryptone glucose yeast extract (TGE) broth and kept in shaker incubator (Labronics, India) (300 rpm) for 48 h at 30° C. After incubation, cells were separated from the growth medium by centrifugation at 15,000 rpm for 15 min at 4 °C. The cell-free supernatant was adjusted to pH 6.5 by a pH meter. The separation of extracellular bacteriocin from the supernatant was performed by solvent extraction method (Burianek and Yousef, 2000). The crude extract thus obtained was used for further study.

The presence of protein was detected by Millon's test and Biuret assay (Walsh, 1961; Switzer and Garrity, 1999). The partial purification of the protein present in the supernatant was achieved by ammonium sulphate precipitation (Mandala et al., 2014). The amount of protein present in the sample was estimated by Lowry's assay (Lowry et al., 1951). The approximate weight of the expected bacteriocin was determined by SDS PAGE (Schagger and Von Jagow, 1987). Approximately 20 µl (v/v) samples were loaded in the wells prepared in the stacking gel. The sizes of molecular markers used were in the ranges of 2.7–18 kDa (SDS7B2).

2.6. Study of antibacterial potential of the protein extract

The clinical isolates of *E. coli*, *Shigella* spp. and *Streptococcus* spp. were obtained from the Department of Pathology, Sagar Hospitals, Bangalore, India. The antibacterial activity of partially purified protein extract towards these isolates was performed by well diffusion assay. The lawn cultures of the clinical isolates were prepared on Mueller Hinton agar plates (Hi-media, India). Approximately, 25, 50, 75 and 100 µg/l of ethanol extract of the partially purified protein was applied in each well. The pH of the culture media was neutralized before the antimicrobial testing. Ethanol was used as the control. All the plates were incubated at 30 °C for 48 h in a bacteriological incubator. After incubation, the diameter of the zone of inhibition observed against each isolate was measured.

2.7. Study of extension of shelf life

One kilogram (1 kg) of three sets of fresh strawberries, tomatos, fish, button mushrooms, fresh meat and corn samples were separately prepared for shelf life extension studies. The samples were selected based on their importance in various South Indian culinary preparations. Five, ten and fifteen ml of freshly prepared (optical density 0.90) culture of the isolated *Pediococcus* spp. were sprayed on to set 1, set 2 and set 3 respectively, which were kept in zip lock pouches (with holes) after the neutralization and elimination of cells from the growth media. The volume of bacterial culture sprayed was with respect to w/v ratio to the food sample taken for the study. The culture broth used for spaying the food samples was freshly prepared, incubated for 48 h and the viable bacterial count was expected to be 1.98×10^7 CFU/ml. The cells were removed after neutralization as the expected antibacterial protein was extracellular. Three sets of the samples were prepared as test and one

set of sample was maintained as control for comparative analysis and the cell free supernatant was applied uniformly by a sterile sprayer. All the samples were stored at room temperature (approximately 28–30 °C) for a period of 20 days and were monitored on daily basis and observed the physiological changes. Based on the best result observed in the previous step using all the samples, the optimum concentration for enhanced preservation was studied on the three sets of selected samples such as corn and tomato by spreading the cell free supernatant of 5, 10, 15 and 20 ml uniformly by a sterile sprayer for a time period of 20 days at 27–30 °C.

2.8. Study of the preservation efficiency of biopreservatives in comparison with chemical preservatives

Two FDA approved chemicals such as sodium benzoate and sodium sulphite (FDA Sec. 182.3798) were selected to compare the preservation efficiency and storage life of biopreservatives. Approximately 15 mg/l of supernatant with antibacterial protein prepared from the fresh culture of *Pediococcus* spp. and 15 ml (15% w/v) chemical preservatives were separately applied on three set of 1 kg selected food samples after surface sterilization and stored in different zip lock bags and kept for 20 days at room temperature (28–30 °C). The samples without any bacterial culture supernatant or chemical preservatives were used as the control. A mass: volume ratio of the test and control preservatives was maintained with respect to the food samples. The samples and controls were kept at room temperature (28–30 °C) and the physiological changes of the samples and controls were monitored on regular basis.

2.9. Determination of bacterial count in the food samples sprayed with biopreservatives

After the storage period of 20 days, the viable bacterial count (CFU/g) present in the stored food samples that applied with 15 ml (15% w/v) cell free supernatant from *Pediococcus* spp., chemical preservatives and control samples were enumerated. The haemolytic patterns produced by the isolated bacteria from all the food samples were also analysed by plating the samples on blood agar.

2.10. Statistical analysis

The experimental protocols mentioned in the study were replicated as three independent trials and the variations in the experimental data observed during these trials were subjected to statistical analysis by ANOVA at $p \leq 0.05$ by SPSS software.

3. Results and discussion

3.1. Microbial counts and molecular characteristics of Pediococcus spp. in dairy products

The phenotypic characteristics and the viable bacterial count (CFU/ml) obtained on PSM from all the tested samples are shown in Table 1. Among various samples used for the isolation of *Pediococcus*, bacterial colonies were isolated from S1, S2, S3 and S4. The table indicated that the viable bacterial count obtained S1, S2, S3 and S4 were estimated to be $1.38 \pm 0.2 \times 10^3$, $1.47 \pm 0.3 \times 10^3$, $4.4 \pm 0.2 \times 10^3$ and $1.68 \pm 0.3 \times 10^3$ CFU/ml respectively. However, the colonies that resembling *Pediococcus* spp. was found only from S3. The colonies were found to be small, convex, round, off-white or grey to cream coloured in PSM medium, which are the colony characteristics of *Pediococcus* spp. There were no colonies isolated from S5, S6, S7 and S8 at 30 °C for 48 h. This was probably due to the environmental conditions used or non-fermented dairy and non-dairy samples, thus, probability of desired bacterial isolates might have reduced. PSM medium favoured the selective isolation of *Pediococcus* spp. and ampicillin supplemented in the

Table 1
The number of bacterial isolates (CFU/ml) obtained from various dairy products by serial dilution method.

Sample	Code	Source	Number of colonies (CFU/ml) observed in PSM[a,b]	Colony characteristics on PSM[a]
Milk	S1	Pasteurized (supplemented with probiotics)	$1.38 \pm 0.2 \times 10^3$	Big, round shaped, cream coloured convex colonies
Goat milk	S2	Goat (*Capra aegagrus hircus*)	$1.47 \pm 0.3 \times 10^3$	Small, circular, grey coloured colonies
Fermented home made curd	S3[c]	Cow (*Bos taurus*) milk	$4.4 \pm 0.2 \times 10^3$	Small, circular, white to cream coloured convex colonies
Idly batter	S4	Rice grain	$1.68 \pm 0.3 \times 10^3$	Small, smooth, round shaped, white coloured colonies
Buffalo milk	S5	Buffalo (*Bubalus bubalis*)	No growth	–
Fresh cream	S6	Cow (*Bos taurus*) milk	No growth	–
Panner	S7	Cow (*Bos taurus*) milk	No growth	–
Butter	S8	Cow (*Bos taurus*) milk	No growth	

[a] Pedicoccus selective medium.
[b] The variations observed in the independent replicates were found to be statistically significant ($p \leq 0.05$).
[c] Table suggested that the bacteria isolated from sample S3 were resembled with phenotypical characteristics of *Pediococcus* spp.

Table 2
Morphological and biochemical features of the isolates from the sample S3, fermented home-made curd from cow *Bos taurus*.

Samples	Percentage of isolates in PSM	Growth	Mor[a]	Mo[b]	I[c]	Mr[d]	Vp[e]	C[f]	U[g]	O[h]	Ct[i]	Mf[j]	Ts[k]	Hs[l]	Nr[m]	Sh[n]	Ah[o]
Home-made curd	90%	[aa]FA	Gram Positive cocci in tetrad[cc]	Non-motile	–	–	–	–	–	–	–	–	+	–	–	–	+

[a] Morphology.
[b] Motility.
[c] Indole.
[d] Methyl red.
[e] Vogus Proskauer.
[f] Citrate utilization.
[g] Urease.
[h] Oxidase.
[i] Catalase.
[j] Mannitol fermentation.
[k] Triple sugar (G,L, S) fermentation.
[l] Hydrogen sulphide production.
[m] Nitrate reduction.
[n] Starch hydrolysis.
[o] Amino acid hydrolysis (Lysine, Arginine, Ornithine).
[aa] Facultative anaerobic.
[cc] Positive for Arginine and lysine hydrolysis.

medium favoured the inhibition of *Lactobacillus* spp., a major Gram positive bacilli probably present in the selected dairy sample. Previous studies revealed that PSM is one of the best selective media for the isolation of *Pediococcus* spp. (with the colony characteristics such round shaped, off-white to cream coloured colonies) and the media supported the rapid isolation of this bacteria than growth observed on the MRS agar (Leuschner et al., 2003). Previous studies reported various lactic acid bacteria of similar kinds were isolated from milk samples such as cow, goat, sheep, camel and buffalo (Sharma et al., 2013) and various fermented foods (Cai et al., 1999).

The biochemical characteristics of the isolate are shown in Table 2 which suggested that the bacteria belonged to the genus *Pediococcus*. The morphological features of isolates from S3 was found to be Gram positive and non-motile cocci arranged in tetrad, characteristic morphological features of *Pediococcus* spp. On mannitol salt agar, small, circular, convex and yellow coloured colonies were observed which suggested that the bacteria were able to ferment mannitol. Most of the *Pediococcus* spp. ferment carbohydrates such as mannitol (Hawaz, 2014). When plated on blood agar, there were non-haemolytic activities exhibited by the isolates which suggested that these were non-haemolytic bacteria, most of the pediococci belonged to non-haemolytic bacteria (Dhas and Hena, 2012). Thus, the isolated bacteria morphologically related to *Pediococcus* spp. (Nikita and Hemangi, 2012; Karska-Wysocki et al., 2010).

The similarity searching performed by BLAST depicted that 16S rDNA sequence of the bacteria showed 99% identity to the 16S rRNA sequence of *Pediococcus acidilactici* strain LH9. The phylogenetic relationship identified by neighbour joining (NJ) approach and boot strap analysis suggested that there was high homologous relationship between the isolate described in this study and 16S rRNA sequences of various *Pediococcus* spp. deposited in the database (Fig. 1). Hence, the 16S rRNA characterisation suggested that the isolated bacterium for the biopreservation study probably was a novel strain and *Pediococcus* sp. dscebt was proposed as the strain designation (Genbank accession KY673793).

The antimicrobial protein produced by *Pediococcus* spp. was extracted by standard protocol, which yielded three layered complex namely supernatant, interfacial and bottom layers. In the current study, the protein obtained in the supernatant was found to be the antimicrobial protein as reported previously (Reichardt and Eckert, 1991). The protein was further detected in the supernatant by Biuret assay and Millon's test.

When the extracted compound subjected to ammonium sulphate (which was removed later) precipitation at 45% saturation showed increase in the purity of the protein. Studies revealed that the inhibitory activity of the protein isolated from malted barley was precipitated from cell-free supernatant using 40% ammonium sulphate saturation and purified by chromatography (Vaughan et al., 2001). Similar studies revealed that, Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088 (NCK88) was purified by ammonium sulphate precipitation at 40% saturation and resulted in a 474 fold increase in specific activity. The protein estimated from the supernatant and pellets were subjected to ammonium sulphate precipitation that increased the purity. Lowry's assay (Lowry et al., 1951) confirmed the proteinnaceous nature of the extracted compound in which the amount of protein present in supernatant was estimated to be approximately 87 μg/ml. The protein can be further purified by gel filtration and affinity chromatography.

The SDS PAGE revealed that weight of the protein obtained was found to be approximately 3 kDa (Fig. 2), a low molecular weight protein (Stoffels et al., 1992; Tulini et al., 2011) which is probably a bacteriocin, which are low molecular weight proteins. However, it is suggested that the PCR based screening for the structural genes needed to be performed to analyse the nature and presence of bacteriocin. There are studies suggested that low molecular weight peptides produced by many organism were found to be bacteriocin especially pediocins. The fold purification achieved in the current study might be lesser in comparison with previous findings (Muriana and Klaenhammer, 1991; Tulini et al., 2011) and the methodology can be probably modified to achieve better result.

3.2. Antibacterial potential of the protein extract

The diameter of zone of inhibition around the well which applied with 100 μg/ml of protein extract was measured to be 21 ± 2 and 18 ± 2 mm against the clinical isolates of *Shigella* and *E. coli*, respectively. However, there was no zone of inhibition observed around the well which was applied with 100 μg/ml of protein extract against the clinical isolate of *Streptococcus* spp. When the statistical analysis was performed, the variations observed in the measured zone of inhibition during independent trails found to be statistically significant ($p \leq 0.05$) (Table 3). The control plates were not showed zone of inhibition as observed in test samples, however, demonstrated a narrow zone diameter (3 mm) against test isolates which probably due to the antimicrobial activities of solvent used. Nevertheless, the current study revealed the antimicrobial potential of the protein extract isolated from

Fig. 1. 16S rDNA characterisation of the isolate from dairy sample S3. The phylogenetic tree obtained from the 16S rDNA genes of best homologous sequences by BLAST suggested that the isolate used in this study demonstrated 99% sequence identity to *Pediococcus acidilactici* strain LH9.

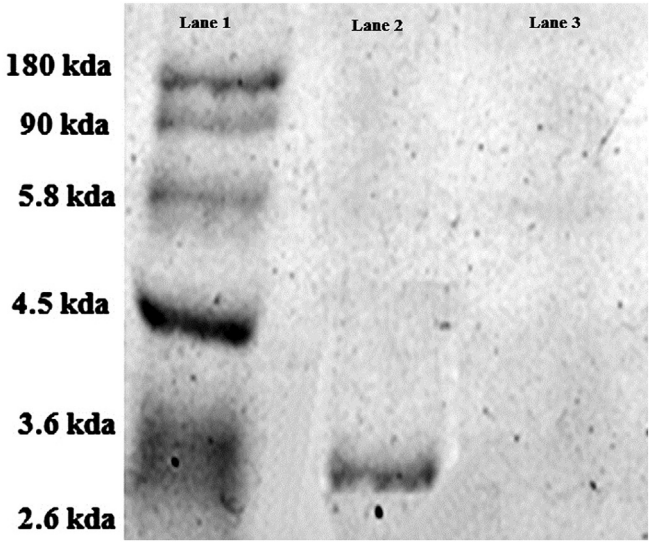

Fig. 2. The SDS page image of the antimicrobial protein obtained from *Pediococcus* spp. Lane A: molecular markers of size of 2.7–18 kDa (SDS7B2) Lane 2: supernatant; Lane 3: pellet. The figure suggested that the approximate size of antimicrobial protein present in the supernatant was approximately 3 kDa which is expected to be bacteriocin.

Pediococccus spp. The findings in current study are similar to that of the previous studies which showed the zone of inhibition obtained when the culture supernatant of *Bacillus mycoides* tested against *Leuconostoc mesenteroides* (Sharma and Gautam, 2007) and *Pediococcus pentosaceus* T1 (Porto et al., 2017). The purification steps and solvent systems are the major parameters that influence the antibacterial activities of various proteins (Joshi et al., 2006).

3.3. Study of shelf life extension of selected food samples

The best preservation was observed for the samples such as strawberries, tomato, corn, fresh meat and button mushroom that were applied with 15 ml of cell free supernatant of *Pediococcus* spp. isolated from sample S3. The fresh meat showed shelf life of 4 days after the application of the cell free supernatant in comparison with control (n = 1), which was spoilt within 24 h with green coloration. Strawberries, tomatoes, corn and button mushrooms were showed the shelf life post application of 15 ml culture cell free supernatant of *Pediococcus* spp. in comparison with controls (n = 4) (Fig. 3). Fungal growth was observed on tomato control on fourth day followed by loss in the firmness. However, the tomato samples applied with cell free supernatant of *Pediococcus* spp. showed preservation efficiency until thirteen days at room temperature in comparison with the control. Although the low pH in the tomato can contribute the preservation capacity, the present study showed that tomato applied with the cell free supernatant of *Pediococcus* spp. showed enhanced preservation in comparison with the tomato control. Among, all the food samples tested, tomato samples demonstrated the best preservation.

The microbial counts enumerated in control samples (n = 5) were found to be high in comparison with the samples applied with cell free supernatant of *Pediococcus* spp. which was found to be statistically

Table 3
Antimicrobial potential of ethanol extract of protein extract on selected clinical isolates.

Test bacteria	Ethanol extract of protein (µg/ml)	Diameter of the zone of inhibition (mm)[a]	
		Test	Control
E. coli	25	0	0
	50	4.0 ± 1.0	0
	75	8.0 ± 2.0	0
	100	18.0 ± 2.0	3
Shigella sp.	25	0	0
	5	3.0 ± 2.0	0
	75	8 0.0 ± 3.0	0
	100	21.0 ± 2.0	4
Streptococcus sp.	25	0	0
	50	0	0
	75	0	0
	100	0	0

[a] The variations observed in the independent replaces were found to be significant ($p \leq 0.05$).

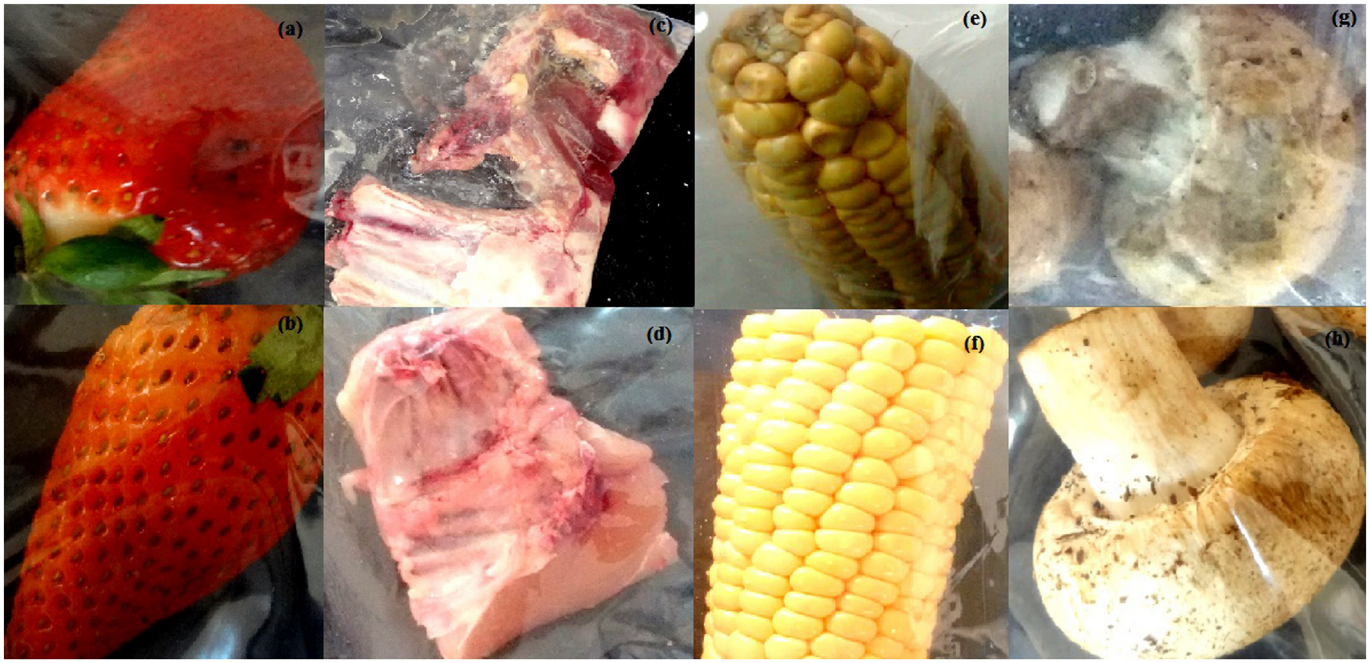

Fig. 3. The preservation efficiency of cell free supernatant of *Pediococcus* spp. on selected food samples. Figure a, c, e, g indicates control samples and b, d, f and h indicate the physical features and freshness of the food samples supplemented with the cell free supernatant of *Pediococcus* spp. This picture was taken after the study duration mentioned in the methodology.

Table 4
Microbiological quality of the food samples supplemented with 15 ml of cell free supernatant of *Pediococcus* spp.in comparison with 15 ml (15% w/v) chemicals applied food samples.

Sample	[a]Number of viable bacterial count (CFU/g)	Hemolysis in blood agar
Tomato sample applied with cell free supernatant of *Pediococcus* spp. (15 ml/kg)	$1.4 \pm 0.2 \times 10^3$	- - -
Corn sample applied with cell free supernatant of *Pediococcus* spp. (15 ml/kg)	$1.73 \pm 0.2 \times 10^3$	- - -
Strawberry applied with cell free supernatant of *Pediococcus* spp. (15 ml/kg)	$1.9 \pm 0.2 \times 10^3$	- - -
Mushroom sample applied with cell free supernatant of *Pediococcus* spp. (15 ml/kg)	$1.6 \pm 0.3 \times 10^3$	- - -
Meat samples applied with cell free supernatant of *Pediococcus* spp. (15 ml/kg)	$1.8 \pm 0.2 \times 10^3$	- - -
Control samples (15 ml/kg)		
Tomato	$6.1 \pm 0.4 \times 10^5$	+ + +
Corn	$7.61 \pm 0.4 \times 10^5$	+ +
Strawberry	$8.4 \pm 0.5 \times 10^6$	+ + +
Mushrooms	$6.14 \pm 0.4 \times 10^6$	+ + +
Meat	$8.9 \pm 0.3 \times 10^6$	+ + +
Tomato samples applied with sodium benzoate (15 ml; 15% w/v)	$7.4 \pm 0.4 \times 10^5$	- - -
Tomato samples applied with sodium sulphite (15 ml; 15% w/v)	$6.98 \pm 0.2 \times 10^5$	- - -

+ + + High level.
+ + Moderate level.
_ _ _No hemolysis.
[a] The variations observed in CFU/g in independent replaces were found to be significant ($p \leq 0.05$).

significant ($p \leq 0.05$) (Table 4). Among all food samples tested with biopreservative, the best shelf life enhancement was observed for corn and tomato samples post application of biopreservatives. The corn control remained fresh for six days, after which black coloration along with loss in the firmness was observed in few corn kernels. Further, the samples were completely degraded and spoiled. The corn samples applied with 5, 10 and 15 ml of cell free supernatant of *Pediococcus* spp. were found to be fresh for seven, sixteen and twenty days, respectively. However, the samples sprayed with 20 ml of culture supernatant were spoiled after 17 days. This is probably due to the increased water content that might have an influence on the spoilage. The amount of culture supernatant of *Pediococcus* required for the extension of the storage life for 1 kg of tested samples was found to be 15–20 ml. As it is not accurate information, the bacterial count (CFU/ml) and the amount of antimicrobial protein present in the culture supernatant needed to be investigated and further studies are required to make precise conclusion. In the case of tomato, fungal growth was observed on the control after six days. In comparison with various quantities, the application of 15 ml/kg cell free supernatant of *Pediococcus* spp. was found to be ideal for tomato sample as it preserved tomato for thirteen days (Fig. 4). The tomato sample sprayed with 10 and 20 ml/kg culture showed storage life of 11 and 10 days, respectively. Hence, it can be suggested that culture supernatant of *Pediococcus* spp. required for the activity mainly depends on type and quantity of the food sample (Mcauliffe et al., 1999). Studies suggested that the application of *Pediococcus pentosaceus* T1 caused the inhibition of *Listeria* sps in Salmon fillets and controlled the maturation of Kimchi (Porto et al., 2017). As *Pediococcus* is a non- pathogenic bacterium, probably probiotic, the direct application of the protein content in the supernatant might not cause any issues in nutritional quality of the food (Kaur et al., 2014; Mikulski et al., 2012). Studies demonstrated that antibacterial protein from lactic acid bacteria enhanced the shelf life of various fruit products

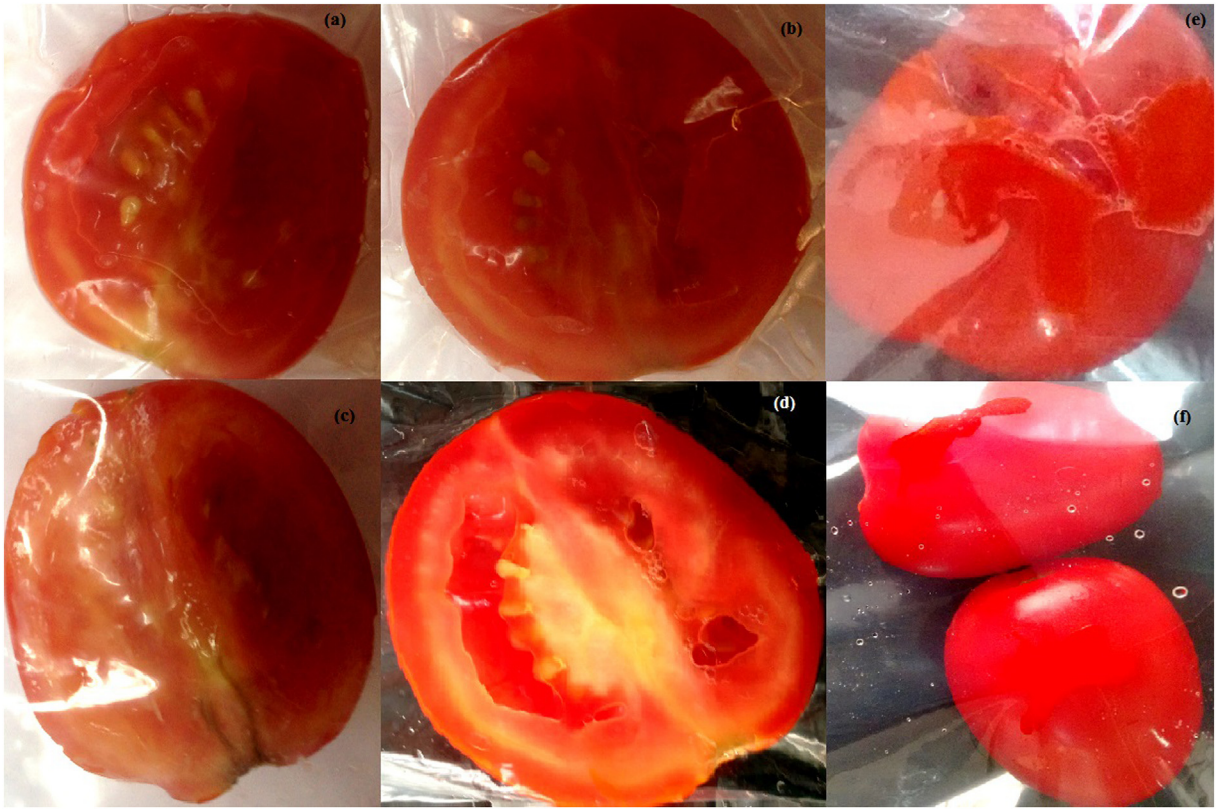

Fig. 4. The preservation efficiency of 15 ml cell free supernatant of *Pediococcus* spp. towards selected food sample (per kg) in comparison with chemical preservatives. (a) The tomato sample applied with sodium sulphate (15% w/v) spoiled within 10 days. (b) The tomato sample applied with sodium benzoate (15% w/v) spoiled within 8 days. Figure (c) and (d) shows that control sample spoiled within 4 days. (e) and (f). The tomato samples applied 15 ml cell free supernatant of *Pediococcus* spp. showed an increased shelf life of 20 days.

and corn stover silage (Barbosa et al., 2017; Li et al., 2015). The current study drawn conclusion based on the observation in the physical changes of selected food samples post application of cell free supernatant followed by preliminary bacterial enumeration (CFU/ml). Hence, more accurate and scientific methods are required to appreciate the preservation potential of biopreservatives prioritised din this study.

3.4. Comparative studies of preservation efficiency against chemical preservatives

The tomato samples (per kg) sprayed with 15 ml/kg of cell free supernatant of *Pediococcus* spp. remained fresh for 14 days in comparison with the tomato samples sprayed with 15 ml (15% w/v) of sodium sulphite and sodium benzoate (Table 4). The tomato samples sprayed with sodium sulphite and sodium benzoate were spoiled within 10 and 8 days, respectively at room temperature. The control was spoilt within 4 days (Fig. 4). When the experiments replicated as independent trails, the data were found to be statistically significant ($p \leq 0.05$). Previous study revealed that application of antimicrobial protein producing *Lactococcus lactis* AP2 towards orange juice demonstrated better preservation in comparison with the application of sodium benzoate (Pratush et al., 2012). Hence, the current study suggests that the cell free supernatant of *Pediococcus* spp. possess better preservation efficiency in comparison with chemical preservatives.

3.5. Microbiological quality of biopreservative applied food samples

When tested for the viable bacterial count, the control and test samples applied with 15 ml (15% w/v) chemical preservatives showed greater number of bacterial count in comparison with the food samples applied with 15 ml/kg cell free supernatant of *Pediococcus* spp. The viable count (CFU/g) for all the samples is shown in Table 4. From the microbiological analysis, it was clear that the viable bacterial count estimated for the food samples that were applied with the chemical preservatives was higher in comparison with the food samples treated with cell free supernatant of *Pediococcus* spp. but lesser viable bacterial count than the control samples. This might a probable indication that chemical preservative might not have high microbicidal activities and might contribute the growth of salt tolerant bacteria in food items. There are reports revealed that the continuous application of chemical preservative may promote the growth of resistant bacterial strains (Er et al., 2014). However, the biopreservative applied food samples showed significantly reduced microbial count which revealed the antimicrobial potential of cell free supernatant of *Pediococcus* spp. The lesser number of bacterial counts from biopreservative applied food samples (even though surface sterilization applied) might be due to the presence of other non-spoilage bacterial flora present in the tested food samples. The variations observed in the CFU/g in independent replicates were found to be statistically significant ($p \leq 0.05$). Further, there were hemolytic activities demonstrated by the bacterial isolates from the food samples that were treated with chemical preservative. However, the samples applied with cell free supernatant of *Pediococcus* demonstrated growth inhibition of hemolytic bacteria, which indicate the anti-hemolytic properties of the biopreservative. Although the application of *Pediococcus* spp. considered being safe, additional tests such as antibiotic susceptibility, production of gelatinase, mucin degradation, presence of genes encoding virulence factors and antibiotic resistance, etc. are essential to confirm the safety of the selected strain. The reduced number of viable bacterial count in the cell free supernatant treated food samples might be an indication of the microbiological quality of food samples. Hence, this study emphasizes wide scope and application of antimicrobial protein producing *Pediococcus*

spp. as potential bio-preservative in food industry.

4. Conclusions

The present study suggested that the application of cell free supernatant of antimicrobial protein producing *Pediococcus* spp. which can be used as biopreservative in comparison with traditional chemical preservatives. The current study drawn conclusions based on the direct physical observation and limited microbiological analysis. Hence, other standard approaches are required to confirm the increased shelf life and preservation efficiency by the isolated bacteria towards selected food products. The sensorial changes in the food that received the cell free supernatant which could change the taste and flavor of food needed to be studied.

Declaration of conflicting interest

No potential conflict of interest was reported by the authors.

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