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*Full Length Article*

# Utilization of Fermentatively Produced Antimicrobial Peptide-Silver Nanoconjugates against Selected Bacterial Pathogens

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## Abstract

In an attempt to curb the health and environmental hazards of chemical food preservatives, a continuous search for natural alternatives is going on continuously. Despite many reports on antagonistic activity of antimicrobial peptides (AMPs) or bacteriocins, certain shortcomings including narrow spectrum of activity and requirement of high dosage hinder it from becoming a practicable approach. Bioconjugation of AMPs with silver nanoparticles is proposed as a resourceful means for enhancing the antimicrobial potential of AMPs. The present study was conducted for utilization of biogenically synthesized antimicrobial peptide (AMP)-silver nanoconjugates produced from Lactic Acid Bacteria (LAB). For this purpose, a culture of *Lactobacillus sp.* was isolated, partially purified and its activity was assessed. Biogenic synthesis of AMP-nanoconjugates was carried out along with optimization of concentration ratio, temperature and pH. Lastly, antimicrobial activity of AMP-AgNPs was evaluated using disc diffusion assay against six indicator bacteria including *Escherichia coli*, *Salmonella sp.*, *Staphylococcus aureus*, multi drug resistant (MDR) *Klebsiella sp.*, *Bacillus subtilis* and Methicillin-resistant *Staphylococcus aureus*. Results of in vitro activity showed synergistic antibacterial efficacy of AMP-AgNPs as their activity was considerably greater than that of individual activity of partially purified AMP. The biogenically synthesized AMP-nanoconjugates showed remarkable antibacterial potential against all bacteria especially against MRSA and MDR *Klebsiella sp.*, which is a steppingstone in use of these composites against drug resistant microorganisms in wide ranging industrial applications. © 2024 Friends Science Publishers

**Keywords:** Bacteriocins; Antimicrobial peptides; Bacteriocin-nanoconjugates AgNPs; Green synthesis; Lactic acid bacteria

## Introduction

Microbial food spoilage is not only a fundamental concern with regard to human health but also renders significant economic losses to the food industry. Food borne diseases occur as a consequence of consumption of food spoiled due to contamination with microorganisms such as bacteria, fungi and viruses or the toxins secreted by them. To date, more than 250 food borne infections and intoxications have been identified (Sidhu and Nehra 2020). It has been reported that an estimated 30% of people residing in urban areas suffer from a food borne disease and around 420,000 people lose their lives to these diseases annually (Ghanbari and Jami 2013). Among microbes, bacteria are known to be one of the most common causes for food spoilage and the food borne diseases resulting from it. Some of the major bacterial food borne pathogens include, *Clostridium botulinum*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas* sp., *Salmonella* sp. and *Klebsiella* sp. (Bourdichon and Rouzeau 2012; Wang *et al.* 2017). The prevention and control of bacterial food borne pathogens is not only essential for increasing the shelf life of food items, but also aims to minimize losses to the food industry (Singh 2018).

Conventionally, the use of chemical food preservatives is the most commonly opted preservation approach in the food industry. However, this is associated with a number of injurious effects on human health ranging from headaches, allergies, palpitations, indigestibility to even cancer (Sharma 2015). The modern-day consumers are far more conscious about incorporating natural, healthy and minimally processed foods in their everyday diet to complement their healthy lifestyles (Juodeikiene *et al.* 2012). This growing inclination and demand of consumers for organic and chemical free food coupled with the increasing regulatory demands has urged the food industry to look into innovative solutions in order to

stay relevant in the business. In this context, the rationale of biopreservation comes into play. Naturally occurring, Lactic Acid Bacteria (LAB) and bacteriocins produced by them are of key importance in this respect and have therefore gathered a lot of attention in the scientific community (Singh 2018). LAB possess a GRAS (generally regarded as safe) status and hold major significance in the preservation of foods and fermented items. Additionally, some of them are known to produce small bioactive or antagonistic peptides, called bacteriocins, which have great activity against bacterial food borne pathogens (Deraz *et al.* 2005; Agriopolou *et al.* 2020). Bacteriocins can be defined as, small ribosomally synthesized antimicrobial peptides (AMPs) produced by bacteria which are capable of killing or inhibiting the growth of other bacteria that are either closely related bacteria (Cotter *et al.* 2005). During the past few years, a lot of interest has gathered around bacteriocins and bacteriocin-like inhibitory substances (BLIS) produced by LAB owing to their prospective application as natural antimicrobial for food safety applications (Deraz *et al.* 2005). LAB bacteriocins are seen as a favorable candidate in this respect due to many reasons including their widely known probiotic properties, non-toxicity to eukaryotic cells and a variety of mode of actions (Egan *et al.* 2016). Moreover, they are gene encoded and therefore, highly acquiescent to genetic manipulation as and when required (Field *et al.* 2015).

Even though bacteriocins fulfill the consumer demand for minimally processed and chemical free food, certain limitations including restricted antimicrobial spectrum of bacteriocins with activity against only closely related bacteria (Dhanam *et al.* 2021). Secondly, bacteriocins are reported to show sensitivity towards proteolytic enzymes (Siddiqui *et al.* 2023). Further, a high dose of bacteriocins is necessary especially in order to inhibit resistant bacteria (Saravana and Annalakshmi 2012). Moreover, a low yield is usually obtained due to ineffective recovery after purification steps (Sidhu and Nehra 2019). The aforementioned shortcomings prompt scientists to find effective solutions for overcoming the limitations of bacteriocins in order to tap their full potential as natural food preservatives. In this context, the use of nanotechnology shows a lot of promise. Nanoparticles (NPs) are described as particulate dispersions or solid particles with a size in the range of 10-1000 nm (Feynman 2018). Thus, they are in the similar size range as that of biological molecules and structures and are therefore very efficient for detection and manipulation of biological systems. They possess a large surface area in contact with the microorganisms, this is believed to be their main property contributing to their antimicrobial activity as it permits greater interactions and makes them flexible for multiple applications (Martinez-Gutierrez *et al.* 2010). Among, inorganic NPs, silver nanoparticles have attracted a lot of attention because of its potent antibacterial activity against many pathogenic microorganisms like *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, etc. among many others (Fahim *et al.* 2016).

Silver nanoparticles (AgNPs) are famous for their activity towards most of the Gram-positive and Gram-negative bacteria. In nano form silver has a high affinity towards Phosphorus and Sulphur which is believed to be the major contributing factor for its antimicrobial property (Ravishankar and Jamuna Bai 2011). Bacterial cell membranes contain sulfur-containing proteins which facilitate and promote the interaction of silver nanoparticles with sulfur-containing amino acids present in or on outer cell surface, resultantly impacting the cell permeability (Sidhu and Nehra 2019). Lately, researchers have proposed that conjugation of nanoparticles with bacteriocins may prove effective in increasing the antimicrobial activity, stability and shelf life of food (Fahim *et al.* 2016).

As both bacteriocins and AgNPs display considerable antibacterial potential, therefore their conjugation is aimed at enhancing their activity. Further, this bioconjugation helps to overcome the limitations associated with sole use of bacteriocins and AgNPs. Therefore, the development of bacteriocin nano-conjugates by green synthesis holds a great potential for their use in the food processing and packaging (Fayaz *et al.* 2010). As silver nanoparticles increase the existing potential of bacteriocins owing to their own antimicrobial effectiveness, they have been suggested as the most appropriate candidate for conjugation with bacteriocins (Duncan 2011). The present study predicts that forming the composite of antimicrobial peptides and silver nanoparticles can enhance the overall antibacterial activity by producing a synergistic effect. Thus, the objectives of the current study include fermentative production and partial purification of antimicrobial peptide (AMP), synthesizing AMP-silver nanoparticle conjugates and finally assessing and comparing the antimicrobial efficiency of AMP nano-conjugates and AMP alone against indicator bacterial strains.

## Materials and Methods

### Isolation and basic identification of AMP-producing LAB

A HiFlora sachet (of approximately 2g) was dissolved in 10 mL of sterile Phosphate-buffered saline (PBS) and 100 $\mu$L of it was inoculated onto MRS agar plates and incubated at 37°C for 48 h under anaerobic conditions created using candle jar. The obtained colonies were checked by Gram staining and catalase test and streaked multiple times onto MRS agar supplemented with 0.05% w/v L-cysteine Hydrochloride, incubated at 37°C for 48 h in anaerobiosis to obtain pure culture which was maintained at 4°C. Stock cultures were maintained at -80°C in LB medium containing 20% v/v glycerol.

### Fermentative production of bacteriocins

**Inoculum preparation:** Loopfuls of a fresh culture of *Lactobacillus sp.* was inoculated in 30 mL MRS broth and shaken at 37°C and 150 rpm for approximately 3 h to obtain

log phase culture. Bacterial growth was measured hourly by determining turbidity of the culture and in terms of optical density (OD) at 600nm by spectrophotometer until it attained $OD_{600nm}$ value of 0.6.

**Selection of optimum medium for bacteriocin production:**1 mL (2%) of the prepared inoculum (OD 0.6) was transferred aseptically in 50 mL of growth medium and shaken at 37°C and 150 rpm for 24 h (Sharma *et al.* 2010). For the purpose of selecting the best medium for bacteriocin production, different media were tested including, MRS broth, Elliker's broth, LAPTg broth, Brain Heart Infusion (BHI) broth, Tryptic Soy Broth (TSB) and Tryptone Glucose Extract (TGE) broth. at 37°C and 150 rpm for 24 h followed by determination of bacteriocin's antibacterial activity (Abo-Amer 2011).

**Optimization of temperature for bacteriocin production:**50 mL of sterile MRS broth and incubated at different temperatures including 25, 30, 37, 40 and 45°C in shaking incubators at 150 rpm for 24 h. Bacteriocin production at different temperatures was observed by testing against the sensitive strains (Malheiros *et al*. 2015).

**Optimization of pH for bacteriocin production:** 50 mL of MRS broth adjusted separately to different pH values including 4.0, 4.5, 5.0,5.5, 6.0, 6.5, 7.0, 8.0, 8.5 and 9.0 and incubated at 37°C and 150rpm for 24 h followed by determination of bacteriocin's antibacterial activity at each pH value (Iyapparaj *et al*. 2013).

**Antibacterial activity determination**

**Cell free supernatant (CFS) preparation:** After fermentative production of bacteriocins was carried out for 24 h, the culture broths were pH-neutralized to 7.0 in order to eliminate the inhibitory effect of organic acids. These were centrifuged at 6000 RPM and 4°C for 15 min to obtain clear supernatant, which was then filter sterilized using 0.20µm syringe filter to prepare CFS samples (Arakawa 2019).

**Broth dilution assay:** To determine the antibacterial activity of bacteriocins, macro broth dilution was carried out using method described by Cuozzo *et al*. (2001) with some modifications. For this, 1 mL of filter sterilized CFS, 200 $\mu$L of exponentially growing indicator (adjusted at OD600 0.6) and 800 $\mu$L of Nutrient broth were added to each tube, mixed well and incubated at 37°C for 18 h, after which the change in OD values was recorded using spectrophotometer. Ampicillin sodium salt and Streptomycin sulfate salt were prepared and used as positive control for Gram-positive and Gram-negative bacteria respectively. Distilled water was used as negative control.

**Partial purification of bacteriocins**

AMP was partially purified by Ammonium Sulfate precipitation and four fractions at 20,40,60 and 80% saturation were obtained by adding solid Ammonium Sulfate to the prepared CFS at 4°C under continuous slow stirring conditions using a magnetic stirrer. The precipitates obtained were centrifuged at 8000 rpm for 20 min at 4°C and the resultantly obtained pellet represented the crude AMPs which was completely dissolved in 20 m*M* phosphate buffer having pH 6.5 (Sidhu and Nehra 2020). In order to remove salt and other impurities from the crude bacteriocin, the resuspended pellets were dialyzed through pre-treated 1 kDa dialysis membrane, against 20 m*M* phosphate buffer overnight at 4°C and with the application of gentle stirring.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

The molecular weight of partially purified antimicrobial peptide was determined by SDS-PAGE. For preparing the sample, method described by Sidhu and Nehra (2021) was used with some modifications, briefly, 15 $\mu$L of partially purified antimicrobial peptide was mixed with 5 $\mu$L of loading dye and heated at 100°C for 10 min on boiling water. This was followed by allowing it to cool on crushed ice for 10 min. Finally, the sample was spun shortly in microcentrifuge. After complete polymerization of both gels, samples were loaded into the wells. The voltage was set across in the range of 80-120 Volts. After the full run, gel was removed carefully from within the plates and was placed in staining solution for 15 min. The stained gel was then washed well with distilled water and lastly it was allowed to stand overnight in de-staining solution. The de-stained gel was visualized on a clean SDS tray and the results were recorded.

**Biosynthesis of AMP-AGNP conjugates**

A 2 m*M* solution of silver nitrate ($AgNO_3$) was prepared by uninterrupted stirring for 3-4 h on a magnetic stirrer at room temperature under dark conditions. For the biogenic synthesis of AMP-AgNPs, the partially purified bacteriocin was mixed with the freshly prepared $AgNO_3$ solution in 1:9 ratio (v/v). The resultantly obtained solution was incubated under UV light of laminar air flow cabinet for a time period of 50 min at room temperature. After incubation, the reaction showed a color change occurring from light yellow to reddish brown which gave the preliminary indication for the successful formation of bacteriocin-nanoconjugates.

**Recovery of AMP-AgNPs**

After overnight incubation, the reaction mixtures were centrifuged at 10,000 rpm for 20 min using a tabletop centrifuge in order to completely pellet down the AMP-nanconjugates. The supernatant was discarded and the pellet was collected and washed 2-3 times using deionized water for ensuring complete removal of any unbound antimicrobial peptide or silver nitrate (Sidhu and Nehra 2020). Following this, bath sonication cycle of 1 h was carried out to attain a homogeneous mixture which was used for characterization and analysis.

**Optimization of synthesis conditions**

**Effect of concentration ratio:** The partially purified AMP was mixed separately with silver nitrate in different ratios including 0.5:9.5, 1:9, 1.5:8.5 and 2:8(v/v) and was then incubated for 24 h at room temperature.
**Effect of pH:** For studying the effect of pH, the partially purified AMP was mixed with fresh $AgNO_3$ solution in the ratio of 1:9 (v/v) and then pH adjustment of these mixtures was done at varying pH values, including 2, 4, 6, 8, 10 and 12 by using 1 N NaOH and 1 N HCl. These pH-adjusted solutions were irradiated in UV light for 50 min and then incubated at room temperature for 24 h.
**Effect of temperature:** UV- irradiated AMP-AgNPs reaction mixtures were incubated at different temperatures such as 25, 35, 45, 55, 65 and 75°C for a time period of 24 h.

**Characterization of AMP-AgNPs**

**UV-Visible Analysis:** The homogeneous sample was analyzed using UV-Visible Spectroscopy performed in the wavelength range of 200-800nm. Finally, the resultantly obtained spectrum were plotted on Origin Pro 2018 software.
**Fourier transform infrared (FTIR) spectroscopy:** Washed pellets were dried on a watch glass and were scraped off after drying. This dried sample of AMP-AgNPs was scanned using FTIR spectrophotometer in the range of 4000-500 $cm^{-1}$.

**Evaluation of antibacterial activity of AMP-AgNPs**

**Disc diffusion assay:** A 100 $\mu$L of each of the six indicator bacteria was inoculated evenly on pre poured MHA plates using sterilized cotton swab. Four discs were added per plate including that of partially purified AMP alone, AMP-nanoconjugates (70 $\mu g/\mu L$), antibiotic and autoclaved distilled water. The assay was performed in triplicates and the plates were incubated at 37°C for 24 h. Finally, the zone of inhibition obtained was recorded using a scale (Savadgo *et al*. 2004).

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

**MIC test:** The indicator organisms were grown overnight on nutrient agar slants at 37°C and this fresh growth was used to inoculate 8 mL each of sterile nutrient broth which placed in shaking incubator of 37°C to attain bacterial growth corresponding to an $OD_{600nm}$ value of 0.4±0.001, which served as inoculum. For determining MIC of antimicrobial peptide-nanoconjugates against the selected indicator microorganisms, the macro broth dilution method was used. Series of pre-sterilized test tubes were set in a test tube stand and labelled appropriately. For each indicator bacteria, firstly 2 mL of sterile nutrient broth was added in all the test tubes. Next, 2 mL of AMP-NPs homogenized suspension was added in the first test tube and two-fold serial dilution was carried out, wherein the media was mixed with suspension in the first tube and then 2 mL from this was transferred to the next tube and the same was done for all the consecutive tubes. 2 mL of this mixed solution was discarded from the last test tube. At last, all the test tubes were inoculated with 50 $\mu$L of the indicator bacteria and they were incubated at 37°C for 24 h. MIC test for all the selected pathogens was performed in replicates of three sets each. After incubation, microbial growth was observed both visually and optical density values were also recorded at 600nm using spectrophotometer from which the corresponding MIC values were determined.
**MBC test**: 100 $\mu$L of solution from each test tube was spread evenly on nutrient agar plates which were incubated at 37°C for 24 h after which the results were observed. The solutions were plated in triplicates. Following incubation, the lowest concentration with no visible bacterial growth was recorded as MBC value (Pandit *et al*. 2017).

**Statistical analyses**

All experiments were done in triplicates, and the data has been presented as mean±SD.

## Results

In the current study, isolation of AMP producing Lactic acid bacteria was done from commercially available lyophilized probiotic for diarrhea management. Profusely grown pure culture of *Lactobacillus sp*. with small, creamy white to white colored colonies was obtained after culturing on MRS agar supplemented with L-cystine HCl. As part of preliminary identification, microscopy confirmed Gram-positive long and slender rods. On the other hand, basic biochemical profiling showed catalase negative and oxidase negative results. Moreover, a strong fermentative odor was obtained which is characteristic of *Lactobacillus sp.*, owing to the production of organic acids.
**Optimization of growth parameters:** Three key growth parameters (growth media, temperature and pH) were optimized in order to identify optimum conditions for AMP production. Amongst all the six tested media, AMPs produced in MRS broth displayed the most potent antagonistic activity against all the four indicator bacteria, wherein the most effective inhibition was observed against *S. aureus*. (Fig. 1). Next, a range of temperatures from 25°C to 45°C was examined and the AMPs produced at 37°C displayed the strongest antibacterial activity against all the tested bacteria (Fig. 2). Finally, a range of pH values from 4-9 were examined by pH adjustment of MRS broth. The AMPs produced in the medium having pH 6.5 were seen to be most active against the indicator bacteria (Fig. 3). A sharp decrease in activity was noted as the pH increased further beyond 6.5 up till pH 9.
**Partial purification of AMP and determination of its molecular weight:** The culture broth was subjected to ammonium sulfate precipitation. Four fractions were obtained at 20, 40, 60 and 80% and their antagonistic activity

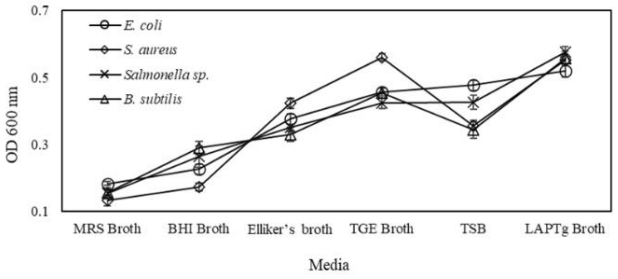


**Fig. 1:** Antibacterial activity of bacteriocins produced in different culture media determined by broth dilution assay
Volume of each medium 50ml, initial pH 6.0, size of inoculum 1 mL (2%) v/v and incubation time 24 h
The error bars indicate standard deviation (±SD) value amongst three replicates run parallel

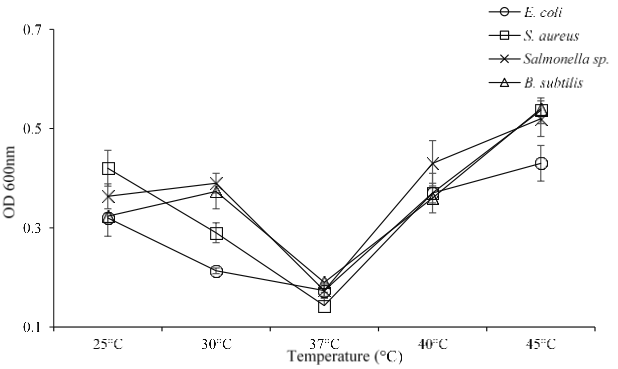


**Fig. 2:** Antibacterial activity of bacteriocins produced after incubation at different temperatures determined by broth dilution assay
Media volume 50 mL, initial pH 6.0, size of inoculum 1ml (2%) v/v and incubation time 24 h
The error bars indicate standard deviation (±SD) value amongst three replicates run parallel

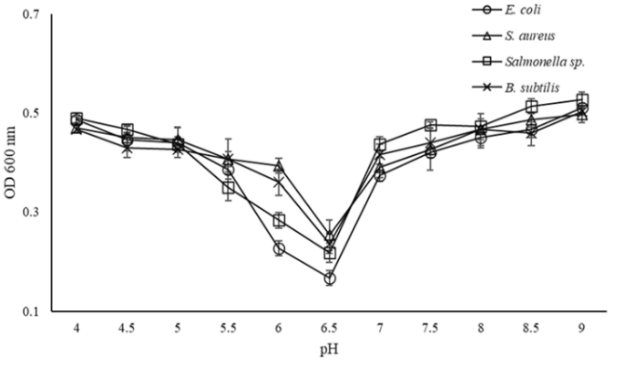


**Fig. 3:** Antibacterial activity of bacteriocins produced under varying initial pH values determined by broth dilution assay.
Media volume 50 mL, size of inoculum 1ml (2%) v/v, temperature 37°C and incubation time 24 h
The error bars indicate standard deviation (±SD) value amongst three replicates run parallel.

and protein content were determined by broth dilution assay and Bradford's assay respectively. For each fraction, both the protein content and he bacteriocinogenic activity against *S. aureus* was determined (Table 1). The AMPs precipitated in

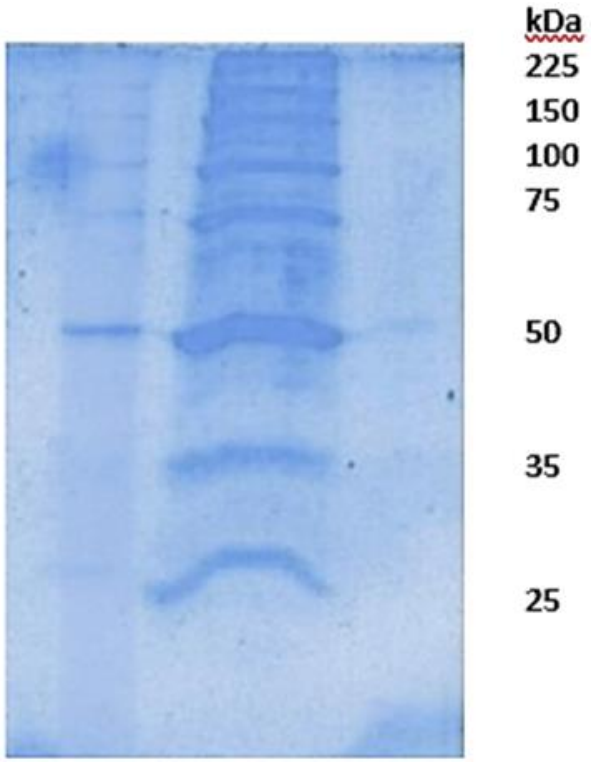


**Fig. 4:** Gel image of partially purified AMP visualized by SDS-PAGE (Left); Thermo Fischer Scientific ® Protein Ladder (Right)

40% fraction exhibited the strongest activity as well as highest protein content (123.90 $\mu$g/mL). Therefore, it was selected for further processing and was dialyzed overnight against 20 m*M* Potassium Phosphate buffer at 4°C. The molecular weight of partially purified AMP was determined by SDS-PAGE and protein band of 50 kDa was observed (Fig. 4).

**Optimizing the bioconjugation of AMPs and AgNPs:** The bioconjugation of AMPs and AgNPs is carried out by mixing the two components (see detail in Methods) followed by UV-irradiation, causing a color change from yellow to brownish red (Fig. 5) which confirms the formation of the composites. Various parameters are considered crucial in effective biosynthesis of AMP-AgNP conjugates and can make a marked difference in the stabilization of the conjugates. In the case of concentration ratios of the two constituents, AMP-AgNPs obtained after mixing of partially purified AMP and silver nitrate in 1:9 ratio gave the sharpest peak with an intense absorbance at a wavelength of 427 nm (Fig. 6a.). A pH range of 2 to 10 was examined and the UV-Visible analysis of AMP-AgNPs produced from each of the pH-adjusted reaction mixtures enabled the visualization of the sharpest peak at pH 10 (Fig. 6b.). It is evident from the UV-Visible spectrum that an apparent direct relationship between temperature and absorbance was found (Fig. 6c). The AMP-AgNPs synthesized under temperatures of lower range depicted low absorbance and broader peaks which are indicative of ineffective synthesis. The temperature of 65°C showed an intense peak at 428 nm.

**Table 1:** Antimicrobial activity and protein content of different Ammonium Sulfate fractions

| AS-fraction (% saturation) | Broth dilution assay against *S. aureus* (OD 600 nm) | Bradford assay (OD 595 nm) | Protein content (µg/mL) |
|---|---|---|---|
| 20% | 0.446 ± 0.02 | 0.828 | 90.60 |
| 40% | 0.193 ± 0.01 | 1.132 | 123.90 |
| 60% | 0.275 ± 0.01 | 1.021 | 111.70 |
| 80% | 0.332 ± 0.03 | 0.694 | 75.76 |

± indicates standard deviation value amongst three replicates

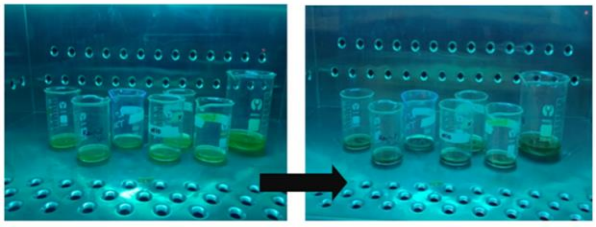

**Fig. 5:** Color change from yellow to brownish red obtained post UV-incubation indicating the effective biosynthesis of AMP-AgNPs conjugates

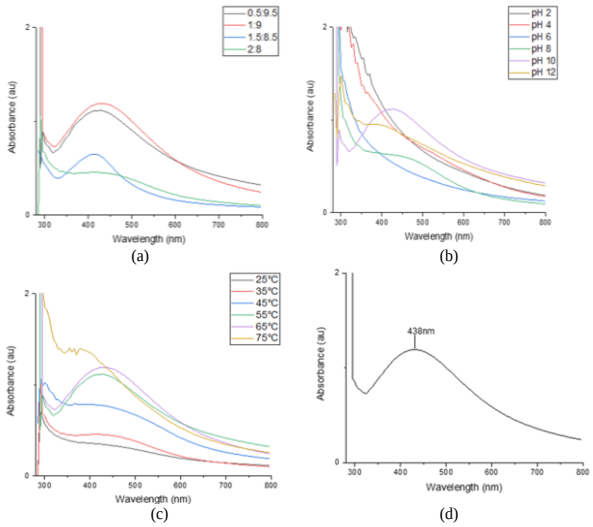


**Fig. 6:** (a) UV-Visible spectra of AMP-AgNPs obtained (a) at different ratios; (b) at different pH values; (c) at varying incubation temperatures; (d) A sharp peak at 438nm at optimized conditions (concentration ratio 1:9, pH 10 temperature 65°C). depicted successful formation of AMP-AgNPs
*UV-VIS Spectra plotted using Origin Pro 2018

**Characterization of AMP-AgNPs:** The final AMP-AgNPs synthesized as per optimum values were also analyzed by analyzed by UV-visible spectroscopy and showed an enhanced absorbance and well-defined peak at 438 nm (Fig. 6d). The bioconjugation of the partially purified AMP and silver nanoparticle, resulting in capping or adsorption of AMP on the surface of the nanoparticles was authenticated using FTIR spectroscopy in the range of 4000-500 $cm^{-1}$ and thus obtained spectrum is presented in Fig. 7. The spectrum shows different peaks which were found for AMP-AgNPs.

**Antibacterial activity determination:** Results of in vitro antibacterial activity show MRSA to be the most susceptible to the action of AMP-AgNPs, whereas no activity was produced by AMP alone. On the other hand, *S. aureus* was observed to be least susceptible to the action of AMP-nanoconjugates, with only slight improvement in activity in comparison to individual activity of partially purified AMP (Fig. 8). The results are noteworthy as, in vitro activity against multi drug resistant *Klebsiella sp.* was produced by both the AMP alone and in conjunction with silver nanoparticles. Individually, the AMP displayed low level of activity, which was improved significantly when AMP- nano conjugates were used. Moreover, the least susceptibility was recorded against *E. coli* as shown in Fig. 9. The MIC and MBC obtained for biosynthesized AMP-AgNPs against all six indicator bacteria is given in Table 2. The lowest MIC value was obtained for MRSA at 2 µg/mL, which indicates that the highest activity of AMP-nanoconjugates was observed against it. The highest MIC value was observed against *E. coli* at 64 µg/mL which indicates the lowest activity of AMP-AgNPs towards it. Similarly, with regard to the MBC, the lowest value was obtained for MRSA at 4 µg/mL. However, the highest MBC values were recorded for *E. coli* and *Salmonella sp.* at 64 µg/mL.

## Discussion

Over the years, isolation of LAB has been vastly carried out from dairy and fermented food products (Abo-Amer 2011; Adebayo-Tayo *et al.* 2017; Khadam *et al.* 2009). However, little evidence of utilization of lyophilized preparations is available in the literature. In the present study, a pharmaceutical sachet of was acquired from a local pharmacy in Lahore and its homogenized suspension was used for isolation. MRS agar was used as it is a selective media for Lactobacilli whereas L-cysteine HCl served as a reducing agent. A candle jar setup was created to mimic anaerobiosis to accommodate the facultative anaerobic nature of LAB.

Optimization of three basic growth parameters was done in order to identify optimum production conditions for the AMP. Out of the six media tested, MRS broth was found to be optimum, based on the results of its antagonistic activity against all the tested indicator bacteria. The suitability of MRS broth for antimicrobial peptide production can be attributed to the presence of a soluble blend of peptidic sources, salts, multiple energy sources and apt supply of micronutrients. A range of temperature from 25°C to 45°C was also examined. The results revealed 37°C to be the optimum temperature as shown by the pattern of antibacterial activity in Fig. 2. A trend of increase in antibacterial activity of crude AMPs with increasing temperature was noticed up till 37°C. However, increasing the temperature beyond 37°C resulted in drastic reduction of antibacterial activity of the crude AMPs. The results obtained can be attributed to the mesophilic nature of *Lactobacillus sp.* Similar results were

**Table 2:** MIC and MBC of AMP-AgNPs

| Indicator Bacteria | Minimum Inhibitory Concentration (MIC) (µg/mL) | Minimum Bactericidal Concentration (MBC) (µg/mL) |
|---|---|---|
| *E. coli* | 64 | 64 |
| *S. aureus* | 16 | 32 |
| *Klebsiella sp.* | 16 | 32 |
| *Salmonella sp.* | 32 | 64 |
| *B. subtilis* | 8 | 16 |
| MRSA | 2 | 4 |

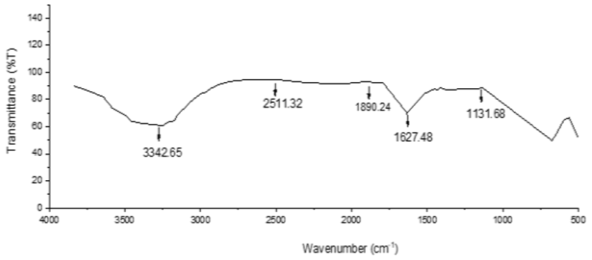


**Fig. 7:** FTIR spectrum of AMP-AgNPs

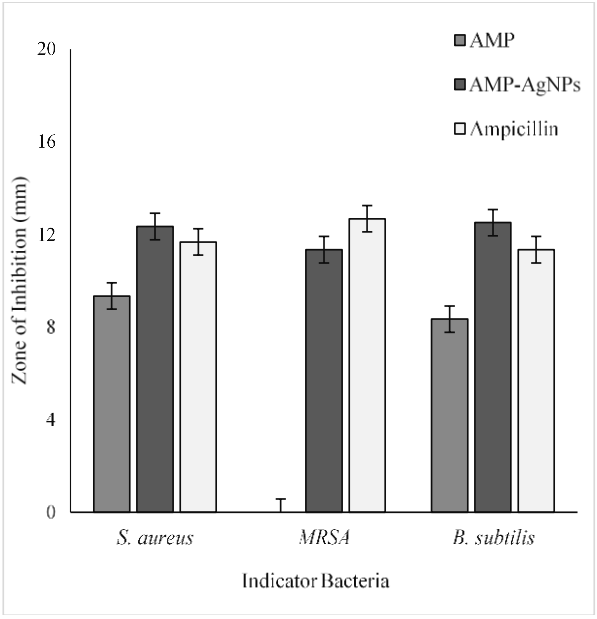


**Fig. 8:** Antibacterial activity of partially purified AMP and AMP-AgNPs against three Gram positive indicator bacteria determined by disc diffusion assay. Standard antibiotic disc (Ampicillin) was used as positive control

*Y-error bars (I) indicate standard deviation (±SD) among three replicates run parallel
*AMPs (Antimicrobial peptides) and AMP-AgNPs (Antimicrobial peptide-silver nanoparticle conjugates)

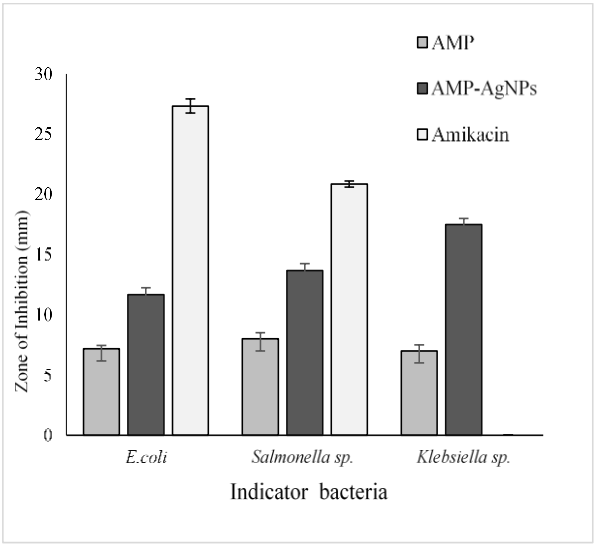


**Fig. 9:** Antibacterial activity of partially purified AMP and AMP-AgNPs against three Gram negative indicator bacteria determined by disc diffusion assay. Standard antibiotic disc (Amikacin) was used as positive control

*Y-error bars (I) indicate standard deviation (±SD) among three replicates run parallel
*AMPs (Antimicrobial peptides) and AMP-AgNPs (Antimicrobial peptide-Silver nanoparticle conjugates)

reported by Cheigh *et al.* (2002) who studied the influence of various parameters on the production of bacteriocin similar to nisin. The effect of initial pH was studied in a range of 4.0 to 9.0. Maximum activity against all the four indicators was produced in medium adjusted to pH 6.5 as depicted by the $OD_{600nm}$ values obtained post incubation. A relatively constant pattern of activity was observed up till pH 5.5, after which the activity increased with a pH of 6.0 and reached its maximum at 6.5. A substantial decline in activity was observed when initial pH of the medium was increased from 7.0 to 9.0. Abo-Amer (2011) also reported pH 6.5 as optimum for the biomass and bacteriocin production of *L. acidophilus*. He also reported a reduction in activity upon adjusting culture media to pH 7.0 and 4.5 which is similar to the present study.

Partial purification of the AMP was carried out using Ammonium sulfate precipitation method which involves graded fractionation. The fraction of 40% was found to have the greatest activity. *S. aureus* which was used as an indicator for broth dilution assay gave an $OD_{600nm}$ value of $0.193 \pm 0.01$ after treatment with the crude fraction. Additionally, this fraction had a protein content of 123.90 $\mu$g/mL which was the highest amongst all the other fractions. As the precipitation via Ammonium sulfate adds salt to the protein of interest, therefore, it becomes mandatory to subject the crude sample to overnight dialysis for the purpose of desalting and removal of other impurities that may have been added during the process. The molecular weight of dialyzed protein was determined by SDS-PAGE and was found to be 50kDa,

which hints that the obtained AMP is potentially a member of class III bacteriocins. Similar results have been obtained by Dave and Shah (1997) who used *L. acidophilus* and recovered a bacteriocin of approximately 50 kDa after two stage precipitation using ammonium sulfate. Goh and Philip (2015) also observed significant antagonistic activity in 5 to 50 kDa fraction of bacteriocin produced from *E. faecium* after two step purification.

Biogenic synthesis of AMP-nanoconjugates was carried out by mixing the partially purified AMP and freshly prepared $AgNO_3$ solution followed by UV irradiation. The UV light was primarily used as a photocatalyst that results in photoreduction of $Ag^+$ ions into silver nanoparticles causing the partially purified AMPs to adsorb on the surface of the nanoparticles in the form of a cap or a coat, thereby acting as a natural stabilizing agent. Following incubation, a change in color from light yellow to brownish red gave the preliminary indication of successful formation of AMP-AgNP conjugates. Similar findings have been reported by Filip *et al.* (2019) conducted UV-mediated green synthesis of both silver and gold nanoparticles using fruit extract and examined its effects on inflammation. Sidhu and Nehra (2021) also synthesized AMP-nanoconjugates by UV-irradiation and studied their activity against common food borne pathogens.

Three synthesis parameters viz, concentration ratio, pH and temperature were optimized to ensure efficient formation and activity of AMP-nanoconjugates. The ratio of 1, 9 mL gave the sharpest and most intense peak at 421nm amongst all the other ratios. A reasonably good absorption was also noted for the ratio of 0.5:9.5 at a wavelength of 410 nm. However, increasing the ratio further to 1.5: 8.5 and 2: 8 resulted in reduction in absorbance and the resultant appearance of broad peaks on the spectrum. Jamdagni *et al.* (2018) who used plant extract for green synthesis of silver nanoparticles and also reported the ratio of 1:9 to be optimum. The UV-Visible analysis of AMP-AgNPs produced from each of the pH-adjusted reaction mixtures enabled the visualization of the sharpest peak at pH 10. A prominent trend of enhanced absorbance for pH values in the basic range was observed. In case of pH values in the lower and acidic range (2, 4 and 6) flatter and broader peaks were observed. The pH 8 showed a reasonably sharp peak but with comparatively low absorbance. However, beyond pH 10, a further increase in pH resulted in decrease in absorbance and corresponding broadened peak. The change in color, indicating AMP-AgNPs successful formation that occurs after an incubation period, occurred rapidly on the addition of NaOH. Similar results have been obtained by Ndikau *et al.* (2017) who found pH of 10 to be optimum for the production of silver nanoparticles from fruit rind extract. Lastly, temperature of incubation of reaction was optimized for which a range of temperature from 25 to 75°C was evaluated. The UV-Visible spectrum shown in Fig. 6 (c) indicates an apparent direct relationship between temperature and absorbance. The AMP-AgNPs synthesized under temperatures of lower range depicted low absorbance and broader peaks which are indicative of ineffective synthesis. The temperature of 65°C showed an intense peak at 427 nm. At temperature >65°C the absorbance was still fairly high but the broadened peak indicated poor synthesis. Therefore, 65°C was considered to be optimum in this case. Song and Kim (2009) also reported results of similar nature in their optimization of silver nanoparticle biosynthesis. The final AMP-AgNPs synthesized as per optimum conditions showed an enhanced absorbance and well-defined peak at 438 nm. Patil *et al.* (2015) reported similar results where absorbance was increased and a peak at 420nm was observed.

The capping or adsorption of AMP on the surface of the nanoparticles was authenticated using FTIR spectroscopy in the range of 4000-500 $cm^{-1}$ and thus obtained spectrum is presented in Fig. 6. The spectrum shows various peaks which were found for AMP-nanoconjugates. The first peak at 3342.65 $cm^{-1}$ indicates the stretching of both OH and $NH_2$ group. The next peak at 2511.32 $cm^{-1}$ portrays the C ≡ C stretch. While the peak arising at 1890.24 $cm^{-1}$ can be attributed to the presence of peptide linkage occurring within amide group. C-H stretch is exhibited through the peak at 1627.48 $cm^{-1}$. Finally, the peak occurring at 1131.68 $cm^{-1}$ may be a consequence of bending at C-N and C-O. The results indicate the predominant occurrence of -OH and amide groups and these results are in accordance with Moodley *et al.* (2018). Moreover, the results depict no observable change in the AMP occurred after interaction with nanoparticles. Sharma *et al.* (2012) and Rasheed (2015) have reported similar findings previously.

The comparative analysis of antagonistic activity clearly shows that the biogenically synthesized AMP-AgNP conjugates portrayed a synergistic effect with significantly enhanced antibacterial activity when compared to individual activity of partially purified AMP. Results of in vitro antibacterial activity show MRSA to be the most susceptible to the action of AMP-AgNPs with a ZOI of 13.1± 0.2 mm, whereas no activity was produced by AMP alone. Ampicillin gave a ZOI of 12.83± 0.28 mm against MRSA, which is comparable to but still lesser than that of AMP-AgNPs. On the other hand, *S. aureus* was observed to be least susceptible to the action of AMP-nanoconjugates, with only 1.36-fold improvement in activity in comparison to individual activity of partially purified AMP. The results are noteworthy as, in vitro activity against multi drug resistant *Klebsiella sp.* was produced by both the AMP alone and in conjunction with silver nanoparticles. On its own the AMP displayed low level of activity with a ZOI of 7.33± 0.2 mm. However, this activity was improved significantly when AMP- nano conjugates were used and the ZOI was recorded to be 17.5±0.5 mm, indicating an approximate increase of 2.4- fold in activity. The least susceptibility was recorded against *E. coli*, for which, AMP gave a ZOI of 7.16± 0.28 mm, whereas, AMP-nano conjugates gave an inhibition zone of 11.67± 0.5 mm, indicating a 1.62- fold increase in activity. The present study shows a greater activity of AMP-AgNPs towards Gram-negative pathogens, which indicates that the bioconjugation

was able to overcome the limitation of restricted antimicrobial spectrum that is associated with bacteriocins. Sharma *et al.* (2012) and Pandit *et al.* (2017) have published similar results reporting enhanced spectrum of activity.

The lowest MIC value was obtained for MRSA at 2 $\mu$g/mL, which indicates that the highest activity of AMP-nanoconjugates was observed against it. This was followed by *B. subtilis* against which minimum inhibitory concentration was obtained at 8 $\mu$g/mL. MIC value of 16 $\mu$g/mL was obtained for both *S. aureus* and *Klebsiella sp.* and *Salmonella sp.* had MIC value of 32 $\mu$g/mL. The highest MIC value was observed against *E. coli* at 64 $\mu$g/mL which indicates the lowest activity of AMP-AgNPs towards it. Similarly, with regard to the MBC, the lowest value was obtained for MRSA at 4 $\mu$g/mL. However, the highest MBC values were recorded for *E. coli* and *Salmonella sp.* at 64 $\mu$g/mL depicting it to be least affected by AMP-AgNPs. The results of the present study show that MRSA is highly susceptible towards the antimicrobial effect exerted by AMP-AgNPs. Whereas, *E. coli* has the lowest susceptibility. Thirumurugan *et al.* (2013) and Dhanam *et al.* (2021) have reported similar results of bacteriocin nanoconjugates.

The results give solid evidence that the conjugation of AMPs or bacteriocins from LAB with silver nanoparticles proves to have a synergistic antibacterial action against some common pathogens and these AMP-nanoconjugates have great potential to be utilized in the food sector in preservation and enhancing the shelf life of perishable food items. Moreover, the significant activity against drug resistant pathogens like MRSA and MDR bacteria like *Klebsiella sp.* offers applications even beyond the food industry.

## Conclusion

The current study was conducted to evaluate the effect of conjugating antimicrobial peptides from LAB with silver nanoparticles. AMPs from isolated *Lactobacillus sp.* were produced according to optimized growth conditions (MRS broth, initial pH 6.5 and temperature 37°C), partially purified and visualized by SDS-PAGE to have ~50kDa protein. AMP-silver nanoconjugates were biosynthesized using partially purified AMP. UV-Visible analysis of optimization parameters revealed ratio of 1:9, pH 10 and incubation temperature of 65°C as optimum conditions for efficient biosynthesis. FTIR analysis depicted effective stabilization of AgNPs through AMP by adsorbing on the surface of NPs in the form of a cap or coat. Evaluation of the antagonistic activity of AMP-AgNPs was done against six indicator bacteria. The results prove that this bioconjugation results in a synergistic effect, whereby antimicrobial potential is significantly improved as compared to non-conjugated AMPs. Methicillin-resistant *S. aureus* was found to be the most susceptible towards the action of AMP-AgNPs and depicted an MIC value as low as 2 $\mu$g/mL. Significant antibacterial activity was also seen against multi drug resistant *Klebsiella sp.* in case of which 2.4-fold increase in antibacterial potential was recorded as compared to AMP alone. The results of this study are significant as they hold promise to be utilized practically within the food sector in biopreservation and bioactive innovative packaging of perishable food products. However, in future robust and detailed toxicological studies are required before the incorporation of these composites is regularized in foods. Moreover, the activity against the two drug resistant bacteria used, opens doors for exploration of this formulation in biomedical applications where antibiotic resistance is emerging as a massive threat.

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## Author Contributions

MUA and KW planned and performed the experiments, MUA and SA supervised the experimental work. FA and SS[2] facilitated interpreting the results, KW and SS[1] helped in article write-up and formatting. MUA and SA performed final review and proof reading.

## Conflict of Interest

All authors declare no conflict of interest.

## Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

## Ethics Approval

Not applicable.

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## References

Abo-Amer AE (2011). Optimization of bacteriocin production by *Lactobacillus acidophilus* AA11, a strain isolated from Egyptian cheese. *Anal Microbiol* 61:445–452

Adebayo-Tayo BC, AO Popoola, OM Ajunwa (2017). Bacterial synthesis of silver nanoparticles by culture free supernatant of lactic acid bacteria isolated from fermented food samples. *Biotechnol J Intl* 1:1–13

Agriopoulou S, E Stamatelopoulou, M Sachadyn-Król, T Varzakas (2020). Lactic acid bacteria as antibacterial agents to extend the shelf life of fresh and minimally processed fruits and vegetables: Quality and safety aspects. *Microorganisms* 8:952

Arakawa K (2019). Basic antibacterial assay to screen for bacteriocinogenic lactic acid bacteria and to elementarily characterize their bacteriocins. *Meth Mol Biol* 1887:15–22

Bourdichon F, K Rouzeau (2012). Microbial food spoilage: A major concern for food business operators. *New Food* 15:54

Cheigh CI, HJ Choi, H Park, SB Kim, MC Kook, TS Kim, JK Hwang, YR Pyun (2002). Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus lactis subsp. lactis* A164 isolated from kimchi. *J Biotechnol* 95:225–235

Cotter PD, C Hill, RP Ross (2005). Bacteriocins: Developing innate immunity for food. *Nat Rev Microbiol* 3:777–788

Cuozzo SA, FJ Sesma, AAPDR Holgado, RR Raya (2001). Methods for the detection and concentration of bacteriocins produced by lactic acid bacteria. *Food Microbiol Protoc* 14:141–146

Dave RI, NP Shah (1997). Characteristics of bacteriocin produced by *Lactobacillus acidophilus* LA-1. *Intl Dairy J* 7:707–715

Deraz SF, EN Karlsson, M Hedström, MM Andersson, B Mattiasson (2005). Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079. *J Biotechnol* 117:343–354

Dhanam S, T Arumugam, AM Elgorban, N Rameshkumar, M Krishnan, M Govarthanan, N Kayalvizhi (2021). Enhanced anti-methicillin-resistant *Staphylococcus aureus* activity of bacteriocin by encapsulation on silver nanoparticles. *Appl Nanosci* 13:1301–1312

Duncan TV (2011). Applications of nanotechnology in food packaging and food safety: Barrier materials, antimicrobials and sensors. *J Colloid Interface Sci* 363:1–24

Egan K, D Field, MC Rea, RP Ross, C Hill, PD Cotter (2016). Bacteriocins: Novel solutions to age old spore-related problems? *Front Microbiol* 7:461

Fahim HA, AS Khairalla, AO El-Gendy (2016). Nanotechnology: A valuable strategy to improve bacteriocin formulations. *Front Microbiol* 7:1385

Fayaz AM, K Balaji, M Girilal, R Yadav, PT Kalaichelvan, R Venketesan (2010). Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: A study against gram-positive and gram-negative bacteria. *Nanomed Nanotech* 6:103–109

Feynman RP (2018). There's plenty of room at the bottom. *Resonance* 16:890–905

Field D, PD Cotter, RP Ross, C Hill (2015). Bioengineering of the model lantibiotic nisin. *Bioengineering* 6:187–192

Filip GA, B Moldovan, I Baldea, D Olteanu, R Suharoschi, N Decea, L David (2019). UV-light mediated green synthesis of silver and gold nanoparticles using Cornelian cherry fruit extract and their comparative effects in experimental inflammation. *J Photochem Photobiol B Biol* 191:26–37

Ghanbari M, M Jami (2013). Lactic acid bacteria and their bacteriocins: A promising approach to seafood biopreservation. In: *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*, pp:381–394. Ghanbari M, M Jami (Eds.). IntechOpen, London

Goh HF, K Philip (2015). Isolation and mode of action of bacteriocin BacC1 produced by nonpathogenic *Enterococcus faecium* C1. *J Dairy Sci* 98:5080–5090

Iyapparaj P, T Maruthiah, R Ramasubburayan, S Prakash, C Kumar, G Immanuel, A Palavesam (2013). Optimization of bacteriocin production by *Lactobacillus sp.* MSU3IR against shrimp bacterial pathogens. *Aquat Biosyst* 9:1–10

Jamdagni P, P Khatri, JS Rana (2018). Biogenic synthesis of silver nanoparticles from leaf extract of *Elettaria Cardamomum* and their antifungal activity against phytopathogens. *Adv Mater Proc* 3:129–135

Juodeikiene G, E Bartkiene, P Viskelis, D Urbonaviciene, D Eidukonyte, C Bobinas (2012). Fermentation processes using lactic acid bacteria producing bacteriocins for preservation and improving functional properties of food products. *Adv Appl Biotechnol* 63–100

Khadam ZA, ZO Shatti, NN Mahmood, MF Al-Marjani, JAS Salman, RA Aziz, KW Qassim (2009). Combined effect of nanoparticles and *Leuconostoc mesentroides* ssp. cremoris bacteriocin against *Listeria monocytogenes* isolated from locally soft cheese. *J Global Pharm Technol* 9:519–524

Malheiros PS, V Sant'Anna, SD Todorov, BD Franco (2015). Optimization of growth and bacteriocin production by *Lactobacillus sakei* subsp. sakei2a. *Braz J Microbiol* 46:825–834

Martinez-Gutierrez F, PL Olive, A Banuelos, E Orrantia, N Nino, EM Sanchez, Y Av-Gay (2010). Synthesis, characterization, and evaluation of antimicrobial and cytotoxic effect of silver and titanium nanoparticles. *Nanomedicine* 5:681–688

Moodley JS, SN Krishna, K Pillay, P Govender (2018). Production, characterization and antimicrobial activity of silver nanoparticles produced by *Pediococcus acidilactici*. *Dig J Nanomater Biostr* 13:77–86

Ndikau M, NM Noah, DM Andala (2017). Green synthesis and characterization of silver nanoparticles using Citrullus lanatus fruit rind extract. *Int J Anal Chem* 161:200–210

Pandit R, M Rai, CA Santos (2017). Enhanced antimicrobial activity of the food-protecting nisin peptide by bioconjugation with silver nanoparticles. *Environ Chem Lett* 15:443–452

Patil SD, R Sharma, T Bhattacharyya, MP Kumar, M Gupta, BS Chaddha, NK Navani, R Pathania (2015). Antibacterial potential of a small peptide from *Bacillus sp*. RPT-0001 and its capping for green synthesis of silver nanoparticles. *J Microbiol* 53:643–652

Rasheed QJ (2015). Synthesis and optimization of nisin–silver nanoparticles at different conditions. *J Eng Technol* 33:331–341

Ravishankar Rai V, A Jamuna Bai (2011). Nanoparticles and their potential application as antimicrobials. In: *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*, pp:197–209. A Méndez-Vilas A (ed.). Formatex, Mysore, India

Saravana KP, A Annalakshmi (2012). Enhancing the antimicrobial activity of nisin by encapsulating on silver nanoparticle synthesized by *Bacillus sp. Intl J Pharma Biol Arch* 3:406–410

Savadogo A, CA Ouattara, IH Bassole, AS Traore (2004). Antimicrobial activities of lactic acid bacteria strains isolated from Burkina Faso fermented milk. *Pak J Nutr* 3:174–179

Sharma S, AP Garg, G Singh (2010). Optimization of fermentation conditions for bacteriocin production by Lactococcus lactis CCSULAC1 on modified MRS medium. *Int J Dairy Sci* 1:1–9

Sharma S (2015). Food preservatives and their harmful effects. *Intl J Sci Res Pub* 5:1–2

Sharma TK, M Sapra, A Chopra, R Sharma, SD Patil, RK Malik, R Pathania, NK Navani (2012). Interaction of bacteriocin-capped silver nanoparticles with food pathogens and their antibacterial effect. *Intl J Green Nanotechnol* 4:93–110

Siddiqui AJ, M Patel, M Adnan, S Jahan, J Saxena, MM Alshahrani, A Ouhtit (2023). Bacteriocin-nanoconjugates (Bac10307-AgNPs) biosynthesized from *Lactobacillus acidophilus*-derived bacteriocins exhibit enhanced and promising biological activities. *Pharmaceutics* 15:403

Sidhu PK, K Nehra (2019). Bacteriocin-nanoconjugates as emerging compounds for enhancing antimicrobial activity of bacteriocins. *J King Saud Univ-Sci* 31:758–767

Sidhu PK, K Nehra (2020). Bacteriocin-capped silver nanoparticles for enhanced antimicrobial efficacy against food pathogens. *IET Nanobiotechnol* 14:245–252

Sidhu PK, K Nehra (2021). Purification and characterization of bacteriocin Bac23 extracted from *Lactobacillus plantarum* PKLP5 and its interaction with silver nanoparticles for enhanced antimicrobial spectrum against food-borne pathogens. *Food Sci Technol* 139:110546

Singh VP (2018). Recent approaches in food bio-preservation – a review. *Open Vet J* 8:104–111

Song JY, BS Kim (2009). Rapid biological synthesis of silver nanoparticles using plant leaf extracts. *Bioproc Biosys Eng* 32:79–84

Thirumurugan A, S Ramachandran, A Shiamala Gowri (2013). Combined effect of bacteriocin with gold nanoparticles against food spoiling bacteria-an approach for food packaging material preparation. *Intl Food Res J* 20:1909–1912

Wang H, X Zhang, G Wang, K Jia, X Xu, G Zhou (2017). Bacterial community and spoilage profiles shift in response to packaging in yellow-feather broiler, a highly popular meat in Asia. *Front Microbiol* 8:2588