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Application of Bacteriocin and Silver Nanoparticles against Bacteria Associated with Selected Vegetables

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Abstract

The combined and individual effects of Bacterocin of L. plantarum and silver nanoparticles synthesized using goat milk at different concentrations (50, 75, 100 µL) was tested against food spoilage organisms (Enterobacter cloacae AS10, Escherichia coli 2013C-3342 and Staphylococcus aureus CIP 9973) isolated from selected vegetables. Results showed that silver nanoparticle/bacteriocin combination exerted notable influence against Enterobacter cloacae AS10 (25 ± 0.16 mm, 27 ± 0.22 mm, 30 ± 0.5 mm), Staphylococcus aureus CIP 9973 (24 ± 0.2 mm, 27 ± 0.27 mm, 30 ± 0.29 mm) and Escherichia coli 2013C-3342 (24.07 ± 0.4, 26.27 ± 0.25 and 30 ± 0.2 mm) at aliquots of 50, 75 and 100 µl respectively with increasing concentration as compared to the individual effects of bacteriocin and silver nanoparticles. Besides the use of bacteria, fungi and other macromolecules, goat milk could also be harnessed in the synthesis of silver nanoparticles while also exploring its use in the nearest future as an antibacterial and preservative agent.

Keywords: *Antibacterial; Bacteriocin; Food spoilage organisms, Preservatives, Silver Nanoparticles.*

Introduction

Preservation and safety are presently two major challenges of the food industry because, huge economic losses are sustained yearly due to food spoilage while numerous consumers have been reported to develop adverse sensitivity reactions to chemical based preservatives (Ahmed et al., 2017). In

order to achieve improved food safety against food-spoilage and food-borne pathogen, food industry makes use of chemical preservatives or physical treatments (Perumal et al., 2016). These preservation techniques have many drawbacks which includes the proven toxicity of the chemical preservatives, the alteration of the organoleptic and nutritional properties of foods, and especially recent consumer demands for safe but minimally processed products without additives (Pei et al., 2017). Lactic acid bacteria and their metabolites are envisaged to be potential alternatives to the use of chemicals since they exhibit antimicrobial effects against spoilage and pathogenic bacteria (Sri and Tri, 2009). Over the last decade, bacteriocins of Lactic acid bacteria have gained considerable attention due to their potential applications in the food industry as natural bio-preservatives, and more recently in the health industry as antimicrobial agents (El-Gendy et al., 2013). However, in spite of these promising advantages, nisin is the only bacteriocin generally recognized as safe by the Food and Drug Administration and is currently used as a food preservative in several countries (Kanchan et al., 2015). Nano particles are one of the promising and useful antibacterial agents that could possibly be applied in the food industry (Agharkar et al., 2014). While scientists are searching for efficient strategies to overcome the limitations of bacteriocins, the use of nanotechnology is a potential approach to maximize the use of these peptides (Allémann et al., 1998); Nanotechnology has the potential to revolutionize the global food system (El-Batal et al., 2015). In this study the combined effect of bacteriocin produced by *L. plantarum* NRIC 0383 along with silver nanoparticles synthesized using goat milk against the pathogens of selected vegetables was studied.

MATERIALS AND METHODS

Bacterial Strains

Bacterial species (*Staphylococcus aureus* CIP 9973, *Enterobacter cloacae* AS10, and *Escherichia coli* 2013C-3342) previously identified using 16S rRNA gene sequencing, were isolated from selected vegetable (Tomato *(Solanum lycopersicum*), Eggplant (*Solanum melongena*) and Pumpkin (*Telfairia occidentalis*) samples and were referred to as the indicator strain. Bacteriocin producing *L. plantarum* strain NRIC 0383 from fermented cow milk was cultivated in Man Ragosa Sharpe broth (Oxoid Ltd, Basingstoke, Hampshire, England) at 30°C for 24 hours.

Production and Purification of Bacteriocin of *Lactobacillus plantarum* NRIC 0383

Maximum production of bacteriocin by *Lactobacillus plantarum* NRIC 0383 was done according to the method of Palanisamy et al. (2013). The strain was propagated in MRS broth (1000 mL) seeded with 10% inoculum (108 CFU/mL) of overnight culture and incubated at 35 °C for 48 hours in an orbital shaker (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) at 120 rpm. Cell-free supernatant was then obtained by centrifuging the culture broth at 10,000 rpm for 30 mins at 40oC. After centrifugation the supernatant was collected in fresh sterile tubes and the pellets discarded. The CFS was adjusted to pH 6.5 using 1N NaOH, 5 mL catalase (C-100 bovine liver, Sigma) was also added to eliminate peroxides and lactic acids effect before filter sterilization using whatman® membrane nylon filter (0.2 µm) to eliminate the probability of the presence of viable cells. The Cell-free supernatant was tagged as bacteriocin crude extract (BCE) and purified using 80% ammonium sulphate.

Determination of the Antibacterial activity of Purified Bacteriocin on the Indicator Strains

Antibacterial activity of bacteriocin of *L. plantarum* NRIC 0383 against the indicator strains (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973 and *Enterobacter cloacae* AS10) was determined using the well diffusion assay as described by *Ivanova* et al. (2004). Hundred microliter (100 µL) suspension of each indicator strain containing 1.5×108 cfu/mL were first grown on Muller-Hinton agar (Hi Media, India) at 37°C for 12 hours. Purified bacteriocin (pH 6.5) at concentration of (50, 75 and 100 µL) was then added in 8 mm wells on the different agar plates. The plates were incubated for 24 hours at 37°C. After incubation the plates were examined for zone of clearance around the individual wells. The diameter of the zone of clearance, if any thus formed, was calculated as a measure of bacteriocin activity against the indicator strain. The test was carried out in triplicates

Biogenic Synthesis of Silver nanoparticles using Goat milk

Milk extracted from Alpine Goat Breed was sterilized using whiteman® membrane nylon filter (0.2 µm). Analytical grade silver nitrate ($AgNO_3$) (Sigma-Aldrich (USA) was used. Synthesis of silver nanoparticle using goat milk (GM) was carried out under sterile conditions to maintain milk

sterilization. Ten milliliter (10 mL) of GM was mixed with 90 mL of 1M $AgNO_3$ solution, and the resulting mixture incubated for 24 hours in a rotary shaker (200 rpm) under ambient conditions of room temperature (30 ± 2°C). Reduction of silver ions in the reaction mixture was monitored by observing the color change of the reaction mixture. The reaction product was then separated by centrifugation at 10,000 rpm for 30 min and purified by re-dispersion of the pellet in sterile water. Biosynthesized Silver nanoparticles were finally collected by centrifugation at 10,000 × g for 30 minutes, washed twice with distilled water and unbound proteins removed by treating with 80% (v/v) ethanol. The nanoparticles were then used for further antibacterial assay.

Antibacterial Activity of Biosynthesized AgNPs using the Agar Well Diffusion Method

Antibacterial activity of biosynthesized AgNPs against the indicator strains (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973 and *Enterobacter cloacae* AS10) isolated from Tomato (*Solanum lycopersicum*), Eggplant (*Solanum melongena*) and Pumpkin (*Telfairia occidentalis*) was carried out using the agar well diffusion method (AWD) according to the method of Dahikar, (2018). Turbidity of inoculums was matched with 0.5 McFarland turbidity standard. Inoculum (2 mL) was then spread over Muller-Hinton agar (Hi Media, India) plate using sterile cotton swab in order to get uniform microbial growth. Wells of 8 mm diameter were bored on the plates with the aid of a sterile cork borer and the wells were loaded with different concentrations (50, 75 and 100 µL) of GM synthesized silver nanoparticles and then incubated for 18 hours at 37°C. The antibacterial activity was then evaluated by measuring the diameter of inhibition zones. Silver nitrate used for the synthesis of silver nanoparticles was used as control. The Experiment was carried out in triplicate and the average diameter of the zones of inhibition recorded.

Synthesis of Combined Bacteriocin and Silver Nanoparticles (CBSNPs)

Synthesis of Combined Bacteriocin and silver nanoparticles was done according to the method of Sharma et al. (2012) with slight modification. Ten milliliter (10 mL) concentration of Silver nanoparticles synthesized using Goat milk was slowly added to equal concentration of purified bacteriocin of *L. plantarum* NRIC 0383 contained in 80 mL of distilled water in sterile bottles with

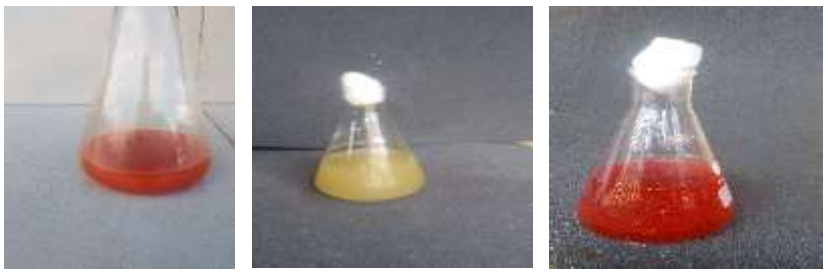
continuous stirring at room temperature (28ºC). The mixture of combined bacteriocin and silver nanoparticles (CBSNPs) was monitored visually for change in color within 72 hours incubation period. After incubation the concentration mixture was centrifuged at 10,000 rpm for 30 minutes using a high-speed centrifuge (Supra 22 K, Hanil Science Industrial, Republic of Korea).

Determination of the Effect of Combined Bacteriocin and Silver nanoparticles on the Indicator Strains

The effect of combined bacteriocin and silver nanoparticles on the indicator strains (*Escherichia coli* 2013C-3342, *Staphylococcus aureus* CIP 9973 and *Enterobacter cloacae* AS10) was measured using the agar diffusion technique. Aliquots 50, 75 and 100 µL concentration was taken into 8 mm diameter wells bored on Muller-Hinton agar (Hi Media, India) plates previously spread with inoculum of the various indicator strains (adjusted to 0.5 McFarland turbidity standard) using sterile cotton swab in order to achieve uniform microbial growth. The plates were then incubated for 18 hours at 37ºC. The effect of combined bacteriocin and silver nanoparticles expressed in terms of antibacterial activity was evaluated by measuring the diameter of inhibition zones. The experiment was carried out in triplicate and the average diameter of the zones of inhibition recorded.

RESULTS

The color of silver nanoparticles previously synthesized using goat milk (GSNPs) in the Nanotechnology Laboratory of the Federal university of Agriculture Makurdi was observed as light reddish brown as seen in Figure 1. After combination of equal concentration of GSNPs with and bacteriocin (10 ml), the combined mixture (CBSNPs) was observed to be dark red in color after 72 hours incubation period (figure 1).



A B C

Figure 1: Synthesis of Combined Bacteriocin and Silver Nanoparticles after 24 hours Incubation. A: Silver nanoparticles synthesized using goat milk; B: Purified *L. plantarum* NRIC 0383 Bacterioicn; C: Combination of bacteriocin and silver nanoparticles (A+B)

Antimicrobial effect of bacteriocin on *Enterobacter cloacae* AS10 at the different concentration was higher (10.00 ± 1.04, 13.00 ± 0.16 and 15.00 ± 0.93 mm) than that observed for silver nano particles (AgNPs) synthesized using goat milk with inhibitory activity measured at 8.50 ± 0.44, 11.00 ± 0.14 and 12.00 ± 0. 73 mm all at 50, 75 and 100µl respectively on the other hand the antimicrobial activity of combined bacteriocin and silver nanoparticles (CBSN) against *E. cloacae* AS10 evaluated as the mean diameter at the different concentrations (50, 75 and 100µl) was recorded as 25.00 ± 0.16, 27.00 ± .22 and 30.00 ± 0.50 mm respectively, as obvious these figures measured as the zones of inhibition were higher when compared to the effect of AgNPs and bacteriocin alone (Figure 2).

The effects of AgNPs and bacteriocin on *Escherichia coli* 2013C-3342, showed that inhibitory effect of AgNPs on *Escherichia coli* 2013C-3342 was 14.20 ± 1.23, 14.99 ± 0.24 and 17.0 ± 0.64 mm which were higher than that observed for bacteriocin at 9.00 ± .82, 11.0 ± 0.34 and 13.00 ± 1.20 mm at 50, 75 and 100 µl respectively (Figure 34). Also, antimicrobial activities of CBSN against *E. coli* 2013C-3342 were 24.07 ± 0.4, 26.27 ± 0.25 and 30 ± 0.2 mm at 50, 75 and 100 µl. This inhibitory activity was found to be higher than that observed for AgNPs and bacteriocin at same aliquot (Figure 3).

Combined bacteriocin and silver nano particles (CBSNPs) inhibited the growth of *S. aureus* CIP 9973 to a greater extent with inhibition zones of 24.00 ± 0.20, 27.00 ± 0.27 and 30.00 ± 0.29 mm at aliquots of 50, 75 and 100 µl respectively (Figure 38). Invariably the zones of inhibition by AgNPs which was 13.00 ± 1.86, 17.00 ± 0.29, 18.00 ± 0.73 mm against *S. aureus* CIP 9973 was higher than that of bacteriocin recorded as 10.0 ± 0.44, 11.00 ± 0. 20 and 12.00 ± 1.23 mm at 50, 75 and 100 µl respectively (Figure 4).

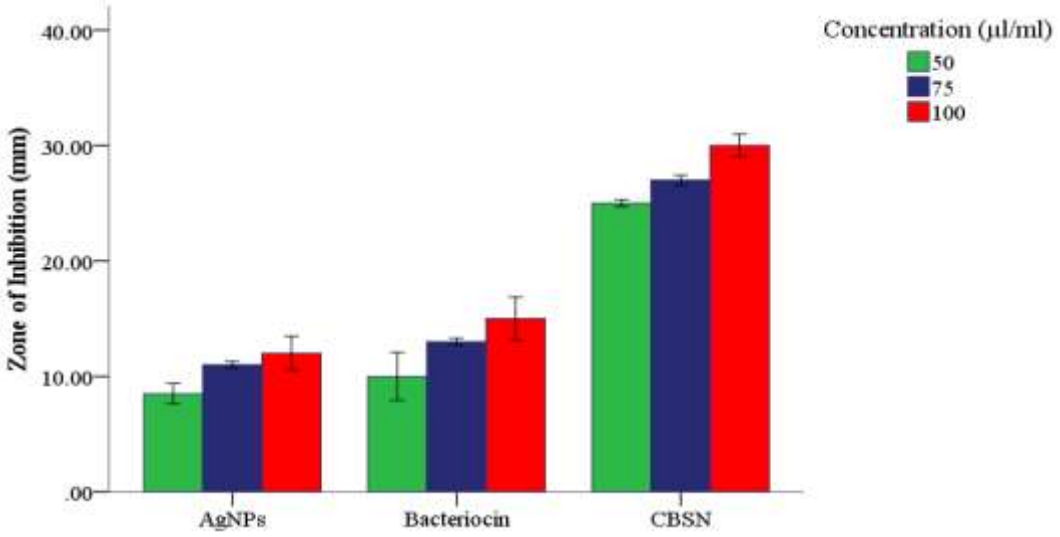


Figure 2: Comparison of the Antimicrobial Activity of Bacteriocin, AgNPs and CBSN against *Enterobacter cloacae* AS10. AgNPs: Silver nano particles synthesized using goat milk
Bacteriocin: Bacteriocin of *L. plantarum* NRIC 0383; CBSN: Combined bacteriocin and silver nanoparticles

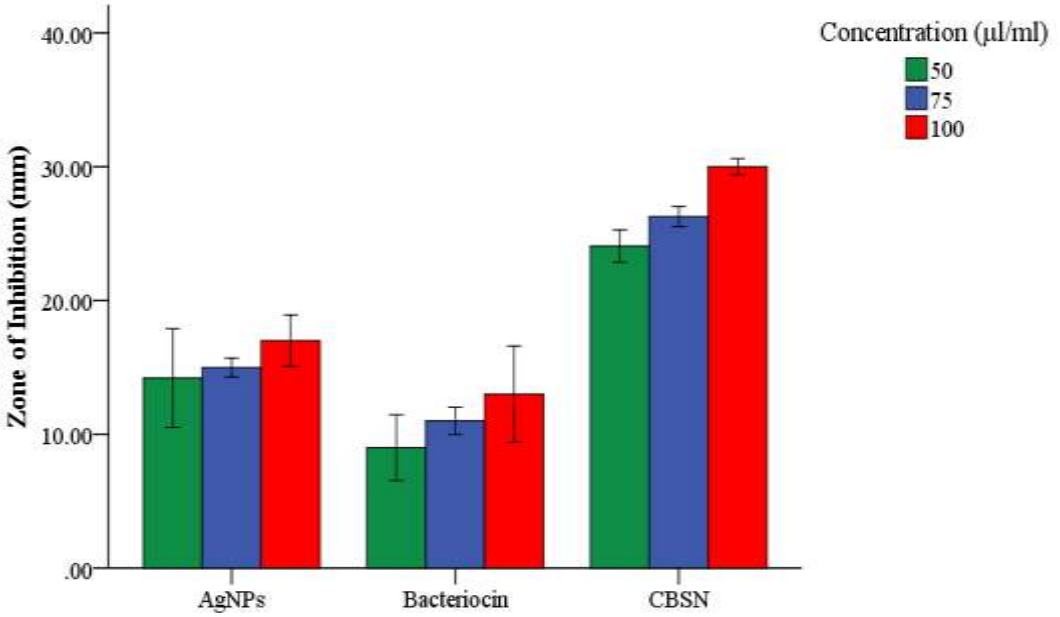


Figure 3: Comparison of the Antimicrobial Activity of Bacteriocin, AgNPs and CBSN against *Escherichia coli* 2013C-3342. AgNPs: Silver nano particles synthesized using goat milk Bacteriocin: Bacteriocin of *L. plantarum* NRIC 0383; CBSN: Combined bacteriocin and silver nanoparticles

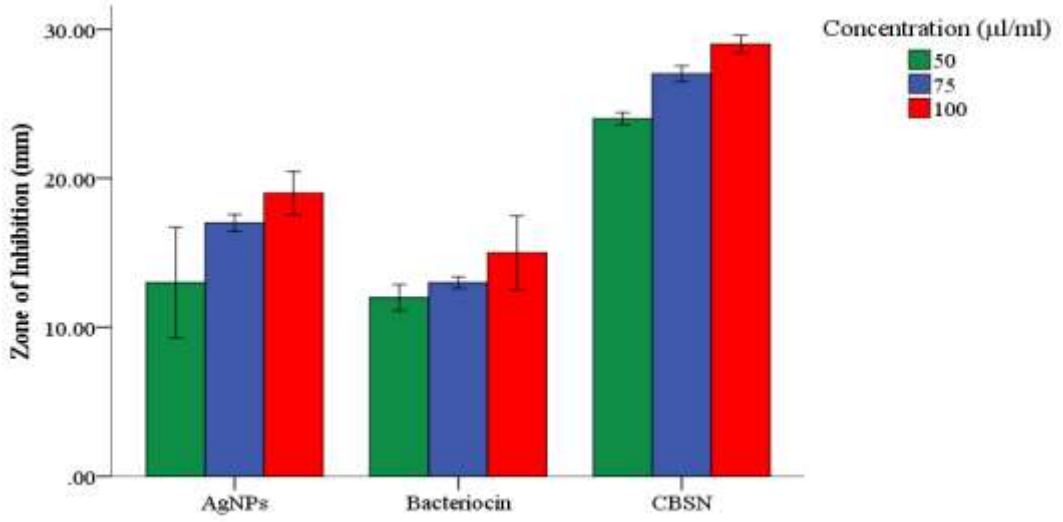


Figure 4: Comparison of the Antimicrobial Activity of Bacteriocin, AgNPs and CBSN against *Staphylococcus aureus* CIP 9973. AgNPs: Silver nano particles synthesized using goat milk; Bacteriocin: Bacteriocin of *L. plantarum* NRIC 0383; CBSN: Combined bacteriocin and silver nanoparticles

DISCUSSION

Increase in antimicrobial activity of CBSNPs against *E. cloacae* AS10, *E. coli* 2013C-3342 and *S. aureus* CIP 9973 at the different concentrations as compared to when *L. plantarum* NRIC 0383 bacteriocin and silver nanoparticles were used separately showed that the combined interaction of both antimicrobial agents on the test bacteria was more effective and target specific. However, different types of interactions can occur between two or more substances given simultaneously but the most desirable is synergism. This type of interaction (synergism) occurs when the effect of the antimicrobial in combination is much higher than would be expected from the individual activities of each component (Cokol *et al*., 2011). This was evident in the antibacterial activity of CBSNPs against *E. cloacae* AS10, *E. coli* 2013C-3342 and *S. aureus* CIP 9973 respectively. Therefore, it is suffice to say that the effect of CBSNPs on *E. cloacae* AS10, *E. coli* 2013C-3342 and *S. aureus* CIP 9973 was synergistic. Several advantages associated with synergistic combinations include increase in efficacy of therapeutic effect, especially when antimicrobials in sequence differ in their mechanism of action, but give similar effects. Secondly, using a combination of antimicrobials, allows for decreasing dosages of each

component and thus their toxicity, which results in less severe adverse effects of each; the third being that it minimizes the development of microbial resistance (Vivian, 2014). Thirumurugan et al. (2013) reported the combined effect of bacteriocin produced by *Lactobacillus plantarum* ATM11 along with gold nanoparticles synthesized using *Bacillus subtilis* on food blemishing microorganisms (*Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Micrococcus luteus*). Some reports have also discussed the combined effect of bacteriocin and nanoparticles (Sharma et al., 2012; Pissuwan et al., 2007; Huang et al., 2007; Maryam et al., 2010; Tahar et al., 2010; Shende et al., 2016; Sharma et al., 2006 and Thirumurugan et al., 2013) and its increased antimicrobial effect against the target organism. Owing to their small sizes and higher surface-to-volume ratio, silver (Ag) nanoparticles possess a wider contact area with microorganisms (Lateef and Adeeyo, 2015). This property can enhance biological and chemical activity, hence provides Ag nanoparticles with high antibacterial activity (Prabhu and Poulose, 2012). Silver nanoparticles have the ability to disturb functions of cell membranes such as permeability and respiration (Bhupindersingh, 2014). Furthermore, upon penetration of the bacteria cell, Ag nanoparticles can react with the functions of Sulphur containing proteins and phosphorus containing compounds such as deoxyribonucleic acid (DNA) (Zohri et al., 2013). Silver nanoparticles are able to destroy the permeability of the bacterial membranes via the generation of many pits and gaps (Gurunathan et al., 2015). Increase in antimicrobial activity of combined bacteriocin and silver nanoparticles (CBSNPs) against *E. cloacae* AS10, *E. coli* 2013C-3342 and *S. aureus* CIP 9973 could be attributed to silver nanoparticles acting as a good anchor carrying more amounts of bacteriocin on the surface via electrostatic attraction between the amine groups of bacteriocin and nanoparticles which gives a better activity. According to Pissuwan et al. (2007) and Thirumurugan et al. (2013) nanoparticles are able to exert their antibacterial properties by attaching to the surface of the bacterial cell. This study suggests, that Silver nanoparticles in combination with bacteriocin *L. plantarum* NRIC 0383 can be used as preservative as well as an antimicrobial agent against food spoiling microbes. It is recommended that further toxicological studies be carried out on the product. Although a number of studies have been conducted on combinations of bacteriocins with other antimicrobials, to circumvent the development of antimicrobial resistance and increase antimicrobial potency, to the best of the authors' knowledge this is a

novel report on the synthesis of silver nanoparticles using goat milk and its combination with *L. plantarum* NRIC 0383 bacteriocin as an antibacterial agent. In agreement with this findings, some studies have shown that encapsulation of bacteriocins in nanoparticles have enhanced the activity of the peptides against food-spoiling microorganisms and multidrug-resistant bacteria (Arthur *et al*., 2014; Mossallam *et al*., 2014). Through this integration, effective delivery, targeting, protection from degradation, in addition to improving drug potency and physicochemical properties can all be achieved. Combinations of bacteriocins with antibiotics can also decrease the concentration of antibiotics required to kill a target pathogen, and broaden the spectrum of activity, which may be useful in treating clinical infections of unknown etiology.

CONCLUSION

The study showed that combined bacteriocin incorporated with Silver nano particles exhibited greater inhibitory activity on isolates from selected vegetables (*Enterobacter cloacae* AS10, *Escherichia coli* 2013C-3342 and *Staphylococcus aureus* CIP 9973) than when bacteriocins or silver nanoparticles was used individually. Combination of silver nano particles synthesized using goat milk and bacteriocin of *Lactobacillus plantarum* NRIC 0383 is therefore recommended as an effective antibacterial agent against common food spoilage organisms especially for target strains (*E. cloacae* AS10, *E. coli* 2013C-3342 and *S. aureus* CIP 9973).

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