Antibacterial activity of fusion from biosynthesized acidocin/silver nanoparticles and its application for eggshell decontamination

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Bacteriocins from lactic acid bacteria (LAB) are useful to control the persistent development of pathogenic microorganisms in food and medicine fields. The bacteriocin acidocin was extracted from *Lactobacillus acidophilus* M1 that was isolated from fermented milk, purified using ammonium sulphate fractionation, and gel filtration column chromatography using Sephadex matrix and applied as a potential antibacterial agent. The molecular weight of the purified acidocin was estimated using SDS–PAGE to be 6.6 kDa. The acidocin was compared with silver nanoparticles (SNPs), biosynthesized by *Aspergillus brasiliensis* (niger) ATCC 16404, against two bacterial strains *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923. Both acidocin and SNPs showed significance antibacterial effects using disc and well diffusion methods; the maximum antibacterial activity was proved against *B. cereus* from acidocin/SNPs composite using a ratio of 1/1 from each agent. The application of acidocin/SNPs composite as immersion solution, for disinfecting chicken eggshells, resulted in remarkable reduction in microbial load on the shells of 5.53 log10 CFU/eggshell. Results could provide an eco-friendly approach for potential antimicrobial composites to be used in food preservation and other health protection researches.

**KEYWORDS**
*Aspergillus brasiliensis*, bacteriocins, food preservation, *Lactobacillus acidophilus*, purification

# 1 | INTRODUCTION

Lactic acid bacteria (LAB) refer to a large group of Gram-positive, acid-tolerant, and bile resistant bacteria [1]. Interest in health benefits associated with the genus *Lactobacillus* has been concentrated on, for instance, cancer control [2] and serum cholesterol reduction [3], although other diseases and conditions have been examined. *Lactobacillus reuteri*, a probiotic that highly enzymatically transforms linoleic acid into conjugated linoleic acid [4]. *Lactobacillus acidophilus* La-5 has been reported to be associated with the production of conjugated linoleic acid during the fermentation of milk [5].

LAB are generally recognized as safe (GRAS), and have a significant role in food preservation and fermented products. They can be used as natural competitive microbes or as specific starter cultures under controlled conditions [6].

Chemical and synthetic preservatives of food could have dangerous side effects; they are characterized as theatrically disinfectants [7], using several antibiotics has been extensively proposed, but these attempts have developed resistance strains from pathogens, e.g. *Helicobacter pylori* [8].

*Bacillus cereus* is a microbial pathogen responsible for a minority of food borne illnesses (2–5%), causing severe nausea, vomiting and diarrhea [9], while *Staphylococcus aureus* is one of the most common causes of bacteremia and infective endocarditis. Additionally, it can cause various skin and soft tissue infections [10].
On the other hand, natural food preservatives are more safety for consumers’ health, e.g. bacteriocins [11]: fermented foods can be used as bactericidal against several pathogens [12]. LAB could decrease food borne pathogens spread throughout the food chain, with broad spectrum of activity and beneficial compounds production in the food matrix during food fermentation.

Some of these bacteria produce antagonistic proteins called bacteriocins. Most LAB bacteriocins are cationic peptides or proteins [13] and are very active against pathogenic microorganisms [14]. Bacteriocins produced by LAB can penetrate the outer membrane of Gram-negative bacteria and forming pores in it [15]. In addition to their GRAS characteristics, the bacteriocins produced by LAB are nontoxic to eukaryotic cells, which make them suitable for several food and medical purposes.

Bacteriocins are normally named based on the genus or species of the lactic acid strain producing it; for instance, L. plantarum produce “plantaricin,” Lactococcus spp. “lacticiin,” Enterococcus spp. “enterocin” and Leuconostoc spp. “leucocin”… and so on.

Methods of extracting bacteriocins are depend also on their affinity to organic solvents, variation in solubility in concentrated salt solutions, and pH value specified [16].

Acidocins are secreted into the culture medium; most strategies start with a step to concentrate acidocin from the culture supernatant, using for example diatomite calcium silicate or ammonium sulfate precipitation [17]. Although these procedures are used principally to reduce the working volume, they do not provide a high degree of purification [18]. Acidocin produced by L. acidophilus DSM 20079 has been characterized as one-component peptide with low molecular weight of 6.6 kDa [19]; this acidocin was purified by ammonium sulphate precipitation.

Optimal parameters, for growth of Lactobacillus spp., and medium composition might interfere with production and purification processes to large-scale of bacteriocins. The yield of extracted acidocin from L. acidophilus may be higher than 50% with a purity of approximately 90%. In general, the production of acidocin by LAB involves several steps; the first is the removal of the cells from the growth medium by filtration followed by fractionated precipitation of the proteins from the culture supernatant by addition of ammonium sulfate, the precipitated proteins are subsequently dissolved in deionized water or in a weak buffer followed by gel filtration column chromatographic purification [20].

Nanotechnology has been recognized to organize and functionalize molecular materials, which is applicable to different fields. Nanotechnology research is significant area in nanoparticles such as silver nanoparticles (SNPs). Silver ions are capable of acting as bacteriostatic or even as bactericidal [21].

The present study, however, was planned to produce purified acidocin from L. acidophilus M1, to make a composite between the purified acidocin and silver nanoparticles, to evaluate the composite antimicrobial activity against two pathogenic microorganisms and to apply the formed acidocin/nano silver composite for the control of chicken eggshells’ contaminating microorganisms.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Unless other source is mentioned, all chemicals and bacteriological media were purchased from Sigma–Aldrich (St. Louis, MO).

2.2 | Microorganisms

The indicator microorganisms, viz., B. cereus ATCC 14579, Staphylococcus aureus ATCC 25923, and Aspergillus brasiliensis (niger) ATCC 16404 were procured from Microbial Type Culture Collection (MTCC, Cairo, Egypt).

2.3 | Isolation and screening of LAB

The isolates from fermented milk were selected and transferred to MRS agar medium (Oxoid, Basingstoke, UK). The isolates were tested for their ability to produce acidocin. These were inoculated into MRS broth and incubated at 37 °C for 48 h. Initial pH of cell free supernatants was adjusted to pH 6.0 with 2 M NaOH, sterilized by passing through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) and evaluated for antimicrobial activity by disk diffusion method against B. cereus, and S. aureus as indicator strains. After that, the isolates were grown in MRS agar media to recognize on the morphological, physiological, and biochemical characteristics of the lactobacilli as described in Bergey’s Manual of Determinative Bacteriology [22].

2.4 | Purification of acidocin from Lactobacillus spp.

The acidocin-producing strain was propagated in MRS broth for 48 h at 37 °C. Cells were removed by centrifugation at 6000 rpm for 20 min. The supernatant fluid was precipitated with 80% saturated ammonium sulfate (NH₄)₂SO₄. The precipitate was left to settle overnight at 4 °C with gentle stirring. After centrifugation, the precipitate was collected and dissolved in 15 ml sodium phosphate buffer (SPB), pH 6.5. The suspension was dialyzed for 24–48 h at 4 °C with a continuous flow of the SPB buffer. The acidocin was further purified with gel
filtration column chromatography using Sephadex matrix. The crude acidocin fraction was loaded on a column (35 × 3 cm) of Sephadex G-100, equilibrated previously with 5 mM SPB, pH 7.2 containing 0.2 M NaCl. The column was eluted with the same buffer at a flow rate of 100 ml/h. The fractions were collected using fraction collector (Gilson® FC-203, Gilson, Middleton, WI), the absorbance of fractions was monitored spectrophotometrically at 280 nm. The fractions were collected together at room temperature as a bold purified sample and the protein concentration was determined [23].

2.5 | Gel electrophoresis and molecular weight determination

Sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS–PAGE) of acidocin was carried out using a discontinuous buffer system; 10% separating and 5% stacking gels [24]. One hundred microlitter of fraction and an equal volume of sample buffer (0.5 M Tris–HCl pH 6.8) were added. The protein was denatured by heating in a boiling water bath for 5 min, and immediately cooled on ice. After electrophoresis, the gel was stained in 50 ml of staining solution using Coomassie Brilliant Blue R-250 for 30–45 min with shaking at 25 °C and destained in destaining solution. The molecular weights were determined by comparison with standard protein marker.

2.6 | Preparation of acidocin capped silver nanoparticles

Aspergillus brasiensis was maintained in nutrient broth medium at 25 °C for 4 days, then the culture was filtered through Whatman No. 1 filter paper. Exactly 10 ml of the culture filtrate was added to 90 ml of silver nitrate (AgNO3) solution to have a final concentration of 10−3 M (170 mg/L AgNO3), the mixture was then incubated at room temperature in dark [25]. The preparation of acidocin/Ag-NPs (acidocin/SNPs) was carried out according to the method described by Sergeev et al. [26]. Ten microgram from purified acidocin were added into 100 ml of 10−3 M freshly prepared solution from SNPs with continuous stirring at room temperature for 24 h and color changes were observed. The acidocin/SNPs emulsion obtained were kept at 4 °C and the prepared composite was characterized morphologically by transmission electron microscopy (TEM).

2.7 | Antibacterial activity of acidocin

The antimicrobial activity of purified acidocin and acidocin/SNPs were evaluated against two bacterial strains (B. cereus and S. aureus), using different antimicrobial assays.

2.7.1 | Well diffusion assay

Inoculated nutrient agar plates were incubated overnight at 30 °C for 24–48 h and zones of inhibition around each well (with 6 mm diameter) were measured using a precisely calibrated ruler (All experiments were conducted in triplicates). Serial combinations from acidocin/SNPs solutions (10, 20, 30, 40, and 50%) were prepared to a final volume of 100 μl, using freshly aqueous solution of SNPs, and then pipetted to each well.

2.7.2 | Minimal inhibitory concentration (MIC) determination

The acidocin titer was expressed as arbitrary or activity unit/ml (AU/ml), toward B. cereus and S. aureus. One arbitrary unit (AU) of acidocin is defined as the reciprocal of the last serial two-fold dilutions demonstrating significant inhibitory activity [27].

2.8 | Eggshell sterilization

The acidocin/SNPs complex was applied for disinfecting eggshells throughout different treatment period. A percentage of 50/50, from both acidocin (100 μg/ml) and SNPs (170 μg/ml), was applied for preparing the eggshells disinfecting solution (EDS). Fresh grade A chicken eggs were obtained clean from the laying farm, USC, Egypt, and subjected to EDS treatment. Treated groups (5 eggs each) was immersed from the laying farm, USC, Egypt, and subjected to EDS treatment. Treated groups (5 eggs each) was immersed in 50 ml from sterilized buffered peptone water (BPW), well stirred, plated onto nutrient agar (NA) plates and incubated at 37 °C for 24–28 h to enumerate the total viable microbial load on eggshells after each exposure time. Distilled water was used for the immersion of control egg group.

3 | RESULTS

3.1 | Isolation and identification of acidocin producing LAB from fermented milk

The isolated strain was identified to be Gram positive rod shaped bacilli, non-motile, positive for methyl red test, and indole production (Table 1). The strain has the ability to grow well in MRS agar plates. Also the isolates were identified as Lactobacillus spp. following criteria described in the Bergey’s Manual of Determinative Bacteriology.

The ability of these isolated strains to produce acids from different carbohydrates was determined. The identities of isolated strains were finally designated as: L. acidophilus M1, L. fermentum M2, and L. casei M3.
3.2 | Purification and molecular weight of acidocin from *L. acidophilus* M1

In the present work; the protein concentration, purification steps and the recovery values of acidocin produced from *L. acidophilus* M1 are summarized in Table 2. The acidocin was recovered using saline solution and 80% saturation of the culture broth with (NH₄)₂SO₄. As can be seen from Table 2, the total acidocin activity that produced from *L. acidophilus* M1 against *B. cereus* was 300.000 AU/ml with specific activity of 78.95 AU/mg protein and after dialysis and gel filtration were 45.000 and 27.000 AU/ml with an increase in specific activity of 30 and 135 AU/mg protein, respectively. The total protein content was decreased in each treatment. On the other hand, the purification processes were carried out and resulted in an approximately 4.5-fold.

In addition, the purification steps and the recovery values of produced acidocin against *S. aureus* are shown in Table 2. The total acidocin activity was 360.000 AU/ml with specific activity of 120 AU/mg protein and after dialysis and gel filtration were 72.000 and 36.000 AU/ml, respectively after dialysis and gel filtration, with an increase to specific activity of 51.43 and 194.6 AU/mg protein. Alternatively, after final purification step, the acidocin was purified to 3.80-fold.

The molecular weight for the purified acidocin of *L. acidophilus* M1 was estimated using gel electrophoresis (SDS–PAGE) for various steps including saline solution; ammonium sulfate and gel filtration column chromatography (Fig. 1). From the SDS-electrophoresis pattern of the sephadex G-100 purification step, only one band was observed with characterized molecular weight of 6.6 kDa.

3.3 | Morphology study

TEM image of the SNPs formed by *A. brasiliensis* (niger) after 48 h of reaction time is shown in Fig. 2A. The TEM technique used to visualize size and shapes of biosynthesized silver nanoparticles have predominantly shown spherical shape structures with size ranging between 18.62 and 32.50 nm. This image shows individual as well as number of aggregates.

Nanoparticles formed were highly stable due to capping agents such as proteins and were not in direct contact even within the aggregates indicating stabilization. On another hand, regarding the TEM image of the SNPs interacted with acidocin shapes, shown in Fig. 2B, SNPs have predominantly shown shape structures with size ranging between 0.65 and 0.69 μm.

3.4 | Inhibitory activity of acidocin

The susceptibilities of pathogenic bacteria to the growth inhibition by the acidocin, SNPs and acidocin loaded onto SNPs are presented in Fig. 3. All treatments showed remarkable inhibitory activities against *S. aureus* and *B. cereus*. The antibacterial activities were stronger against *B. cereus*, from the entire treatments, comparing with *S. aureus*. The maximum activity was recorded from acidocin loaded into SNPs against *B. cereus*, with inhibition zone of 55 mm.

Silver nanoparticles in combination with purified acidocin effectively inhibited *B. cereus*. In case of agar well diffusion method, an attempt was conducted to investigate the effects of different ratios between acidocin and SNPs.

### TABLE 1 | Morphological and biochemical characteristics of *Lactobacillus acidophilus* M1 isolated from fermented milk

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Shape</td>
<td>Rods</td>
</tr>
<tr>
<td>Motility</td>
<td>Non motile</td>
</tr>
<tr>
<td>Indole test</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization test</td>
<td>−</td>
</tr>
<tr>
<td>Catalase test</td>
<td>−</td>
</tr>
</tbody>
</table>

### TABLE 2 | Bioactivity of acidocin from *Lactobacillus acidophilus* M1 before, after dialysis, and after gel filtration column chromatography using sephadex G-100 against *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acidocin (before dialysis)</th>
<th>Acidocin (after dialysis)</th>
<th>Gel filtration (sephadex G-100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td><em>S. aureus</em></td>
<td><em>B. cereus</em></td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>200</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td>Acidocin activity (AU/ml)</td>
<td>1500</td>
<td>1800</td>
<td>1500</td>
</tr>
<tr>
<td>Total acidocin activity (AU/ml)</td>
<td>300.000</td>
<td>360.000</td>
<td>45.000</td>
</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>3800</td>
<td>3000</td>
<td>1500</td>
</tr>
<tr>
<td>Specific acidocin activity (AU/mg)</td>
<td>78.95</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>Purification (fold)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.38</td>
</tr>
</tbody>
</table>

MOHAMMED ET AL.
(10 μL acidocin + 90 μL SNPs, 20 μL acidocin + 80 μL SNPs, 30 μL acidocin + 70 μL SNPs, 40 μL acidocin + 60 μL SNPs, and 50 μL acidocin + 50 μL SNPs) on the activity of acidocin/SNPs composites against B. cereus and S. aureus. The antibacterial activity of acidocin/SNPs composites showed the maximum activity against B. cereus, with 6.6 cm, rather than against S. aureus, with 6.0 cm, at concentration of 0.05 μg/ml (Table 3).

3.5 | Disinfection of eggshells using acidocin/SNP complex solution

The impact of eggshells disinfection, using disinfectant solution (EDS), based on acidocin/SNPs complex, is illustrated in Fig. 4. While the microbial load in control group slightly decreased with the prolongation of washing time with distilled water, for example, from 7.21 to 4.79 log_{10} CFU/eggshell, the decrement of microbial load in treated eggshells with EDS was more severe and remarkable (Fig 4). The recorded total viable counts in disinfected eggs were 6.75, 4.24, 3.01, and 1.22 log_{10} CFU/eggshell after immersion time of 0, 1, 3, and 5 min, respectively.

4 | DISCUSSION

The obtained findings in this study, for the SDS-electrophoresis pattern, were in accordance with other previously reported results, which stated that the bacteriocin produced by L. plantarum was partially purified by ammonium sulphate precipitation [28,29]. Also, they reported that the molecular weight of the purified bacteriocin from L. brevis BK11 was approximately 6.5 kDa. Our results are also agree with another findings that estimated the molecular weight of acidocin produced by L. acidophilus DSM 20079 to be in the range of 6.6 kDa [30].

The SNPs shape and structure, using TEM imaging, was reported in other investigation [31], which indicated that SNPs are typical for the absorption of metallic silver nanocrystals due to surface plasmon resonance, and this confirms the presence of nanocrystalline elemental silver. It was additionally reported that common Lactobacillus strains found in buttermilk could assist the growth of microscopic gold, silver, and gold-silver alloy crystals of well-defined morphology [32].

Regarding the antibacterial activity of SNPs, it has been indicated that the size of silver nanoparticles is an important factor for cytotoxicity and genotoxicity, probably acting through apoptosis and necrosis mechanisms [33]. Silver
nanoparticles must be used with care because their agglomeration behavior and the presence of proteins play important functional roles [34].

It was suggested that study the bond strength between nanoparticles and proteins and their effect on the progress of the equilibrium [35].

Nanoparticle activity against *Salmonella* spp., for example, *S. Typhimurium*, were studied in the presence of mouse blood components, serum albumin is the major component of blood and BSA addition, totally strengthened the antibacterial activity of silver nanoparticles [36].

It was indicated that nanoparticle-protein interactions and morphology role on characterization of protein are essential to the achievement of the desired effects of the nanoparticles in vivo, future studies were recommended to link right morphology according with the required effect [37].

Synthesized SNPs can be produced by biological methods and without the use of any harsh, toxic, and expensive chemical substances [38]. Many studies have reported successful synthesis of SNPs using organisms (microorganisms and biological systems). For instance, it was demonstrated the bioreductive synthesis of SNPs using *Fusarium oxysporum* [39]. Silver nanocrystals of different compositions were successfully synthesized by *Pseudomonas stutzeri* AG259 [40].

Silver nanoparticles have been reported to interact strongly with proteins so that, in *F. oxysporum*, the bioreduction of silver ions was qualified to an enzymatic process involving NADH-dependent reductase. The exposure of silver ions to *F. oxysporum*, resulted in release of nitrate reductase and subsequent formation of highly stable silver nanoparticles in solution [41]. The secreted enzyme was found to be dependent on NADH cofactor. Biosynthesis of SNPs can be done from it easily and applied in various fields such as medicine. Bio silver nanoparticles are not used by acidocin as a loading, and the increasing of activity of acidocin may be due to changes in surface stress. An increase in SNPs leads to a lowering of acidocin activity. As demonstrated in this study, the acidocin activity was influenced greatly by several concentrations. When considering possible future applications of acidocin as a food biopreservative, these factors must be taken into account in the design of more economical acidocin activity processes.

The reduction of microbial count in control eggshell group could be attributed to the difference between the

<table>
<thead>
<tr>
<th>Acidocin/SNPs ratio</th>
<th>Inhibition zone against test organisms (mm)&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><em>B. cereus</em> ATCC 14579</td>
</tr>
<tr>
<td>Purified acidocina</td>
<td>30</td>
</tr>
<tr>
<td>SNPs (10&lt;sup&gt;-3&lt;/sup&gt; M)</td>
<td>38</td>
</tr>
<tr>
<td>10 μL acidocin + 90 μL SNPs (final conc. 0.01 μg/ml)</td>
<td>48</td>
</tr>
<tr>
<td>20 μL acidocin + 80 μL SNPs (final conc. 0.02 μg/ml)</td>
<td>52</td>
</tr>
<tr>
<td>30 μL acidocin + 70 μL SNPs (final conc. 0.03 μg/ml)</td>
<td>56</td>
</tr>
<tr>
<td>40 μL acidocin + 60 μL SNPs (final conc. 0.04 μg/ml)</td>
<td>60</td>
</tr>
<tr>
<td>50 μL acidocin + 50 μL SNPs (final conc. 0.05 μg/ml)</td>
<td>66</td>
</tr>
</tbody>
</table>

<sup>a</sup>Initial concentration (100 μg/ml).
<sup>b</sup>Diameter of the inhibition zone (including the wells diameter of 6 mm).
osmotic pressure of the immersion solution, for example, distilled water, and the interior cell components; this osmotic difference is supposed to disrupt and explode many microbial cells, particularly with the prolongation of immersion time.

Regarding the EDS immersed eggs, the microbial load reduction was a time dependent and could be mainly attributed to the synergistic antimicrobial action of both acidocin and SNPs. The application of SNP-based solution was recommended for microbial pathogen prevention and control [42]. Acidocin produced from \textit{L. acidophilus} was also confirmed as a potent antimicrobial agent against many Gram negative and positive bacteria, especially in food preservation branches [43]. Additionally, it was suggested that the combined interactions between proteinous biomaterials and metal nanoparticles could increase the bioactivity of them through the enhancement of their correlated morphological characteristics [44].

From the attained results in our study, it could be concluded that acidocin produced by \textit{L. acidophilus} have the potential to cover a very broad field of application, including both food industry and medical sectors. Microbial biosynthesis of silver nanoparticles could be conducted to have a potent antimicrobial particles against pathogenic bacteria. Thesis of silver nanoparticles could be conducted to have a potent antimicrobial agent against many Gram negative and positive bacteria, especially in food preservation branches [43]. Additionally, it was suggested that the combined interactions between proteinous biomaterials and metal nanoparticles could increase the bioactivity of them through the enhancement of their correlated morphological characteristics [44].

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REFERENCES


