Antimicrobial activity of chitosan composites against bacterial strains isolated from goat meat and cheese

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Abstract. Chitosan composites based on residual biomass (shrimp shell) and polyvinyl alcohol (PVA) with different concentrations of CuO nanoparticles (NPs) were prepared and tested against bacteria isolated from goat meat and cheese. A casting method was used to prepare composites with 0, 0.05, 0.10, 0.15 and 0.20% CuO in the mixed solution (chitosan+PVA). The CuO NPs were characterized using FTIR spectroscopy and XRD. Additionally, these composites were analyzed by thermogravimetric analysis (TGA). NPs were detected by XRD mainly in the composite with the highest concentration (0.2%). Isolated bacteria from goat meat and milk (15 strains) were used to test the antibacterial properties of the composites, evaluating the effect of CuO NP concentration. All synthesized composites presented antibacterial inhibition against bacteria. However, for some strains of gram-positive and gram-negative bacteria, inhibition was 50% of that observed for the combination of antibiotics, amoxicillin/clavulanic acid (AMC) or trimethoprim/sulfamethoxazole (SXT). Gram-negative bacteria isolated from goat meat were sensitive to PVA composites with CuO NPs. In most of the tested strains, the zone of inhibition increased as the NP concentration in the composite increased.

1. Introduction
Chitosan is a polysaccharide produced from different natural and abundant chitin sources. One of these sources of low-cost raw materials is the shrimp (*Penaeus vannamei*) shell, which is abundant in the north of Peru. Aquaculture is the main economic activity that produces large amounts of shrimp shell in different steps of the production chain, including shrimp cultivation in ponds and processing (conditioning and freezing of shrimp) However, residual biomass is not adequately managed, and a high amount of residue is disposed of in places without proper technical conditions, impacting nearby
populations and environments [1]. A small proportion of the residue is used to produce flour for animal feed; nevertheless, it is not an economical and technical alternative for producers.

Chitosan is a versatile polymer widely used in medical, pharmaceutical and food applications due to its biocompatibility, environmental advantages and antibacterial capacity [2]. Moreover, the antibacterial activity of chitosan might be enhanced by combining it with other antibacterial particles or molecules. CuO nanoparticles are a very effective material against a wide range of bacteria [3], and they have been used in combination with chitosan and other polymers against different bacteria [4-7].

Goat cheese and meat are two important food products in the dry forest areas in northwest Peru. However, due to the inappropriate sanitary conditions of production and the nature of milk products, they are prone to microbial contamination and reduced quality and shelf life. The present study aimed to test the effect of composites of chitosan, polyvinyl alcohol (PVA) and different concentrations of CuO nanoparticles on bacterial strains isolated from goat cheese and meat. This study examines the antibacterial effect of these composites on a wide range of strains that cause deterioration of these food products.

2. Materials and Methods

2.1. Synthesis and characterizations of composites

The composites of chitosan derived from shrimp shells (deacetylation degree of 81%) were prepared using the casting method. Solutions of 1% wt/v chitosan and 1.5% PVA were prepared separately. The CuO nanoparticles were synthetized using a sol-gel method starting from copper sulfate as a precursor. Table 1 depicts the codes of the different composites prepared for this study.

| Samples            | Description                                                                 |
|--------------------|-----------------------------------------------------------------------------|
| SCh-PVA            | Chitosan composites made from shrimp shell /PVA                              |
| SCh-PVA-CuO (0.05) | Chitosan composites made from shrimp shell /PVA plus CuO (0.05% weight in the mixed solution) |
| SCh-PVA-CuO (0.10) | Chitosan composites made from shrimp shell / PVA plus CuO (0.10% weight in the mixed solution) |
| SCh-PVA-CuO (0.15) | Chitosan composites made from shrimp shell / PVA plus CuO (0.15% weight in the mixed solution) |
| SCh-PVA-CuO (0.20) | Chitosan composites made from shrimp shell / PVA plus CuO (0.20% weight in the mixed solution) |

Structure characterization was performed by X-ray diffraction (XRD) using a PANalytical XpertPro diffractometer, and Fourier transform infrared (FTIR) (Shimadzu IR Prestige 21) analyses were carried out to characterize the CuO nanoparticles and composites. Additionally, thermal properties were analyzed on a Q-600 instrument (Universal TA Instruments) to compare the composites with different concentrations of CuO nanoparticles.

2.2. Antibacterial properties of composites

For the purpose of this study, 15 bacterial strains isolated from meat and goat milk cheese were used to test the prepared composites. The strains were taken from the microbial bank of the Laboratory of Microbiology of the National University of Tumbes, which have been identified at the species level using genotypic methods based on 16S rRNA gene sequencing.

To measure the antibacterial properties of the composites, discs of uniform size with a diameter of 6 mm were prepared.

A bacterial inoculum was prepared from the cultures between 18 and 24 hours of incubation on nonselective agar and resuspended in sterile saline solution. The turbidity of the tube was adjusted to
0.5 on the McFarland scale. Plates with Muller-Hinton agar were inoculated using the Kirby Bauer method. The discs were placed aseptically at a distance of 25 mm from each other. Once the procedure was performed, the plates were incubated at 35°C for 18-24 hours. The diameter (mm) of the zone of inhibition around each disc was measured and interpreted according to the data in table 3. In the experiment, the bacteria isolated previously from goat meat and cheese were used.

3. Results and discussion

3.1. Production and characterization of composites

The CuO NPs were characterized by XRD and FTIR prior to making the composites. The XRD characterization of the CuO NPs used in the experiments is shown in figure 1. The pattern in figure 1a presents the typical peaks of CuO [8-10]. Additionally, according to Ahmadian, Bakravi, Hashemi and Namazi [1], the broad intensities are due to the nanosized particles. The FTIR spectrum of the CuO NPs in figure 1b shows typical peaks, which are evidence of the presence of NPs. The intensities of the peaks located at (i) 490, 550 and 601 cm⁻¹ come from Cu-O symmetric stretching, Cu-O asymmetric stretching and Cu-O wagging, respectively [9, 11]; (ii) those at 3388 cm⁻¹ are assigned to OH due to the presence of water[9]; and (iii) those at 1643 cm⁻¹ are assigned to an out-of-plane metal-oxygen (M-O) bond [11]. The intensities of the peaks in the range of 752 to 880 cm⁻¹ might correspond to bending mode vibrations of M-O-M (M = Cu) [12]. According to Ethiraj and Kang [13], when there is no peak between 605 and 660 cm⁻¹, Cu₂O does not exist as an impurity, as in the case of this study. However, Ho, Tay, Qi, Huang, Li and Chen [14] postulated that the peaks at 1124 and 1085 cm⁻¹ correspond to C-O in Cu₂O, which indicates the presence of some impurities.

![Figure 1. XRD (a) and FTIR (b) analyses of the CuO NPs used in this study.](image)

Figure 2 depicts both the XRD and FTIR spectra of the composites without CuO NPs and those with different concentrations of CuO. The peaks assigned to CuO are visible for the composites with the highest concentration of the nanoparticles, sample Sch-PVA-CuO (0.20); however, small peaks are visible for the samples Sch-PVA-CuO (0.15) and Sch-PVA-CuO (0.10). In the case of sample Sch-PVA-CuO (0.05), the peaks did not appear because the concentration of the NPs might be too small for XRD detection.

Figure 2b depicts the FTIR spectra of the composites, and table 2 shows the main peaks with their respective functional groups. The intensities of the peaks at 3212, 2904, 1539, 1406, 1064 and 541 cm⁻¹
shifted to higher or lower wavenumber values when CuO NPs were incorporated into the composites (table 2). This indicates an interaction between the CuO NPs and the chitosan/PVA matrix.

![Figure 2. XRD (a) and FTIR (b) analyses of the composites.](image)

**Table 2. FTIR intensities (cm⁻¹) from the composites with different concentrations of CuO NPs.**

| Functional group [4] | SCh-PVA | SCh-PVA-CuO (0.05) | SCh-PVA-CuO (0.10) | SCh-PVA-CuO (0.15) | SCh-PVA-CuO (0.20) |
|----------------------|---------|--------------------|--------------------|--------------------|--------------------|
| 3212                 | 3244    | 3269               | 3262               | 3262               |
| 2904                 | 2913    | 2940               | 2940               | 2940               |
| 1539                 | 1547    | 1549               | 1551               | 1551               |
| 1406                 | 1404    | 1398               | 1398               | 1398               |
| 1152                 | 1153    | 1152               | 1152               | 1153               |
| 1064                 | 1053    | 1049               | 1049               | 1049               |
| 651                  | 653     | 658                | 658                | 662                |

The mass loss comparison among the composites is shown in figure 3a in these thermogravimetric curves from the prepared composite to identify the thermal behavior for each one. The weight loss in the sample can be grouped into five stages: (i) from room temperature to 140-150°C, reaching weight loss levels of up to 10-12%; (ii) from 140-150°C to 216-295°C, reaching weight loss levels of up to 22-50%; (iii) from 216-295°C to 405-470°C, reaching weight loss levels of up to 55-65%; (iv) from 405-470°C to 450-550°C, reaching weight loss levels of up to 85-97%; and (v) from 450-550°C to 680°C, where the weight loss level remained constant. The first stage corresponded to the vaporization of water and residual acetic acid in the chitosan solution [15, 16], the second to the thermal degradation of chitosan and PVA [16], and the third and fourth to the degradation of the byproducts of PVA (aldehydes and alkene end-groups in the molten state) [15]. The residual mass at 680°C was 3.3, 6.3, 14.4, 15.3 and 15.5% in the case of SCh-PVA, SCh-PVA (0.05), SCh-PVA (0.10), SCh-PVA (0.15) and SCh-PVA (0.20), respectively (see figure 3b). As the amount of CuO NPs in the composite increases, the residual
mass increases. However, the increase in residual mass is not proportional to the CuO NP concentration. This could be due to the possibly inhomogeneous NP distribution in the chitosan matrix.

3.2. Antimicrobial activities of composites

The antibacterial inhibition (mm) of the composites against isolated strains is shown in Table 3. Additionally, the percentage of composite antibacterial inhibition with respect to control amoxicillin/clavulanic acid (AMC) (%AMC) and trimethoprim/sulfamethoxazole (STX) (%STX) was calculated.

In most strains tested, it was observed that the zone of inhibition increased as the nanoparticle concentration increased in the composite. Several studies have shown that microbial activity is associated with the amount of nanoparticles in composites [6, 17]. Various authors have compared the antibacterial activity of chitosan and chitosan combined with CuO NPs, and they concurred that the presence of CuO NPs improved the antibacterial capacity of chitosan [5, 18].

In this test, gram-positive bacteria *Bacillus cereus* and *Macrococcus caseolyticus* isolated from cheese showed zones of inhibition between 52-69% for the composition of SCh-PVA-CuO (0.20%) in relation to the STX control. Similarly, *M. caseolyticus* isolated from meat showed zones of inhibition between 52-57% with respect to the same control. Gram-negative bacteria isolated from meat showed zones of inhibition above 50% in relation to those presented by both controls, in contrast to the strains isolated from cheese. Only for *Methylobacterium lusitanum* were the four concentrations of the composite effective at showing zones of inhibition were 50-100% of those shown by AMC. However, the zone of inhibition was 17% larger for the four concentrations than that observed with SXT. For *Moraxella osloensis* and *Pseudomonas lundensis*, the inhibition zone for the four concentrations was between 50-100% in relation to those presented by AMC.

It has been postulated that the bacterial activity of nanoparticles is diminished mainly in gram-negative bacteria and not in gram-positive bacteria due to the structure of their cellular wall [19], and this is also different according to the species of bacteria [19, 20]. In addition, because the NPs are not acting individually, their diffusion into the cell would be reduced [19].

Antimicrobial activity would be related to the direct electrostatic binding of the cellular cover that is negative and the positive charge of the nanoparticles of the composite, as well as the synergistic activity of the chitosan and the CuO nanoparticle [5, 18]. Another mechanism involves the release of reactive oxygen species that act on the cellular wall of bacteria, which would lead to the alteration of cellular permeability and nanoparticle entry, thus altering physiological and biochemical processes that cause cell death [17]. According to Chatterjee, Chakraborty and Basu [21], Cu-NPs caused combined, multiple toxic effects on bacteria cells: generation of reactive oxygen species, lipid peroxidation, protein oxidation and DNA degradation.
Table 3. Antimicrobial inhibition (mm and % with respect to AMC and SXT) of composites of chitosan/CuO nanoparticles against bacteria isolated from goat meat and cheese.

| Bacteria                          | SCh-PVA-CuO (0.05) | SCh-PVA-CuO (0.10) | SCh-PVA-CuO (0.15) | SCh-PVA-CuO (0.20) | Amoxicillin/clavulanic acid (AMC) (mm) | Trimethoprim/sulfamethoxazole (SXT) (mm) |
|-----------------------------------|--------------------|--------------------|--------------------|--------------------|----------------------------------------|----------------------------------------|
|                                   | mm     | %AMC | %SXT | mm     | %AMC | %SXT | mm     | %AMC | %SXT | mm     | %AMC | %SXT | mm     | %AMC | %SXT |
| From goat meat                    |        |      |      |        |      |      |        |      |      |        |      |      |        |      |      |
| Methylobacterium lusitanum        | 7      | 50   | 117  | 7      | 50   | 117  | 7      | 50   | 117  | 7      | 50   | 117  | 14     | 6     |
| Macrococcus caseolyticus          | 9      | 28   | 39   | 13     | 41   | 57   | 12     | 38   | 52   | 12     | 38   | 52   | 32     | 23    |
| Moraxella osloensis               | 6      | 50   | 24   | 6      | 50   | 24   | 10.5   | 88   | 42   | 11     | 92   | 44   | 12     | 25    |
| Pseudomonas lundensis             | 6      | 50   | 25   | 6      | 50   | 25   | 10     | 83   | 42   | 12     | 100  | 50   | 12     | 24    |
| Bacillus firmus                   | 6      | 38   | 23   | 6      | 38   | 23   | 6      | 38   | 23   | 6      | 38   | 23   | 16     | 26    |
| Staphylococcus kloosii            | 7      | 23   | 21   | 7      | 23   | 21   | 7      | 23   | 21   | 10     | 33   | 29   | 30     | 34    |
| From goat cheese                  |        |      |      |        |      |      |        |      |      |        |      |      |        |      |      |
| Klebsiella oxytoca                | 7      | 23   | 29   | 7      | 23   | 29   | 7      | 23   | 29   | 7      | 23   | 29   | 30     | 24    |
| Lactococcus lactis                | 6      | 32   | 38   | 6      | 32   | 38   | 6      | 32   | 38   | 6      | 32   | 38   | 19     | 16    |
| Kocuria kristinae                 | 7      | 39   | 41   | 7      | 39   | 41   | 7      | 39   | 41   | 6      | 33   | 35   | 18     | 17    |
| Bacillus cereus                   | 6      | 19   | 38   | 7      | 22   | 44   | 7      | 22   | 44   | 11     | 34   | 69   | 32     | 16    |
| Morganella morganii               | 7      | 26   | 30   | 7      | 26   | 30   | 7      | 26   | 30   | 7      | 26   | 30   | 27     | 23    |
| Lactococcus lactis                | 7      | 26   | 30   | 7      | 26   | 30   | 7      | 26   | 30   | 7      | 26   | 30   | 27     | 23    |
| Macrococcus caseolyticus          | 12     | 32   | 38   | 7      | 19   | 22   | 12     | 32   | 38   | 17     | 46   | 53   | 37     | 32    |
| Staphylococcus saprophyticus      | 7      | 23   | 26   | 7      | 23   | 26   | 7      | 23   | 26   | 7      | 23   | 26   | 30     | 27    |
4. Conclusions

Chitosan composites based on residual biomass (shrimp shell), polyvinyl alcohol (PVA) and different concentrations of CuO nanoparticles (NPs) were prepared and tested against 15 bacterial strains isolated from goat meat and cheese. The CuO NPs were detected mainly in the composite with the highest concentration of CuO NPs (0.2%) by XRD. The prepared composites presented antibacterial effects on the bacterial strains isolated from goat cheese and meat. However, in most of cases, the inhibitory effect of the composites was lower than that of the combination of antibiotics, amoxicillin/clavulanic acid (AMC) and trimethoprim/sulfamethoxazole (SXT). In most of the tested strains, the zone of inhibition increased as the nanoparticle concentration increased in the composite. These synthesized composites could be used for other technological applications.

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