

ORIGINAL RESEARCH PAPER

Investigation of synergistic effect of cuo nanoparticles and nisin on genome of Escherichia coli bacteria

Trifa Sheikhaighaiy, Bahram Golestani Eimani*

Department of Biology, Urmia Branch, Islamic Azad University, Urmia, Iran

Received: 2018-07-17

Accepted: 2018-10-01

Published: 2018-10-15

ABSTRACT

Given the gradual development of drug resistance in different bacterial species, it is necessary to search for new drugs with effective broad-spectrum antimicrobial activity. Therefore, recent studies on various nanometal oxides such as copper oxide and on antibacterial peptides including nisin as antibacterial agents are especially important. The present study aimed to investigate the synergistic effect of nisin-conjugated copper oxide nanoparticles (CuO NPs) on the genome of *E. coli* selected as a Gram-negative model. After being cultured in a Nutrient Broth medium, the bacteria were treated with CuO NPs at 15, 30, 40, and 60 µg/mL, with nisin at 30, 60, 90, and 120 µg/mL, and with nisin-conjugated CuO NPs at 10, 20, and 30 µg/mL and were then incubated. The optical densities of the samples were read at 600nm and their DNA was extracted. RAPD-PCR was used to study genomic effects, and statistical analysis was performed employing NTSYS-PC based on the DICE coefficient, the similarity matrix, and the drawn dendrogram. Results showed that the combination of CuO NPs and nisin had synergistic effects and was able to inhibit growth more than either of them used alone. However, this combination had no synergistic effects on the genome and caused minimal changes in the DNA sequence.

Keywords: Copper Oxide Nanoparticles, Escherichia Coli, Nisin, RAPD-PCR, Synergistic

How to cite this article

Sheikhaighaiy T, Golestani Eimani B. Investigation of synergistic effect of cuo nanoparticles and nisin on genomic of Escherichia coli bacteria. J. Water Environ. Nanotechnol., 2018; 3(4): 355-367. DOI: 10.22090/jwent.2018.04.008

INTRODUCTION

Nanotechnology is a leading scientific field because it combines physics, chemistry, biology, medicine, bioinformatics, and engineering. It is an emerging field with a high potential for achieving great success, and it has applications in real life [8]. Nanotechnology is one of the fastest growing industries in human history and has been called the next industrial revolution. For centuries, people have used copper for its antibacterial properties. Copper oxide nanoparticles (CuO NPs) have more antibacterial activity than copper [14]. Antibacterial activity of CuO is associated with a sudden decrease in cell membrane integrity and production of reactive oxygen species (ROS) [15, 16]. Recent studies have shown that some lactic acid bacteria used as bio-preservatives can not

only compete with pathogens for nutrients but also produce antimicrobial metabolites that are harmless to humans [7]. Some bacteriocins have antimicrobial activity against closely related species. Certain strains of *Lactococcus lactis* produce these bacteriocins including nisin [2, 3]. Heat stability of nisin and its stability under acidic and freezing conditions in addition to its inactivation in the digestive system have made this property of nisin more functional. Many studies have shown that the effective suppression of bacteria by nisin depends on a broad spectrum of factors. In particular, its antibacterial activity increases when it is used in combination with other agents such as vegetable oils, green garlic juice, ethanol, sodium fluoride or chlorhexidine, lactoperoxidase, and hydrostatic pressure. Nisin can inhibit the growth of Gram-

* Corresponding Author Email: golestani_bahram@yahoo.com



positive bacteria with a minimal inhibitory concentration in the nanomolar range but is less effective against Gram-negative bacteria [4]. The general mechanisms of nisin activity against bacteria include binding to the cell membrane, insertion into the cell membrane, pore formation and reaction with lipid II [1]. The present study aimed to investigate the synergistic antibacterial and genomic effects of CuO NPs and nisin on *E. coli*.

MATERIALS AND METHODS

Preparation of stock solutions

To prepare the CuO stock solution, 0.106 g of phosphate buffered saline powder was dissolved in 10 mL of distilled water and autoclaved at 121

°C for 15 min. Then, 0.01 g of CuO NPs powder was added (its concentration reached 1000 µg/mL) and the solution was sonicated at 70 volts and a frequency of 20 kHz for 5 min. at 25 °C. The nisin stock solution was prepared in the same way. To prepare the solution of nisin-conjugated CuO NPs, 7 mL of the CuO stock solution was mixed with 7 mL of nisin stock solution and the mixture was incubated overnight in a shaking incubator. It was then poured into 2 mL microtubes and the non-covalent bonds between CuO and nisin were cleaved through washing the solution three times with phosphate buffer and by centrifuging it at 10,000 rpm for 45 min. The sediments were resuspended in 300 µL of phosphate buffer and stored at 4 °C for further use.

Table 1: Nucleotide sequence of RAPD-PCR primers

| Primer name | Primer nucleotide sequence |
|-------------|----------------------------|
| OPS.13 | GTCGTTCCCTG |
| OPA.10 | GTGATCGCAG |
| OPA.09 | GGGTAACGCC |
| OPR.12 | ACAGGTGCGT |
| OPA.11 | CAATCGCCGT |
| OPQ.14 | GGACGTTCA |
| OPS.05 | TTTGGGGCCT |
| OPS.03 | CAGAGGTCCC |
| OPQ.17 | GAAGCCCTTG |
| OPC.09 | CTCACCGTCC |

Table 2: Temporal and thermal cycles used in RAPD-PCR

| Cycle | Stage name | Temperature | Time | Cycles number |
|--------|----------------------|-------------|-------|---------------|
| First | Initial denaturation | 95 °C | 5 min | 1 |
| | Denaturation | 95 °C | 35 s | |
| Second | Annealing | 30 °C | 45 s | 40 |
| | Extension | 72 °C | 45 s | |
| Third | Final extension | 72 °C | 7 min | 40 |

Table 3: Components needed for PCR

| Compounds | Amounts |
|------------------|---------|
| Primer | 1µL |
| Master Mix | 12.5µL |
| H ₂ O | 10.5µL |
| DNA Sample | 1µL |
| Total Volume | 25µL |

Table 4: Mean optical density reflecting antimicrobial activity exhibited by CuO NPs

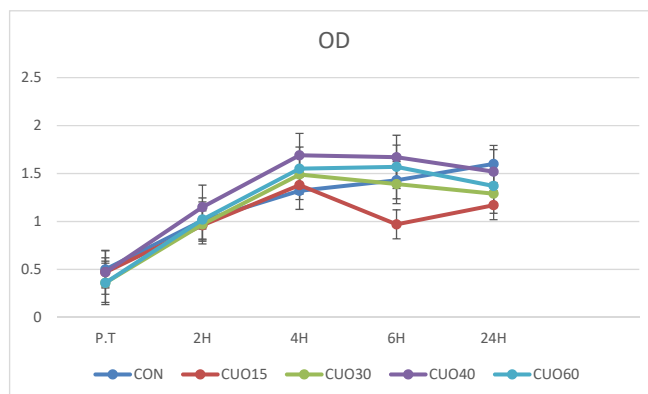
| Concentration | Tube number | Before treatment cell/ml | 2 hours after treatment cell/ml | 4 hours after treatment cell/ml | 6 hours after treatment cell/ml | 24 hours after treatment cell/ml |
|---------------|-------------|--------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------------|
| control | 1 | 400 × 10 ⁶ | 808 × 10 ⁶ | 1.056 × 10 ⁹ | 1.144 × 10 ⁹ | 1.28 × 10 ⁹ |
| 15µg/ml | 2 | 376 × 10 ⁶ | 768 × 10 ⁶ | 1.104 × 10 ⁹ | 776 × 10 ⁶ | 936 × 10 ⁶ |
| 30µg/ml | 3 | 288 × 10 ⁶ | 776 × 10 ⁶ | 1.192 × 10 ⁹ | 1.112 × 10 ⁹ | 1.032 × 10 ⁹ |
| 40µg/ml | 4 | 376 × 10 ⁶ | 920 × 10 ⁶ | 1.352 × 10 ⁹ | 1.336 × 10 ⁹ | 1.216 × 10 ⁹ |
| 60µg/ml | 5 | 288 × 10 ⁶ | 816 × 10 ⁶ | 1.24 × 10 ⁹ | 1.256 × 10 ⁹ | 1.104 × 10 ⁹ |



Bacterial culture and treatment

E. coli strain O157:H7 was first cultured in solid Nutrient Agar and then a single colony of it was passed on a liquid Nutrient Broth culture medium and incubated in a shaking incubator at

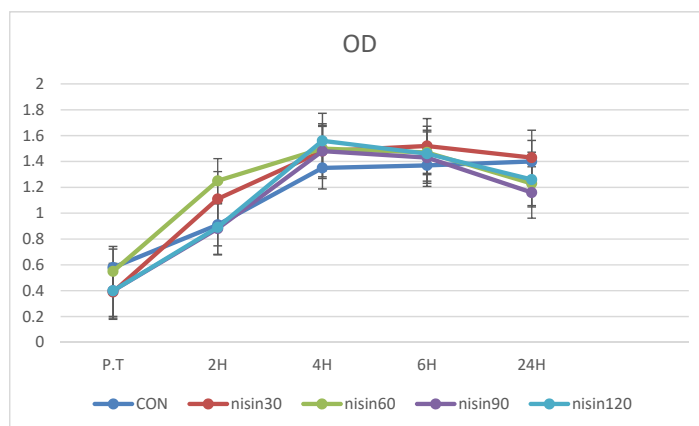
150 rpm for 24 hours at 37 °C. Antimicrobial effects of CuO NPs at 15, 30, 40, and 60 µg/mL, nisin at 30, 60, 90, and 120 µg/mL, and solutions of nisin-conjugated CuO NPs at 10, 20, 30 µg/mL were investigated along with those of the control in two



Line chart1: The CuO nanoparticles addition to the culture medium after 2,4,6,24 hours.

Table 5: Mean optical density reflecting antimicrobial activity exhibited by Nisin

| Concentration | Tube number | Before treatment cell/ml | 2 hours after treatment cell/ml | 4 hours after treatment cell/ml | 6 hours after treatment cell/ml | 24 hours after treatment cell/ml |
|---------------|-------------|--------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------------|
| control | 1 | 464 × 10 ⁶ | 728 × 10 ⁶ | 1.08 × 10 ⁹ | 1.096 × 10 ⁹ | 1.12 × 10 ⁹ |
| 30 µg/ml | 2 | 312 × 10 ⁶ | 888 × 10 ⁶ | 1.184 × 10 ⁹ | 1.216 × 10 ⁹ | 1.144 × 10 ⁹ |
| 60 µg/ml | 3 | 440 × 10 ⁶ | 1 × 10 ⁹ | 1.2 × 10 ⁹ | 1.176 × 10 ⁹ | 984 × 10 ⁶ |
| 90 µg/ml | 4 | 320 × 10 ⁶ | 704 × 10 ⁶ | 1.184 × 10 ⁹ | 1.144 × 10 ⁹ | 928 × 10 ⁶ |
| 120 µg/ml | 5 | 320 × 10 ⁶ | 712 × 10 ⁶ | 1.248 × 10 ⁹ | 1.168 × 10 ⁹ | 1.008 × 10 ⁹ |



Line chart2: The nisin addition to the culture medium after 2,4,6,24 hours.

Table 6: Mean optical density reflecting antimicrobial activity exhibited by nisin-conjugated CuO NPs

| Concentration | Tube number | Before treatment cell/ml | 2 hours after treatment cell/ml | 4 hours after treatment cell/ml | 6 hours after treatment cell/ml | 24 hours after treatment cell/ml |
|---------------|-------------|--------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------------|
| control | 1 | 440 × 10 ⁶ | 720 × 10 ⁶ | 992 × 10 ⁶ | 1 × 10 ⁹ | 1.168 × 10 ⁹ |
| 10 µg/ml | 2 | 288 × 10 ⁶ | 784 × 10 ⁶ | 1.256 × 10 ⁹ | 1.32 × 10 ⁹ | 1.52 × 10 ⁹ |
| 20 µg/ml | 3 | 352 × 10 ⁶ | 712 × 10 ⁶ | 1.144 × 10 ⁹ | 1.224 × 10 ⁹ | 1.032 × 10 ⁹ |
| 30 µg/ml | 4 | 296 × 10 ⁶ | 640 × 10 ⁶ | 1.056 × 10 ⁹ | 872 × 10 ⁶ | 920 × 10 ⁶ |

replications. Optical densities of all the samples were measured before and after treatment at 2, 4, 6, and 24-hour intervals.

DNA extraction and RAPD-PCR

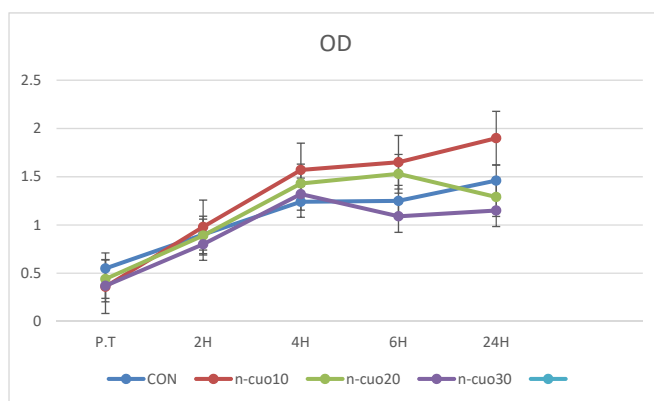
DNA of the control and of the treated bacteria was extracted using DNA extraction kits (Exir Azma Company) according to the manufacturer's instructions. The extracted DNA quality was analyzed by spectrophotometry and electrophoresis on 1% agarose gel. RAPD-PCR was then used to investigate the effects of the prepared solutions on the bacterial genome. To this end, 10 random primers were used with characteristics shown in Table 1. The temperature program and the components of PCR are shown in Tables 2 and 3, respectively.

Results concerning bacterial growth inhibition by CuO NPs were obtained through measuring OD_{600nm} of treatments and different times that reflect bacterial density and growth. The inhibitory effects of CuO NPs and nisin on bacterial growth at all treatment concentrations were observed 4 to 24 hours after treatment. The related information is shown in Tables 4 and 5 and line chart 1 and 2, respectively. Among the treatments of nisin-conjugated CuO NPs, inhibitory effects on bacterial

growth were observed only at concentrations of 20 and 30 µg/mL 6 to 24 hours after the treatments. That information is also presented in Table 6 and line chart 3. Comparison of antibacterial effects of CuO NPs, nisin, and nisin-conjugated CuO NPs at the same concentration (30µg/mL) based on OD read 6 to 24 hours after treatment showed that CuO had a better inhibitory effect than nisin and nisin-conjugated CuO NPs. Further details are presented in Table 7 and Chart 4.

Results of RAPD-PCR using 10 primers were examined by performing electrophoresis on its products on 2% agarose gel (Tables Fig. 1-4). Based on the presence and absence of bands, the products were given the score of zero and one and analysis were then performed using the NTSYS-PC software.

Based on the results of similarity matrix calculations in Tables 8-10 and of dendrograms No. 5-7, the CuO NPs had the lowest and highest genomic effects at 30µg/mL and 60µg/mL, respectively. In addition, nisin had the lowest genomic effect at 30µg/mL and the highest at 60µg/mL. Results related to the genomic effects of these three solutions at the same concentration of 30 µg/mL, which similarity matrix and dendrogram are respectively depicted in Table 11 and Dendrogram



Line chart3: The nisin-conjugated CuO NPs addition to the culture medium after 2,4,6,24 hours.

Table 7: Mean optical densities reflecting antimicrobial activity shown by CuO, nisin, and nisin-conjugated CuO NPs at the same concentration (30µg/mL)

| Concentration | Tube number | Before treatment cell/ml | 2 hours after treatment cell/ml | 4 hours after treatment cell/ml | 6 hours after treatment cell/ml | 24 hours after treatment cell/ml |
|---------------|-------------|--------------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------------|
| control | 1 | 400× 10 ⁶ | 808× 10 ⁶ | 1.056× 10 ⁹ | 1.144× 10 ⁹ | 1.28× 10 ⁹ |
| CuO | 2 | 288× 10 ⁶ | 776× 10 ⁶ | 1.192× 10 ⁹ | 1.112× 10 ⁹ | 1.032× 10 ⁹ |
| Nisin-CuO | 3 | 296× 10 ⁶ | 640× 10 ⁶ | 1.056× 10 ⁹ | 872× 10 ⁶ | 920× 10 ⁶ |
| Nisin | 4 | 312× 10 ⁶ | 888× 10 ⁶ | 1.184× 10 ⁹ | 1.216× 10 ⁹ | 1.144× 10 ⁹ |



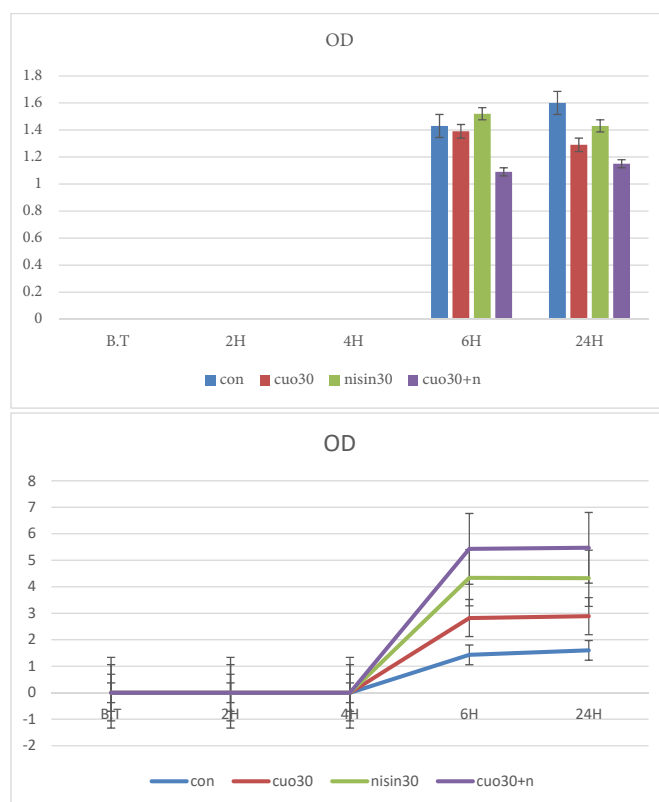
No. 8 respectively, show that the longest and shortest created genetic distances compared to the control belonged to the bacteria treated with CuO NPs and the solution of nisin-conjugated CuO NPs, respectively.

Analysis of variance of ODs measured at different times with different concentrations of copper oxide nanoparticles:

Analysis of variance of OD measured at different times under the influence of different antibacterial

concentrations of copper oxide nanoparticles showed that F calculated with 4 and 15 degrees of freedom was larger than F in the (Table 12). In addition, the calculated Sig was smaller than the assumed α of 5% (Table 13). Therefore, there was a significant difference at 5% level between the amounts of bacteria grown at different times measured.

The OD table obtained from the Tukey's test for analyzing the means at different times from the mean of 4 concentrations of copper oxide



Column chart and line chart 4: Mean optical densities reflecting antimicrobial activity exhibited by CuO, nisin, and nisin-conjugated CuO NPs at the same concentration



Fig. 1: Electrophoresis results of non-conjugated products obtained from RAPD-PCR

Column 1: Marker, Columns 2 to 5: Primer OPQ14, Column 6: Marker, Columns 7 to 10: OPQ17, Column 11: Marker, Columns 12 to 15: OPR12, Column 16: Marker, Columns 17 to 20: OPS03, Column 21: Marker, Columns 22 to 25: OPA11

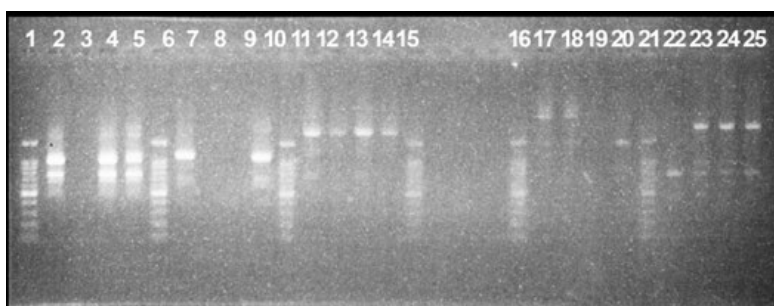


Fig. 2: Electrophoresis results of non-conjugated products obtained from RAPD-PCR

Column 1: Marker, Columns 2 to 5: Primer OPA09, Column 6: Marker, Columns 7 to 10: OPA10, Column 11: Marker, Columns 12 to 15: OPC09, Column 16: Marker, Columns 17 to 20: OPS13, Column 21: Marker, Columns 22 to 25: OPS05

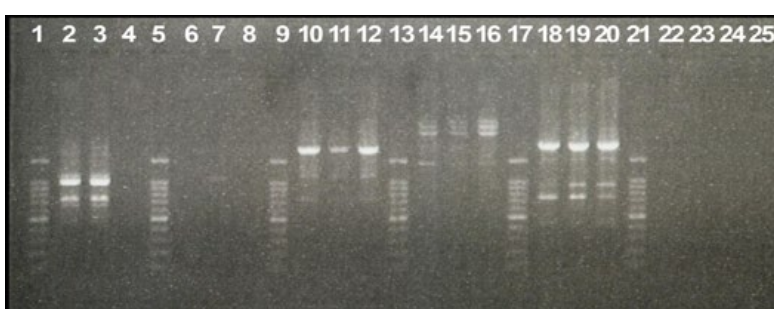


Fig. 3: Electrophoresis results of conjugated products obtained from RAPD-PCR

Column 1: Marker, Columns 2 to 4: Primer OPA09, Column 5: Marker, Columns 6 to 8: OPA10, Column 9: Marker, Columns 10 to 12: OPC09, Column 13: Marker, Columns 14 to 16: OPS13, Column 17: Marker, Columns 18 to 20: OPS05, Column 21: Marker



Fig. 4: Electrophoresis results of conjugated products obtained from RAPD-PCR

Column 1: Marker, Columns 2 to 4: Primer OPQ14, Column 5: Marker, Columns 6 to 8: OPQ17, Column 9: Marker, Columns 10 to 12: OPR12, Column 13: Marker, Columns 14 to 16: OPS03, Column 17: Marker, Columns 18 to 20: OPA11, Column 21: Marker

nanoparticles showed a significant difference between subsets 1 and 2 (before and 2 hours after treatment), so that the mean OD increased from 0.4150 to 1.0250, indicating a lack of inhibitory effect of copper oxide nanoparticles in these two time periods. In the fourth subset, the mean OD increased for 4 hours after treatment to 1.5275, and finally, in the third subset, the mean OD decreased from 1.3800 to 1.3400, indicating that the inhibitory

effect of antimicrobial concentrations of copper oxide nanoparticles occurred 4 to 6 after treatment, after which it was almost constant (Diagram 1).

Analysis of variance of ODs measured at different times with different concentrations of nisin:

Analysis of variance of OD measured at different times under the influence of different antibacterial concentrations of Nisin showed that F calculated

Table 8: Similarity matrix for the control samples and samples treated with CuO NPs

| Case | Dice (Czekanowski or Sorenson) Measure | | |
|-------|--|-----------|-----------|
| | 1:ctr | 2:t30 | 3:t60 |
| 1:ctr | 1.000 | | |
| 2:t30 | 0.5294118 | 1.0000000 | |
| 3:t60 | 0.4516129 | 0.7037037 | 1.0000000 |

This is a similarity matrix

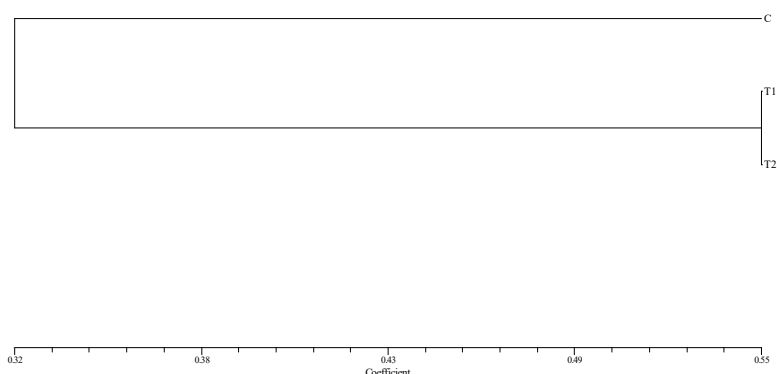


Fig. 5: Dendrogram obtained from analysis based on a rapid test using the UPGEMA method by NTSYS-PC

Table 9: Similarity matrix for the control samples and samples treated with Nisin

| Case | Dice (Czekanowski or Sorenson) Measure | | |
|-------|--|-----------|-----------|
| | 1:ctr | 2:t30 | 3:t60 |
| 1:ctr | 1.000 | | |
| 2:t30 | 0.8235294 | 1.0000000 | |
| 3:t60 | 0.7500000 | 0.7692308 | 1.0000000 |

This is a similarity matrix

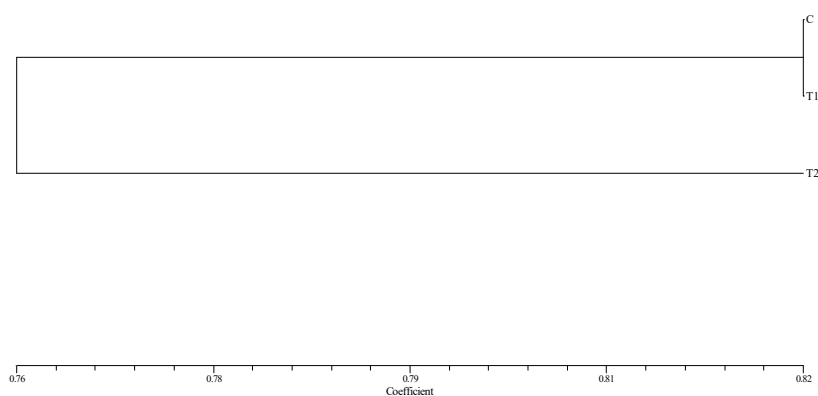


Fig. 6: Dendrogram obtained from analysis based on a rapid test using the UPGEMA method by NTSYS-PC

with 4 and 15 degrees of freedom was larger than F in the (Table 14). In addition, the calculated Sig was smaller than the assumed α of 5% (Table 15). Therefore, there was a significant difference at 5% level between the amounts of bacteria grown at different times measured.

The OD table obtained from the Tukey's test for analyzing the means at different times from the mean of 4 concentrations of nisin showed a significant difference between subsets 1 and 2 (before and 2 hours after treatment), so that the mean OD increased from 0.4350 to 1.0325,

Table 10: Similarity matrix for the control samples and samples treated nisin-conjugated CuO NPs

| Case | Dice (Czekanowski or Sorenson) Measure | | |
|-------|--|-----------|-----------|
| | 1:ctr | 2:t20 | 3:t30 |
| 1:ctr | 1.000 | | |
| 2:t20 | 0.7500000 | 1.0000000 | |
| 3:t30 | 0.6666667 | 0.3636364 | 1.0000000 |

This is a similarity matrix

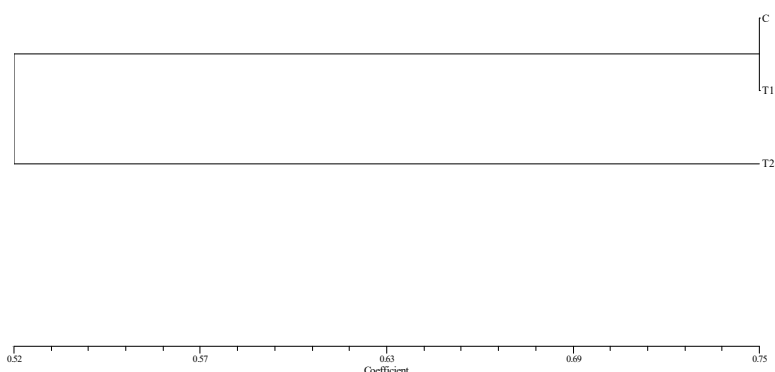


Fig. 7: Dendrogram obtained from analysis based on a rapid test using the UPGEMA method by NTSYS-PC

Table 11: Similarity matrix for nisin, CuO, and CuO NPs conjugated with nisin at the same concentration of 30µg/mL

| Case | Dice (Czekanowski or Sorenson) Measure | | | |
|------|--|-----------|-----------|-----------|
| | C | T1 | T2 | T3 |
| C | 1.0000000 | | | |
| T1 | 0.1818182 | 1.0000000 | | |
| T2 | 0.6666667 | 0.5000000 | 1.0000000 | |
| T3 | 0.4000000 | 0.0000000 | 0.3333333 | 1.0000000 |

This is a similarity matrix

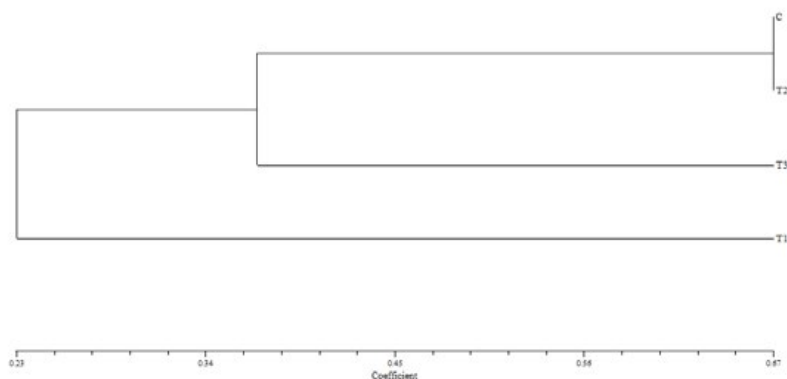


Fig. 8: Dendrogram obtained from analysis based on a rapid test using the UPGEMA method by NTSYS-PC

C: Control; T1: CuO NPs; T2: CuO NPs conjugated with nisin; T3: Nisin

indicating a lack of inhibitory effect of nisin in these two time periods. In the third subset, including the time intervals of 4, 6, and 24 hours after treatment, the mean ODs were different, but this difference was not significant. Up to 4 hours after treatment, the mean ODs increased to 1.5175, but at 4 to 6 hours after treatment, they decreased to 1.4700, indicating the inhibitory effects of these

concentrations at this time interval (Diagram 2). Therefore, it can be concluded that the antibacterial activity and inhibitory effect of nisin occurred only 4 to 6 hours after treatment.

Analysis of variance of ODs measured at different times with different concentrations of copper oxide-nisin complexes:

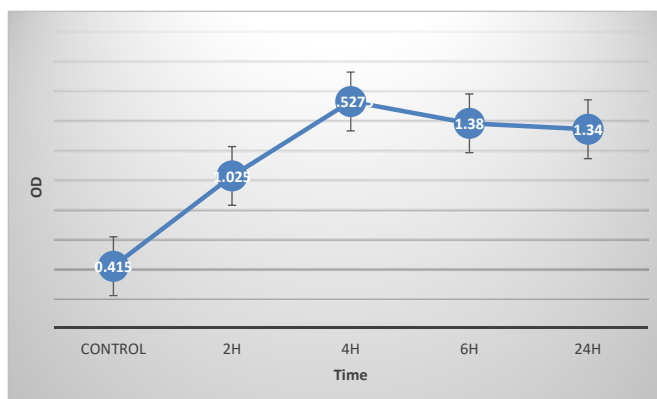
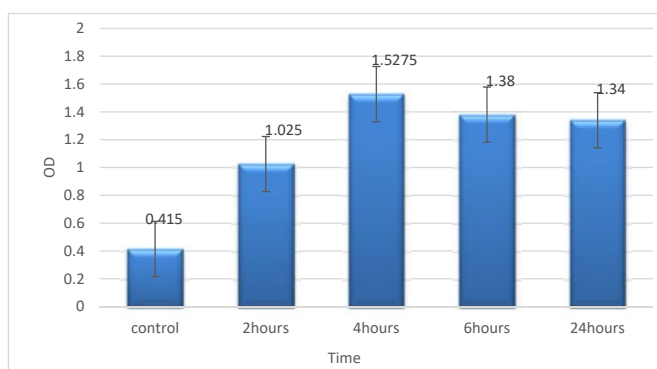


Table12: Analysis of variance of OD at different antibacterial concentrations of copper oxide nanoparticles

| Source of variations | Sum of squares | Degree of freedom | Mean of squares | F | Sig. |
|----------------------|----------------|-------------------|-----------------|--------|-------|
| Intergroups | 3.146 | 4 | 0.787 | 23.095 | 0.001 |
| Intragroups | 0.511 | 15 | 0.034 | | |
| Total | 3.657 | 19 | | | |

Table13: Tukey's test

| Treatment concentrations | Treatment | α subsets at the level of 0.05% | | |
|--------------------------|-----------|--|--------|--------|
| | | 1 | 2 | 3 |
| 1 | 4 | 0.4150 | | |
| 2 | 4 | | 1.0250 | |
| 5 | 4 | | 1.3400 | 1.3400 |
| 4 | 4 | | 1.3800 | 1.3800 |
| 3 | 4 | | | 1.5275 |
| Sig. | | 1.000 | 0.098 | 0.615 |



Diagrams 1: Means of ODs obtained at different concentrations of copper oxide nanoparticles in the Tukey's test

Analysis of variance of OD measured at different times under the influence of different antibacterial concentrations of copper oxide-nisin complex showed that F calculated with 4 and 15 degrees of freedom was larger than F in the (Table 16). In addition, the calculated Sig was smaller than the assumed α of 5% (Table 16). Therefore, there was a significant difference at 5% level between the amounts of bacteria grown at different times measured.

The OD table obtained from the Tukey's test

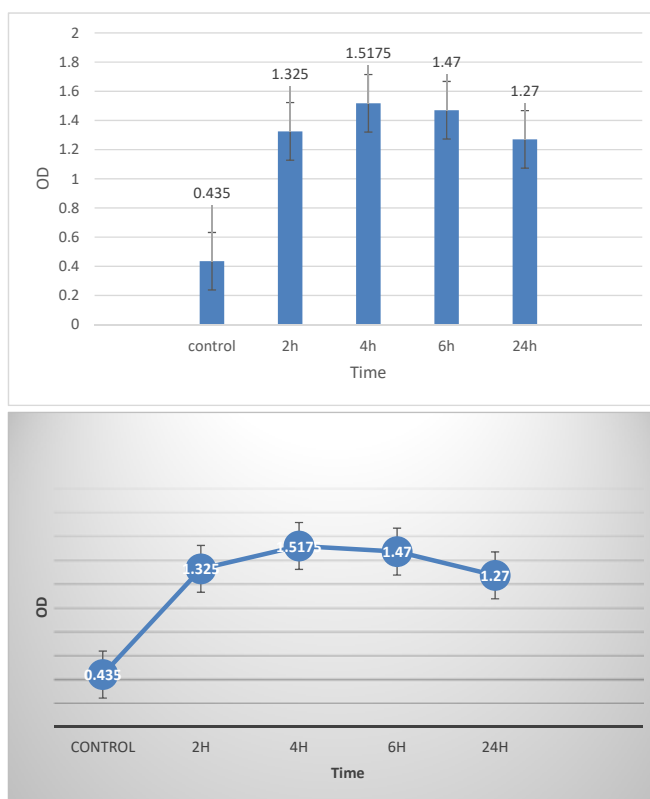
for analyzing the means at different times from the mean of 3 concentrations of copper oxide-nisin complexes showed a significant difference between subsets 1 and 2 (before and 2 hours after treatment), so that the mean OD increased from 0.4267 to 0.9167, indicating a lack of inhibitory effect of copper oxide nanoparticles in these two time periods. In the second subset, including the time intervals of 4, 6, and 24 hours after treatment, the mean ODs were different, but this difference was not significant. At 4 and

Table14: Analysis of variance of OD at different antibacterial concentrations of nisin.

| Source of variations | Sum of squares | Degree of freedom | Mean of squares | F | Sig. |
|----------------------|----------------|-------------------|-----------------|--------|-------|
| Intergroups | 3.107 | 4 | 0.777 | 71.110 | 0.001 |
| Intragroups | 0.164 | 15 | 0.011 | | |
| Total | 3.271 | 19 | | | |

Table15: Tukey's test

| Treatment concentrations | | α subsets at the level of 0.05% | | | |
|--------------------------|---|---------------------------------|--------|--------|--------|
| | | 1 | 2 | 3 | 4 |
| 1 | 4 | 0.4350 | | | |
| 2 | 4 | | 1.0325 | | |
| 5 | 4 | | | 1.2700 | |
| 4 | 4 | | | 1.4700 | 1.4700 |
| 3 | 4 | | | | 1.5175 |
| Sig. | | 1.000 | 1.000 | 1.000 | 0.965 |



Diagrams 2: Means of ODs obtained at different concentrations of Nisin in the Tukey's test

6 hours after treatment, the mean ODs increased from 1.4300 to 1.4900, but at 6 to 24 hours after treatment, they decreased to 1.3567 (Diagram 3). Therefore, it can be concluded that the inhibitory effect of copper oxide-nisin occurred only 6 to 24 hours after treatment.

DISCUSSION

Comparison of antibacterial activities of nisin, CuO, and nisin-conjugated CuO NPs at 30µg/mL at different durations after treatment showed that the optical densities of CuO and nisin decreased

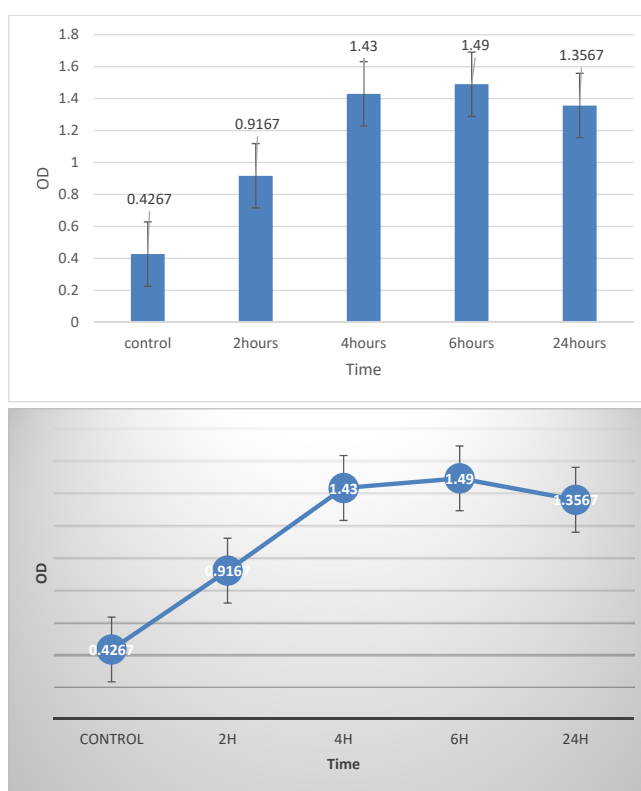
6 to 24 hours after treatment whereas that of the nisin-conjugated CuO NPs increased. This indicates that CuO NPs had antibacterial activity but lacked synergistic effect when they were conjugated with nisin. The importance of the present research is that study of the synergistic effect of CuO NPs and nisin on the *E. coli* genome as a model for Gram-negative bacteria determined the possible mechanisms of the effects that CuO NPs had on the bacteria, and showed that use of these NPs at relatively low concentrations and with fewer harmful effects could be a good

Table16: Analysis of variance of OD at different antibacterial concentrations of the solution of nisin-conjugated CuO NPs

| Source of variations | Sum of squares | Degree of freedom | Mean of squares | F | Sig. |
|----------------------|----------------|-------------------|-----------------|-------|-------|
| Intergroups | 2.433 | 4 | 0.608 | 9.522 | 0.002 |
| Intragroups | 0.639 | 10 | 0.064 | | |
| Total | 3.072 | 14 | | | |

Table 17: Tukey's test

| Treatment concentrations | α subsets at the level of 0.05% | | |
|--------------------------|---------------------------------|--------|--------|
| | | 1 | 2 |
| 1 | 3 | 0.4267 | |
| 2 | 3 | 0.9167 | 0.9167 |
| 5 | 3 | | 1.3567 |
| 3 | 3 | | 1.4300 |
| 4 | 3 | | 1.4900 |
| Sig. | | 0.199 | 0.110 |



Diagrams 3: Means of ODs obtained at different concentrations of nisin-conjugated CuO NPs in the Tukey's test

alternative to employing the common antibiotics and disinfectants. In addition, research has shown that the development of strong bacterial resistance to antibiotics is a major health problem. In this regard, NPs are considered new antimicrobial agents [13, 18].

According to a study by Hazem and Fahimi *et al.* in 2016, bacteriocins are a promising alternative to existing antibiotics and are resistant to increased frequencies of living organisms. However, there are limitations that challenge the use of bacteriocins as bio-preservatives/antibacterial

agents in food and in the pharmaceutical industry. Use of nanoformulations is a promising strategy for overcoming these limitations [7].

In a study conducted by Azam *et al.* in 2012, it was proved that changes in the susceptibility or resistance of bacteria may arise from differences in cellular structure, physiology, metabolism, or the extent of contact between organisms and NPs. Gram-negative bacteria such as *E. coli* have a particular cell membrane structure with substantial ability to withstand antimicrobial agents. They also found that other factors, such

as the spreading rate of NPs, may affect bacterial species [10, 12]. According to the experiments, CuO NPs at concentrations less than 15 µg/mL had no effects on the bacteria but these nanoparticles had better inhibitory effects at higher concentrations. King (2006) showed that the strong binding of NPs to the outer membrane of bacteria could prevent dehydrogenase from functioning. In addition, metal oxide NPs can inhibit the activity of periplasmic enzymes in bacteria, impair transcription and translation, and prevent DNA and RNA function and activity and protein synthesis, which altogether leads to cell lysis [18, 20]. In this study, 10 primers were used in RAPD-PCR to investigate the synergistic effects of CuO NPs and nisin. The presence or absence of bands in the gel image suggests that changes in the DNA sequence were caused by CuO, nisin, and nisin-conjugated CuO NPs. Therefore, a large number of the primers were not able to identify the target sequence, the fragment was not amplified and, hence, it lacked bands on the agarose gel. The difference between bands of the control and of the treated samples indicated that the target sequences of the primers in the treated bacteria underwent changes that led to differences in the binding of primers and in the amplification of PCR fragments. This may arise from the direct or indirect mutation of DNA caused by CuO NPs and nisin. Pal *et al.* (2007) reported that DNA polymerase caused disruption in the replication mechanism so that the replication accuracy was impaired. Therefore, differences in the target sequences that the RAPD primers bound to were caused by changes made in DNA sequence during replication [19]. According to Sharma *et al.*, the use of CuO NPs as a new antimicrobial agent has been investigated recently. Therefore, when copper and silver oxide NPs were compared, CuO was less effective against *E. coli* and Methicillin-resistant *Staphylococcus aureus* but was active against *Bacillus subtilis*, which may be due to the greater interaction of copper with the amine and carboxylic groups at the cellular level of this pathogen [17].

CONCLUSIONS

Copper oxide nanoparticles and nisin used together had synergistic effects and inhibited bacterial growth more than either of them used alone, but their combination had no synergistic effects against the genome and caused minimal changes in DNA sequence.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

REFERENCES

1. Karam L, Jama C, Dhulster P, CHhib NE. Study of Surface Interaction Between Peptides, Materials and Bacteria for Setting up Antimicrobial Surfaces and Active Food Packaging. *Environ.Sci* 2013;4(5): 798-821.
2. Şanlıbaba P, Akkoç N, Akçelik M. Identification and characterisation of antimicrobial activity of nisin produced by *Lactococcus lactis* subsp. *lactis* LL27. *Czech Journal of Food Sciences*. 2009;27(No. 1):55-64.
3. Koral G, Tuncer Y. Nisin Z-Producing *Lactococcus lactis* Subsp. *Lactis* GY132 Isolated from Boza. *Journal of Food Processing and Preservation*. 2012;38(3):1044-53.
4. Zhou L, van Heel AJ, Montalbán-Lopez M, Kuipers OP. Potentiating the Activity of Nisin against *Escherichia coli*. *Frontiers in Cell and Developmental Biology*. 2016;4.
5. Punyappa-path S, Phumkhachorn P, Rattanachaikunsopon P. NISIN: Production and Mechanism of Antimicrobial Action. 2015;7(2)
6. Moosavy MH & Shavisi N. Determination of Antimicrobial Essential Oil Against *ESCHERICHIA COLI O157:H7* Under Various Conditions (PH, Temperature and NaCl Concentration). 2013;19(2):61-62
7. Fahim HA, Khairalla AS, El-Gendy AO. Nanotechnology: A Valuable Strategy to Improve Bacteriocin Formulations. *Frontiers in Microbiology*. 2016;7.
8. Jan J.T.M. Swartjes, Prashant K. Sharma, Theo G. van Kooten, Henny C. van der Mei, Morteza Mahmoudi, Henk J. Busscher and Edward T.J. Rochford. 2014. Current Developments In Antimicrobial Surface Coatings For Biomedical Application. 2014;10:2174/0929867321666140 916121355
9. Grigore M, Biscu E, Holban A, Gestal M, Grumezescu A. Methods of Synthesis, Properties and Biomedical Applications of CuO Nanoparticles. *Pharmaceuticals*. 2016;9(4):75.
10. Azam, Ahmed AS, Oves M, Khan MS, Memic A. 2012. Size-dependent antimicrobial properties of CUO nanoparticles against GRAM-Positive and GRAM-Negative bacteria strains. *International Journal of nanomedicine*. 7:3527-3535
11. Huh AJ, Kwon YJ. "Nanoantibiotics": A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *Journal of Controlled Release*. 2011;156(2):128-45.
12. Liang X, Sun M, Li L, Qiao R, Chen K, Xiao Q, et al. Preparation and antibacterial activities of polyaniline/Cu_{0.05}Zn_{0.95}O nanocomposites. *Dalton Transactions*. 2012;41(9):2804.
13. Bahram GE. An Investigation of the Effect of Copper Oxide and Silver Nanoparticles on E. Coli Genome by Rapid Molecular Markers. *Advances in Biotechnology & Microbiology*. 2016;1(2).

14. Krithiga N, Jayachitra A, Rajalakshmi A. 2013. Synthesis, characterization and Analysis of The Effect of Copper Oxide Nanoparticles in Biological Systems. *Indian Journal Of Nanoscience*. 1(1)
15. Meghana S, Kabra P, Chakraborty S, Padmavathy N. Understanding the pathway of antibacterial activity of copper oxide nanoparticles. *RSC Advances*. 2015;5(16):12293-9.
16. Ananth A, Sunmook Y, Subtamanian D. 2015. Copper Oxide Nanomaterials: Synthesis, Characterization and Structure-Specific Antibacterial Performance. *The Chemical Engineering Journal*. (262)179-188
17. Sharma A, Kumar Arya D, Dua M, Chhatwal GS, Johri AK. Nano-technology for targeted drug delivery to combat antibiotic resistance. *Expert Opinion on Drug Delivery*. 2012;9(11):1325-32.
18. King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, Blumberg HM. Emergence of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* USA 300 Clone as the Predominant Cause of Skin and Soft-Tissue Infections. *Annals of Internal Medicine*. 2006;144(5):309.
19. Pal S, Tak YK, Song JM. Does the Antibacterial Activity of Silver Nanoparticles Depend on the Shape of the Nanoparticle? A Study of the Gram-Negative Bacterium *Escherichia coli*. *Applied and Environmental Microbiology*. 2007;73(6):1712-20.
20. Soenen SJH, Himmelreich U, Nuytten N, Pisanic TR, Ferrari A, De Cuyper M. Intracellular Nanoparticle Coating Stability Determines Nanoparticle Diagnostics Efficacy and Cell Functionality. *Small*. 2010;6(19):2136-45.