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Novel Approaches for Controlling Enteric Bacteria in Dairy Farms

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ABSTRACT

Escherichia coli (*E. coli*), *Staphylococcus aureus*, and *Salmonella enteritidis* are virulent bacterial pathogens that cause serious infections on dairy farms. Nano-based disinfectants are a promising area for antibacterial applications, including chitosan nanoparticles (ChNPs) and silver nanoparticles (AgNPs). This study aimed to evaluate the antimicrobial activity of two commercial disinfectants, ChNPs and AgNPs, against *Escherichia coli* O26 (*E. coli* O26), *Staphylococcus aureus*, and *Salmonella enteritidis* isolated from three dairy farms in the Sharqia governorate, Egypt. After applying 5% hydrogen peroxide, 10% iodine, 5% silver-hydrogen peroxide nanoparticles (Ag-H2O2 NPs), 5% chitosan-hydrogen peroxide nanoparticles (Ch-H2O2 NPs), 10% silver iodide nanoparticles (Ag-I NPs), and 10% chitosan iodide nanoparticles (Ch-I NPs) individually and in combination, the viability of fifty-three strains—comprising 7 *E. coli* O26, 43 *Staphylococcus aureus*, and 2 *Salmonella enteritidis*—was tested using a quantitative suspension method in the presence or absence of organic matter at varying contact times. The tested strains of *E. coli* O26, *Staphylococcus aureus*, and *Salmonella enteritidis* predominated in the samples at 17.07%, 39.45%, and 50%, respectively. Additionally, the in vitro experiments revealed that the most effective (100%) and fastest (less than a minute) bactericidal effect was achieved by the combination of H2O2-Iodine, loaded AgNPs, ChNPs, and their complexes. The results indicated that bactericidal efficacy depended on factors such as organic matter presence, contact time, and disinfectant concentration. Nano-based disinfectants combining silver, chitosan, and the H2O2-Iodine complex proved to be highly effective biocidal agents against pathogenic *E. coli* O26, *Staphylococcus aureus*, and *Salmonella enteritidis*. When two or more antimicrobial agents are combined, they can offer a valuable tool for controlling pathogenic bacteria.

Keywords: Chitosan, Commercial disinfectant, Dairy farm, Nano-based disinfectant, Silver nanocomposite

INTRODUCTION

Dairy farms are an important source of animal protein and economic value in Egypt, providing all the necessary amino acids human beings require (Taha et al., 2023). There were approximately 1.5 million cows in the nation, collectively producing about 3,072 thousand tons of milk annually, with an average yield of 727 kg per cow in 2014 (Sarhan and Damrawi, 2022).

The face several challenges, one of which is the fundamental association between the development of resistant strains of *Escherichia coli O26* (*E. coli O26*), *Salmonella*, and *Staphylococcus aureus* (*S. aureus*) infections and the widespread, uncontrolled, and improper usage of antibiotics in dairy farming (Ashraf, 2023). In three dairy cattle farms in the Sharqia governorate, Egypt, *E. coli* and *Salmonella* spp. were identified from bovine feces and environmental samples. The highest prevalence of *E. coli* (62.2%) was followed by *Salmonella* spp. with a percentage of 0.74% (Zaki et al., 2024). In Egypt, however, *S. aureus* has been detected in 72.5% of the examined samples, including bulk tank milk (100%), lactating cows (72.9%), workers' hand swabs (81.5%), the farm environment (88.9%) and the milking equipment (40%) (Elmonir et al., 2019).

Enteropathogenic strains of *E. coli* associated with foodborne diseases can cause severe and occasionally fatal diarrhea (Behiry et al., 2011). Shiga toxin-producing *E. coli* is another dangerous strain that is related to severe side effects, such as hemolytic uremic syndrome and hemorrhagic colitis (Cleary, 2004). Similarly, *Salmonella* infections can lead to endotoxemia, fever, diarrhea, dehydration, anemia, prolonged pneumonia, joint infections, abortion, and even sudden death from septicemia, particularly in small-scale dairy farms (Langford et al., 2006). Infections with *S. aureus* also pose significant health risks, as they can lead to severe abdominal pain and may develop into potentially fatal effects such as bacteremia, endocarditis, meningitis, pneumonia, and septic arthritis if left untreated (Murillo et al., 2018).

Iodine and hydrogen peroxide are the most widely used disinfectants in the dairy industry due to their ability to generate reactive oxygen species (ROS) that effectively target bacterial cells (Banerjee et al., 2010; Alkawareek et al.,

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2019). Despite their effectiveness, conventional disinfectants are difficult to use due to their toxic nature and quick deterioration. These drawbacks show how urgently safer, more stable, and manageable alternative disinfection techniques are needed to ensure ongoing pathogen protection without the hazards of commercial disinfectants currently available in the market (Dvorak, 2008).

Nanoparticles offer a promising alternative due to their broad-spectrum antibacterial activity and reduced potential for producing disinfection biodegradation products. They are categorized into inorganic nanoparticles, such as silver nanoparticles (AgNPs), and organic nanoparticles, such as chitosan nanoparticles (ChNPs) (Bhardwaj and Saxena, 2017).

Among these, AgNPs have demonstrated antimicrobial and larvicidal properties. Their modes of action include interfering with the permeability of bacterial cell walls and respiration processes by producing reactive oxygen species (Farouk et al., 2020). Silver nanoparticles have been shown to possess broad-spectrum antimicrobial properties in dairy farms, successfully combating multidrug-resistant forms of bacteria, including *Salmonella* and *E .coli* (Costa-Junior et al., 2018). Additionally, they have been effective against *Salmonella enterica* isolates from feces of diarrheic calves collected across five cities in Northern West Egypt (Helmy et al., 2022). The highly bacteriostatic and bactericidal efficacy of AgNPs, particularly in combination with hydrogen peroxide, has been demonstrated against multidrug-resistant bacterial strains, such as *Escherichia coli O157*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* (El-Gohary et al., 2020). Additionally, it has been shown that the combination of silver and iodine nanoparticles (Ag-INPs) has superior antibacterial activity compared to either substance alone (Zhao, 2020).

Organic nanoparticles have no impact on the environment and are cost-effective and easy to use (Hatton et al., 2008). A natural biodegradable polymer known as chitosan nanoparticles (CS-NPs) is created when shellfish waste is recycled into commercially valuable products like chitin. Chitosan has special properties like nontoxicity, biodegradability, and antibacterial activity (No et al., 2002). In dairy farms, chitosan nanoparticles (CS-NPs) have been shown to significantly reduce bacterial counts in raw milk, confirming that they are an efficient disinfectant (Mohamed et al., 2022). Moreover, chitosan nanoparticles (CS-NPs) have exhibited strong antibacterial effects against *Pseudomonas species* and *S. aureus*, which are known to cause bovine mastitis (Rivera et al., 2020; Orellano et al., 2021). When combined with H_2O_2, chitosan nanoparticles enhance their bactericidal efficacy, particularly against resistant bacterial strains such as *Salmonella typhimurium, Escherichia coli O157:H7,* and *Klebsiella pneumoniae,* which are commonly found in dairy environments (El-Gohary et al., 2020). Zhang et al. (2024) discovered that the chitosan-iodine complex had a powerful bactericidal effect on *Escherichia coli* and *S. aureus* with high cytocompatibility and stability.

The purpose of this study was to evaluate the inhibitory efficacy of two commercial disinfectants (iodine and H_2O_2) and nano disinfectants (silver and chitosan) at varying contact times against the tested virulent bacterial strains (*E. coli O26*, *Salmonella enteritidis*, and *S. aureus*) from various dairy farms in the Sharqia governorate, Egypt. On the evaluated virulent bacterial strains, the inhibitory efficacy of the commercial disinfectants and the nano disinfectants was also compared.

MATERIAL AND METHODS

Ethical approval

The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zagazig University (Ref. No: ZU-IACUC/2/F/196/2024). The research was conducted in compliance with local legislation and institutional requirements. Written informed consent was obtained from all participants involved in this study.

Bacterial isolates

This study comprised a total of 53 bacterial strains from *E. coli 026* (n = 7), *Salmonella enteritidis* (n = 2), and *S. aureus* (n = 43). The bacterial isolates were previously found in three sizable dairy farms in the Sharqia Governorate, Egypt, between July 2022 and June 2023. Data from the Veterinary Public Health Department, Faculty of Veterinary Medicine, Zagazig University, were used to select the bacterial isolates under examination. Not all the isolates came from the same source. The glycerinated bacterial strains were refreshed and subjected to standard microbiological techniques recommended by Quinn et al. (1994) to confirm the presumed bacterial isolates**.** Eosin methylene blue (Oxoid, Cambridge, UK) and MacConkey's were utilized to cultivate *E. coli*. However, *Salmonella* species were grown on Rappaport-Vassiliadis Soya broth and Xylose Lysine Deoxycholate (Hi-Media, India). Additionally, *S. aureus* was cultivated on paired Parker agar (Oxoid, UK). The serotyping and molecular identification of all bacterial isolates were previously described by Zaki et al. (2024).

Disinfectant suspension test

The study evaluated the inhibitory efficacy of freshly prepared commercial disinfectants (hydrogen peroxide [H_2O_2] and iodine) and nano-based disinfectants (silver and chitosan-loaded formulations) against *E. coli* O26, *Salmonella enteritidis,* and *S. aureus* at different contact times. The disinfectants were tested both individually and in combination, in the presence and absence of organic matter. On each test day, both the commercial and the nano disinfectants were freshly prepared and diluted following the manufacturer's recommendations.

Preparation of nano-based disinfectants

***Preparation of Silver hydrogen peroxide nanoparticles* (*Ag-H_2O_2 NPs*)**

A ready-to-use solution containing hydrogen peroxide and silver (0.0035-0.0038%) was obtained from SAN Factory, Saudi Arabia.

Preparation of Chitosan hydrogen peroxide nanoparticles (Ch-H_2O_2 NPs)

Five grams of chitosan were dissolved in 1% (v/v) aqueous acetic acid. A sufficient amount of 30% hydrogen peroxide (H_2O_2) was added to produce a final solution of 3% (w/v) chitosan and 1% (w/v) H_2O_2 in 100 mL. The freshly prepared chitosan/H_2O_2 solution was stored at ambient temperatures (Xia et al., 2013).

***Synthesis of Silver iodide nanoparticles* (*AgI NPs*)**

In the presence of 0.2 g sodium dodecyl sulfate, 25 ml of 0.1 M potassium iodide (KI, 0.415 g KI in 25 ml distilled water) was added drop-wise to 25 ml of 0.1 M $AgNO_3$ (0.425 gm $AgNO_3$ in 25 ml distilled water) under ultrasound power. The resulting yellowish-white precipitate was centrifuged at 7500 rpm, repeatedly washed with ethanol and double-distilled water, and then dried at 60°C in an oven. The final product was dissolved for use (Safaei-ghomi and Ghasemzadeh, 2013).

***Synthesis of Chitosan iodide nanoparticles* (*ChI NPs*)**

One gram of chitosan was placed in a 150 mL necked beaker and dissolved in 50 mL of water. The beaker was maintained in a thermostatic water bath at 70°C. Using the funnel, 20 ml of 30% hydrogen peroxide steam generator was distilled. The isothermal reaction period was five hours. Excess hydrogen peroxide was removed by cooling or by adding sodium hydrogen sulfite. The insoluble substance was removed by adding 1.22 g of KI to 4 ml of glycerin, dissolving it inversely, adding 1.5 g of I_2, adding water to 10 ml, mixing, and dissolving to obtain liquid iodine. The resulting iodine solution was mixed with the chitosan solution at a 2:1 volume ratio (Dávila Rangel et al., 2020; Sklyar et al., 2023).

Evaluation of germicidal efficacy of commercial and nano-based disinfectants

The antimicrobial activity of all disinfectants against *E. coli* O26, *Salmonella enteritidis*, and *S. aureus* was assessed using quantitative suspension assays modified from Pilotto et al. (2007) and Aidaros et al. (2022).

The disinfectants were employed in the following concentrations: Two commercial disinfectants separately, each containing 5% hydrogen peroxide and 10% iodine. Combined Iodine (5%) and H_2O_2 (2.5%) were also utilized. However, nano-based disinfectants such as 5% Ag-H_2O_2, 5% Chitosan-H_2O_2, 10% Ag-I, and 10% Chitosan-I were applied separately. Additionally, combined Ag-H_2O_2 (2.5%) plus Ag-I (5%) and lastly, combined Chitosan-H_2O_2 (2.5%) plus Chitosan-I (5%) nanoparticles were used.

An overnight bacterial culture in brain heart infusion (BHI) broth at 37ºC for 18-24 hours was used to prepare bacterial suspensions, adjusted to a turbidity of 0.5 MacFarland standards. To test the disinfection efficacy in the presence of organic waste, 3% dried feces were added to tubes containing 9.5 mL of brain heart infusion broth along with 0.5 mL of each microbial strain. Similar sets of standard saline solutions were used to make the organic matter-free microbial-disinfectant mixture. Various concentrations of tested disinfectants were added to the tubes.

Subcultures were carried out with 0.5 mL of the sample mixture transferred into new tubes with 5 mL of brain heart infusion broth at contact times of 1, 5, 10, 15, and 20 minutes.

The addition of 5 µL of Tween 80 inhibited the effect of the disinfectants. Each plate was labeled according to the bacterial strain and contact time. Incubation of the cultivated plates lasted for 24 hours at 37°C. Plates of nutrient agar showed evidence of microbial development. The efficacy of disinfectants was assessed using the pace at which the microorganism was eliminated and the lack of microbial growth (Pachapur et al., 2016).

Statistical analysis

The IBM Corp SPSS software version 25 (Armonk, NY) was used to analyze all data in this study. The data were expressed as frequency and percentage, and a basic descriptive analysis was used to explain the relationship between the variables (McHugh, 2013).

RESULTS

Prevalence of bacterial pathogens isolated from various sources in dairy farms

Previous research by Zaki et al. (2024) identified *Escherichia coli O26* and *Salmonella enteritidis* as the most common and pathogenic strains in three dairy farms surveyed in Sharqia Governorate. However, samples collected from various sources within the dairy farms under investigation also included *S. aureus* (Table 1). These strains were thus chosen to assess the efficacy of different commercial and nano-based disinfectants. As presented in Table 1, the most prevalent and virulent bacteria among the three dairy farms under study in Sharqia Governorate were 17.07% *E. coli O26* (17 out of 41) and 50% *Salmonella enteritidis* (2 out of 4), according to earlier research documented by Zaki et al. (2024). Nonetheless, 109 of the 612 samples taken from the three cow dairy farms were positive for *S. aureus*, with an overall frequency of 17.81%. Of these, 43 (39.45%) isolates were identified serologically. A variety of sample types were employed, including drinking water, cow milk, feedstuffs, cattle feces, worker hand swabs, and cattle crush swabs. The prevalence of *S. aureus* varied across sample types, ranging from 4.65% to 41.86% (Table 1).

Table 1. The prevalence of *Escherichia coli O26, Salmonella enteritidis,* and *Staphylococcus aureus* isolated from various sources in the dairy farms under investigation

Source	*E. coli O26* (%) *	*S. aureus* (%) **	*Salmonella enteritidis* (%) ***
Drinking water	2 (28.57)	6 (13.95)	-
Cow's milk	4 (57.14)	18(41.86)	-
Feedstuffs	1 (14.28)	3 (6.97)	-
Cattle fecal matter	-	8 (18.60)	2 (50)
Workers hand swabs	-	6 (13.95)	-
Cattle crush swabs	-	2 (4.65)	-
Total	7 (17.07%)	43 (39.45%)	2 (50%)

* Total positive *E. coli* serotypes equals 41 isolates. ** Total positive *S. aureus* equals 109 isolates. ***Total positive *Salmonella enterica* equals 4 isolates

Antimicrobial effect of commercial disinfectants

After 5 minutes, both *E. coli O26* and *S. aureus* were sensitive to the antimicrobial effects of hydrogen peroxide 5%, a common disinfectant, when organic matter was absent (Table 2). The contact period for *Salmonella enteritidis* was 10 minutes. For all the tested strains, the contact time with 10% iodine was 10 minutes. When comparing combined H_2O_2 (2.5%) and iodine (5%), *Salmonella enteritidis* showed no growth after 5 minutes, but *E. coli O26* and *S. aureus* showed a considerable reduction, with no growth found after less than one minute. However, the efficacy of H_2O_2 and iodine declined in the presence of organic matter. At 10 minutes, 5% hydrogen peroxide was efficient against both *E. coli O26* and *S. aureus*, while 15 minutes was required to eliminate *Salmonella enteritidis*. For 10% iodine, a contact time of 15 minutes was necessary for all strains. All strains under investigation showed a considerable decrease with respect to combined H_2O_2 (2.5%)-Iodine (5%), with no growth identified after 5 minutes in the presence of organic matter.

Table 2. The time needed to eliminate *Escherichia coli O26, Staphylococcus aureus,* and *Salmonella enteritidis* following the application of certain commercial disinfectants

Commercial disinfectant	Concentration (%)	In the absence of organic matter			In the presence of organic matter		
		E. coli O26	*S. aureus*	*Salmonella enteritidis*	*E. coli O26*	*S. aureus*	*Salmonella enteritidis*
Hydrogen peroxide (H_2O_2)	5	5	5	10	10	10	15
Iodine	10	10	10	10	15	15	15
Combined H_2O_2-Iodine	2.5 + 5	1	1	5	5	5	5

Antimicrobial effect of silver and chitosan nanoparticle-based disinfectants

The antibacterial properties of Ag-H_2O_2 (5%), Chitosan-H_2O_2 (5%), Ag-I (10%), Chitosan -I (10%), as well as combined Ag-H_2O_2 (2.5%) plus Ag-I (5%) and combined Chitosan- H_2O_2 (2.5%) plus Chitosan-I (5%) nanoparticles were tested against selected pathogenic bacteria, as presented in Table 3. In the absence of organic matter, Ag-H_2O_2 (5%) eliminated *Salmonella enteritidis, S. aureus,* and *E. coli O26* in less than a minute. However, when organic matter was added, a longer time was required to eliminate the bacteria, with no growth after only 5 minutes. Furthermore, *E. coli O26* and *S. aureus* were eliminated by 5% Chitosan-H_2O_2 nanoparticles in less than a minute, but *Salmonella enteritidis* was completely eradicated after 5 minutes. Additionally, adding organic matter lengthened the time needed to eliminate *E. coli O26* and *S. aureus* by 5 minutes and *Salmonella enteritidis* by 10 minutes. *Salmonella enteritidis* was eliminated after 5 minutes of using 10% Ag-I nanoparticles, while *E. coli O26* and *S. aureus* were eliminated in less than one minute. After adding organic matter, it took longer to eliminate *E. coli O26* after 5 minutes. However, neither *S. aureus* nor *Salmonella enteritidis* showed any growth after 10 minutes.

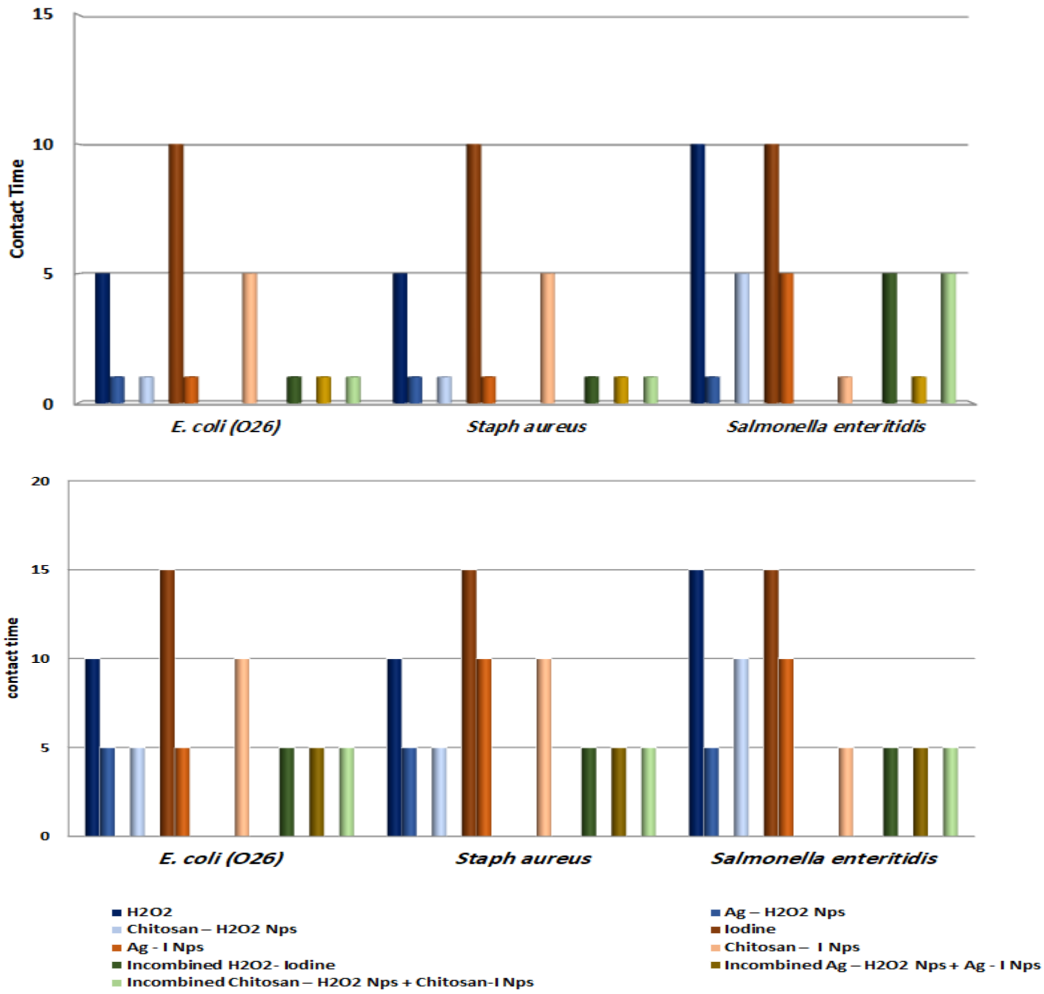
Salmonella enteritidis was eliminated in less than a minute when 10% Chitosan-I nanoparticles were used, and *E. coli O26* and *S. aureus* were eliminated in 5 minutes. Contact periods were increased to 10 minutes for *S. aureus* and *E. coli O26* and 5 minutes for *Salmonella enteritidis* following the addition of organic matter. However, after less than a minute, combined Ag-H_2O_2 plus Ag-I (2.5 + 5%) and combined Chitosan-H_2O_2 plus Chitosan-I nanoparticles (2.5 + 5%) were successfully eliminating *E. coli O26* and *S. aureus.* In contrast, combined Ag-H_2O_2 plus Ag-I (2.5 + 5%)

nanoparticles had effects against *Salmonella enteritidis* similar to 5% Ag-H_2O_2 nanoparticles. Likewise, combined Chitosan-H_2O_2 plus Chitosan-I nanoparticles (2.5 + 5%) were as effective as 5% Chitosan plus H_2O_2 nanoparticles. However, all nano-based disinfectants were affected by the presence of organic matter, leading to increased elimination times.

The inhibitory effects of commercial and nano-based disinfectants against *E. coli O26*, *S. aureus*, and *Salmonella enteritidis* are illustrated in Figure 1. The sensitivity pattern of virulent bacterial strains in the absence of organic matter (Figure 1A) showed that *E. coli O26* and *S. aureus*, Ag-H_2O_2 (5%), Chitosan-H_2O_2 (5%), Ag-I (10%), Chitosan-I (10%), combined H_2O_2 plus I (2.5 + 5%), combined Ag-H_2O_2 plus Ag-I (2.5 + 5%) and combined Chitosan-H_2O_2 plus Chitosan-I (2.5 + 5%) nanoparticles had the most powerful germicidal effects, with no bacterial growth observed in under a minute. *Salmonella enteritidis* exhibited a 100% reduction when treated with 5% Ag-H_2O_2, 10% Chitosan-I, and combined Ag-H_2O_2 plus Ag-I (2.5 + 5%) within a minute. Even in the presence of organic matter (Figure 1 B), nano-based disinfectants demonstrated superior bactericidal effects compared to traditional disinfectants, although elimination times increased by approximately 5 minutes for *E. coli O26*, *S. aureus*, and *Salmonella enteritidis*.

Table 3. Time taken for destroying *Escherichia coli O26, Staphylococcus aureus,* and *Salmonella enteritidis* after applying nano-based disinfectants

Nano-based disinfectant	Concentration (%)	In the absence of organic matter			In the presence of organic matter		
		E. coli O26	***S. aureus***	***Salmonella enteritidis***	***E. coli O26***	***S. aureus***	***Salmonella enteritidis***
Ag–H2O2 Nanoparticles	5	1	1	1	5	5	5
Chitosan–H_2O_2 Nanoparticles		1	1	5	5	5	10
Ag–I Nanoparticles	10	1	1	5	5	10	10
Chitosan –I Nanoparticles		5	5	1	10	10	5
Combined Ag–H_2O_2 + Ag-I Nanoparticles	2.5 + 5	1	1	1	5	5	5
Combined Chitosan–H2O2 + Chitosan-I Nanoparticles		1	1	5	5	5	5



A)

B)

Figure 1. Comparing the efficacy of commercial and nano-based disinfectants against *Escherichia coli O26, Staphylococcus aureus,* and *Salmonella enteritidis.* **A:** The absence of organic matter, **B:** The presence of organic matter.

DISCUSSION

Escherichia coli and *Salmonella* remain significant concerns in food processing environments due to their association with foodborne diseases, which pose major public health problems worldwide. In Egyptian dairy farms, serovars such as *E. coli O26*, *Salmonella enteritidis*, and *S. aureus* have frequently been identified in natural environments, potentially contributing to raw milk contamination (Zaki et al., 2024). In addition, 39.45% of the total samples investigated in the current study included *S. aureus*. Among the identified *S. aureus* isolates, cow's milk had the highest incidence (41.86%). Similarly, Aziz et al. (2022) reported a high prevalence (42%) of *S. aureus* in cow's milk samples in Punjab, Pakistan, supporting these findings. The results recorded in the current study were more conclusive than the values previously reported in Tigray, Ethiopia (15.5%; Abebe et al., 2014), Korea (6.3%; Lim et al., 2013), and São Paulo, Brazil (5.5%; Lee et al., 2012), yet lower than those found in Minnesota, USA (84%; Haran et al., 2012), Egypt (73.3%; Eltokhy and Abdelsamei, 2021) and (52%; Meshref et al., 2019) and North Morocco (40%; Bendahou et al., 2008). As far as public health is concerned, *S. aureus* is a pathogen that can pose serious risks to humans through the consumption of contaminated raw milk or milk byproducts, primarily due to poor personal hygiene practices among milkers, including coughing, sneezing, and improper container sanitation (Kadariya et al., 2014). Abebe et al. (2016) highlighted that cows infected with mastitis serve as persistent sources of *S. aureus* contamination in dairy environments. Given these concerns and the need for improved hygiene practices, it is crucial to investigate potential sources of contamination and implement effective sanitary measures during the milking process.

The aforementioned findings indicate that, in the absence of organic matter, 5% hydrogen peroxide (H_2O_2) demonstrated superior efficacy compared to 10% iodine, eliminating both *E. coli* O26 and *S. aureus* within five minutes, as compared to 10 minutes for 10% iodine. The effectiveness of 5% H_2O_2 and 10% iodine against *Salmonella enteritidis* was equal when the contact time was 10 minutes. One possible explanation for the increased effectiveness of H_2O_2 is that it reacts with O^2 and/or iron (Fe^{++}) from bacteria to generate the extremely harmful hydroxyl radical (OH^{-1}). Nucleic acid splitting by the hydroxyl radical causes dose-dependent production of long-lived ROS, which damages the cells by oxidizing proteins, lipids, and DNA (Zhao and Drlica, 2014). Iodine produces ROS, which inhibits normal biological functions even though it oxidizes the sulfhydryl groups of amino acids in proteins (Kitagawa et al., 2005). Similar findings were reported by Sander et al. (2002), who demonstrated that, in the absence of organic matter, the overall killing times for *Salmonella*, *S. aureus*, and *E. coli* using 3% H_2O_2 were 5-10, 15, and 10 minutes, respectively. In contrast to previous research, hydrogen peroxide was effective against *Salmonella enteritidis* after 120 minutes at concentrations of 2% and 5%. In the absence of organic matter, the effectiveness of hydrogen peroxide depended on concentration and contact time (Abd-Elall et al., 2023). However, Abdallah et al. (2019) used the inhibition method to determine the effect of H_2O_2 against *Salmonella*, *S. aureus*, and *E. coli*. It was found that the diameter of the zone was 26.71 ml for a 2% concentration and 23.90 ml for a 5% concentration. On the other hand, Aksoy et al. (2020) found that utilizing 1:100 and 1:200 dilutions at all contact times from 5 minutes to 24 hours prevented the development of *Salmonella* when iodine and H_2O_2 were used separately. Additionally, the effects of the isolates of *Salmonella typhimurium* and *Salmonella enteritidis* exposed to various concentrations of hydrogen peroxide and iodine at 37°C for 30 minutes, 2 hours, and 4 hours were examined by McLaren et al. (2011). Their results indicated that disinfectant efficacy was dependent on both concentration and exposure duration, with longer contact times and higher concentrations leading to increased bactericidal activity.

The findings of the current study demonstrate that H_2O_2 and iodine required a longer contact time to be effective against all bacterial species tested when organic matter was present. Ruano et al. (2001) reported similar results, showing that after 10 minutes of contact time without organic matter, H_2O_2 eliminated *E. coli*, while in the presence of organic matter, longer contact and or higher disinfectant dosage were needed to maintain effectiveness. Furthermore, Park et al. (2014) found that the presence of organic matter decreased the bactericidal efficacy of povidone-iodine against *Salmonella typhimurium*, which showed significant bactericidal activity at a 400-fold dilution. However, the effective dilution dropped to only 5-fold after the addition of organic matter, necessitating a 30-minute exposure for effective disinfection. Giddey et al. (2015) also pointed out that the presence of organic matter reduced the effectiveness of H_2O_2 against *E. coli* because it oxidized the organic matter, which resulted in fewer bacterial reductions.

In the absence of organic matter, the combined effect of 5% H_2O_2 and 10% iodine in this investigation was quite noticeable. After less than a minute, no growth of *E. coli O26* and or *S. aureus* was found, and after only 5 minutes, *Salmonella enteritidis* was eliminated. In contrast, the contact time was increased when organic matter was present. The current results are consistent with those of Zubko and Zubko (2013), who discovered that while iodine and H_2O_2 showed static inhibitory effects on each other, their combinations were synergistically lethal for *E. coli* due to respiratory-defective mutants, which indicated genotoxic effects. Additionally, Klebanoff (1967) discovered that the combined effects of iodide and H_2O_2 reduced the *E. coli* counts from 6.2×106 to 4.9×106. However, when organic matter is present, for all investigated bacteria, combined H_2O_2-iodine resulted in a slight increase in contact time (5 minutes), which is consistent with Gehan et al. (2009) who claimed that the presence of organic matter required either higher disinfectant concentrations or longer exposure times to achieve effective disinfection.

Reactive metal oxide nanoparticles have been shown to exhibit superior bactericidal activities (Stoimenov et al., 2002). The study of the application of additional inorganic and organic nanoparticles as antibacterial agents is highly desirable. Although only a few uncommon forms of bacteria are silver-resistant, it has long been known that most bacteria are extremely harmful to silver ions and compounds (Aymonier et al., 2002).

The current work used silver and Chitosan nanoparticles at varying contact times to improve the disinfection power of H_2O_2 and iodine to control pathogenic *E. coli O26, S. aureus,* and *Salmonella enteritidis*. Based on the findings of the current study, *E. coli O26* and *S. aureus* were extremely susceptible to 5% Ag-H_2O_2, 5% Chitosan-H_2O_2, and 10% Ag-I nanoparticles in the absence of organic matter, while *Salmonella enteritidis* exhibited greater resistance. Notably, 5% Chitosan-I required a shorter contact time for effective disinfection.

The present study found that the effectiveness of all disinfectants is reduced when organic matter is present. This is consistent with the findings of Mohammed and Abdel Aziz (2019), who pointed out that the use of disinfectants without precleaning in the presence of organic matter led to fewer disinfectants coming into contact with microorganisms, indicating the need for higher disinfectant concentrations and longer contact times. Additionally, according to Yang et al. (2024), organic matter can interact with silver nanoparticles (AgNPs) and change their stability, aggregation, and dissolution in the environment. This can change the production of reactive oxygen species (ROS) and alter the surface properties of AgNPs, which can alter their reactivity and bactericidal efficacy. However, the effectiveness of chitosan as an antibacterial agent can be influenced by environmental factors such as pH and the presence of organic matter, which can modify its charge and diminish its bactericidal efficacy (Lichtenberg et al., 2020). Additionally, when evaluated without organic matter, many disinfection solutions demonstrated effective antibacterial activity within 30 minutes of contact. However, longer contact times were required to show the effects when organic matter was present (Gehan et al., 2009).

The first step in the antibacterial mechanism of AgNPs is the binding of Ag^{+1}, which inhibits the bacterial cell from absorbing vital nutrients and leads to cell death. Ag^{+1} can enter the cell by competitively binding with heavy metals such as Ca^{2+}, Mn^{2+}, and Mg^{2+}, or it may be transported and accumulated irreversibly in the cell by complexing with substrates. Lastly, Ag^{+1} may bind and condense DNA once inside, or it may impede respiration (Ismail et al., 2019). Furthermore, according to Liao et al. (2019), the use of silver nanoparticles to kill bacteria causes DNA fragmentation since it is similar to apoptosis in eukaryotes. Additionally, a study by Raffi et al. (2008) demonstrated that silver nanoparticles at concentrations as low as 60 µg/L adhered to bacterial cell walls, penetrated cells, and exerted cytotoxic effects, completely inhibiting *E. coli* growth and multiplication.

Chitosan can adhere to the bacterial cell wall polyanions via its electrostatic interaction. Moreover, Gram-negative bacteria have a higher inhibitory effect compared to chitosan compounds compared to Gram-positive bacteria due to a higher negative charge on their cell wall (Kong et al., 2010). Additionally, Chitosan-H_2O_2 nanoparticles were highly effective against different bacteria, including *S. aureus*, where the minimum inhibitory concentration was 7.5 to 15 mg/mL (Doan et al., 2021). Also, Chitosan-H_2O_2 showed a greater antibacterial effect when compared to H_2O_2 alone by an approximately two-fold decrease in minimum inhibitory concentration values against *S. aureus* bacteria (Fasiku et al., 2021).

According to Zhang et al. (2024), the chitosan-iodine combination exhibited high cytocompatibility and stability while having a potent bactericidal impact on *S. aureus* and *E. coli.* Furthermore, Banerjee et al. (2010) discovered that when iodine is present, the nanocomposite of chitosan NPs, AgNPs, and iodine performs significantly better against *E. coli* than when iodine, AgNPs, or chitosan are used alone. The chitosan-AgIO nanocomposite also demonstrated significant antibacterial effects against *S. aureus* and *E. coli* by reducing bacterial colony counts as much as 75.69% and 85.1%, respectively (Ahghari et al., 2022). Although Chen et al. (2013) pointed out that the silver iodine complexing antibacterial agent has a disinfecting rate of up to 99.9% and can be added to other materials to enhance their antibacterial qualities, it can also be used to disinfect and sterilize skin surfaces, household equipment, and medical devices and instruments. Furthermore, silver nanoparticles coated with iodine (Ag + iodine) demonstrated twice as much antibacterial efficacy against *E. coli* as AgNPs alone (Ashmore et al., 2018).

The findings of the current study demonstrated that in the presence of organic matter, combined Ag-H_2O_2 plus Ag-I nanoparticles and combined Chitosan-H_2O_2 plus Chitosan-I nanoparticles were equally efficient against all strains of *E. coli O26*, *S. aureus*, and *Salmonella enteritidis*. These findings are in line with those of Davoudi et al. (2012), who discovered that an effective and potent disinfection agent against *E. coli* can be produced by combining hydrogen peroxide and silver ions in the absence of organic matter. Additionally, Alkawareek et al. (2019) demonstrated that even at lower concentrations, H_2O_2 and AgNPs work in combination to significantly decrease bacterial viability over contact time, ultimately destroying *S. aureus* and *E. coli* after 15 and 45 minutes, respectively. However, using AgNPs alone produced a bacteriostatic effect rather than a bactericidal effect.

The results of the current study showed that even when organic matter was present, the bactericidal impact of nano-based disinfectants, either alone or in combination, was superior to that of commercial types against tested virulent bacterial strains. These results align with those of Zhao (2020), who found that combined Ag-I nanoparticles had a more potent antibacterial effect than either material alone. The combination of chitosan NPs plus AgNPs and iodine showed a significantly higher biocide impact against *E. coli* compared to when applied singly, according to Banerjee et al. (2010). However, Ashmore et al. (2018) found that silver nanoparticles coated with iodine (Ag plus iodine) had twice the antibacterial activity on *E. coli* as compared to AgNPs alone. The main explanation was that nano-based disinfectants change the bacterial morphology, metabolism, and cellular membrane integrity. Additionally, the antibacterial activity of their nanostructures may be related to their large inner volume, high surface-to-volume ratio, and specific chemical and physical characteristics (Dizaj et al., 2015).

CONCLUSION

Escherichia coli, *S. aureus*, and *Salmonella enteritidis* can infect dairy animals and contaminate dairy products. In this context, increased antimicrobial resistance can pose risks to both animal and human health. Therefore, the authors recommend developing and implementing a regular cleaning and disinfection program that begins at the animal housing and extends throughout the entire dairy production process to remove organic matter that shields these pathogens and diminishes disinfectant effectiveness. The study showed varying levels of complete bacterial eradication, indicating the need for longer contact times and higher disinfectant concentrations. Additionally, a nano disinfectant combining silver, chitosan, and the H2O2-Iodine complex proved to be an effective antimicrobial agent against *E. coli* O26, *S. aureus*, and *Salmonella enteritidis*. More research is needed to assess the bactericidal effects of the nano disinfectant and its combined formulations against other pathogens to achieve greater biocidal efficacy.

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Author’s contributions
All authors contributed significantly to the study. Mahmoud Samy Ahmed Zaki conceptualized the research, supervised the study, conducted the investigation, developed the methodology, performed formal analysis, and contributed to writing, reviewing, editing, and visualizing. Amr Mohamed Mohamed Abd-El-all conducted the investigation, formal analysis, and writing of the review, and editing. Ayman Megahed contributed to supervision, Amira Samir Attia Attia provided resources and contributed to supervision, investigation, validation, formal analysis, and writing of the review, and editing. Manal Ali Al-Ashery provided resources, conducted investigations, developed methodologies, and contributed to the writing of the original draft, as well as the writing of the review and editing. All authors read and approved the final version of the manuscript.

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The Authors declare that no competing interests exist.

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