Emirates Journal of Food and Agriculture. 2022. 34(8): 696-702
doi: 10.9755/ejfa.2022.v34.i8.2918
http://www.ejfa.me/

RESEARCH ARTICLE

The *in vitro* effect of heat, lactic acid-silver nanoparticle combination on shiga toxigenic *Escherichia coli*

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ABSTRACT

Lactic acid and silver nanoparticle compositions' effects as antimicrobial to reduce Shiga Toxigenic *Escherichia coli* (STEC) contamination were studied in vitro conditions. Four silver nanoparticle sizes, 20, 40, 100, 150 nm with lactic acid (LA) solutions of 1.5%, 2%, 2.5%, were applied and heated in a water bath to 20°C, 40°C and 50°C. Shiga toxigenic *E. coli* O26, O103, O111, O145 and O157 were used as control microorganism. Microbial counts were analyzed using a general linear model univariate test. According to our results, 20, 100, and 150 nm Ag Nanoparticles (AgNPs) in 2% and 2.5% LA density at 40 °C and 50 °C have a decrease of 3 $\log_{10}$ CFU/ml and more. The highest antimicrobial effect was seen at 150 nm AgNP, 2.5% LA at 50°C. Our findings show that AgNP combined with lactic acid can be a low-cost reduction method and promise a novel antimicrobial agent.

Keywords: Lactic acid, Nanoparticles, Shiga Toxigenic *E. coli* (STEC), Silver, Verotoxigenic *E. coli*

INTRODUCTION

Lactic acid (LA) is Generally Regarded as a Safe (GRAS) chemical that has a widespread application in food production for its properties like; antimicrobial activity, acidity regulator, colour stabilizer and flavour enhancer for vegetables. Fermented foods, pickled foods, carcass surfaces, meat products, jellies, fruit syrups, sterilized and UHT creams, whey protein cheese, frozen vegetables, seaweeds, nuts and seeds, salt substitutes, prescription medical infant formulae (excluding generic infant formulae), follow-up formulae and formulae for particular medical purposes for infants are among the foods that lactic acid used (Ameen & Caruso, 2017; Gyawali & Ibrahim 2012; Steinkraus, 1992). As the amount of lactic acid increases, the acceptability of flavour decreases (Ameen and Caruso, 2017). As the acceptable levels of lactic acid application are standardized, additional measures should be applied together with the lactic acid, as heat or nanoparticles.

Nanoparticles (NPs) are different sizes of oxide nanoparticles, metal nanoparticles, and graphene nanoparticles that can be prepared naturally or synthetically, and are dissolved in the particle, trapped, adsorbed, or bonded to the surface (Çırpanlı, 2009; Derman et al., 2013; Rao & Geckeler, 2011). Metallic nanoparticles, which have increased chemical activity due to their large surface to volume ratios and crystallographic surface structure, are among the most promising antibacterial nanomaterials (Morones et al., 2005). For many years, metal nanoparticles have been used as antimicrobial agents. Resistance to metal nanoparticles is less likely than resistance to conventional narrow-spectrum antibiotics (Pal et al., 2007). The antimicrobial properties of nanoparticles are based on their nano size, unique electrical, chemical, mechanical, and optical properties (Dakal et al., 2016; Morones et al., 2005). The fact that they have a large surface area resistant to high heat treatments raises the demand for their use (Baran, 2018). For centuries, silver ion has been used to prevent infections, heal wounds, and eliminate inflammation due to its antimicrobial activity. (Carmona et al., 2012; Durán et al., 2010; Landry et al., 2009; Raffi et al., 2008; Rai et al., 2009). Because AgNP is less reactive than silver ions, its use in clinical and therapeutic applications appears

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Received: 13 November 2021; **Accepted:** 25 July 2022

much more appropriate. The antimicrobial properties of AgNPs are strongly influenced by their colloidal state, concentration, shape, and size. (Dakal et al., 2016).

It has been discovered that AgNPs interferes with cell-membrane function, resulting in the generation of intracellular reactive oxygen species and that silver ions react with protein -SH groups, resulting in an antibacterial effect. (Durán et al., 2010; Morones et al., 2005; Slawson et al., 1992; Yan et al., 2018; Zhao and Stevens, 1998).

Antimicrobial resistance in bacterial pathogens prompts research into alternative antimicrobial agents. The appealing properties of nanoparticles have recently increased the number of studies in this field (Pan et al. 2021; Raza et al. 2016; Wu et al. 2014; Yan et al. 2018). It is critical for public health to develop alternative and more effective strategies for resistant pathogens.

The purpose of this study was to increase the effectiveness of lactic acid for antimicrobial activity in foods using nanoparticle silver with mild heat conditions without increasing the amount of lactic acid used. Could it be an effective tool for using silver nanoparticles combined with lactic acid with mild heat for improving antimicrobial activity and overcoming antibacterial resistance in STEC?

MATERIALS AND METHODS

Preparation of silver nanoparticles

Four AgNPs with sizes 20 (Sigma-Aldrich 730793), 40 (Sigma-Aldrich 730807), 100 (Sigma-Aldrich 730777) and 150 nm (Sigma-Aldrich 484059) were used. Lactic acid solutions of (v/v) 1.5%, 2% and 2.5% were prepared. All lactic acid solutions were enriched with 200 ppm AgNP. Then the solutions were heated in a water bath to 20°C, 40°C and 50°C by checking with a thermometer (Table 1).

Preparation of bacterial cultures

The reference strains of *E. coli* O26, *E. coli* O103, *E. coli* O111, *E. coli* O145, *E. coli* O157 were obtained from ISS Collection, Italy. All strains were inoculated to Brain Heart Infusion Broth (BHI, Oxoid, England) and incubated at 37±2 °C overnight. All enrichments were subcultured twice on Plate Count Agar (PCA, Oxoid, England). Subcultures on PCA were collected in an Eppendorf tube using sterile swabs wetted with sterile saline solution. The collected subcultures were diluted with 1000 µl of sterile saline solution and centrifuged at 3000 rpm for one minute. The supernatant was discharged, and this step was repeated twice.

Application of AgNP-LA and microbiological analyses

For application, 1000 µL was added onto the pellet from each prepared solution, homogenized, and incubated for 3 minutes. After 3 minutes, 1000 µL of the homogenate was serially diluted and 100 µL from each dilution was spread on PCA in two parallels. All petri dishes were incubated at 37±2°C for 24h, and all colonies were counted. For the control group, sterile distilled water was used at room temperature without heating and adding AgNP (Kevenk and Koluman, 2021).

Statistical analysis

The trials were performed in duplicate. The microbial counts were expressed on $\log_{10}$ CFU/ml. STEC was studied in relation to lactic acid concentration, AgNP size, and application temperatures. Microbial counts were analyzed using a general linear model univariate test. The Statistical Package for the Social Sciences was used to perform statistical estimations (SPSS, Chicago, IL, USA, 1997).

RESULTS

In this study, the antibacterial effect of AgNP-LA with different heat combinations was determined. Four different

Table 1: Experimental design of the study (All particles were 200 ppm (w/w) and exposure was 3 min)

AgNP Size (nm)	LA Conc. (w/w)%	Application Temparature (°C)
20	1.5	20
		40
		50
	2.0	20
		40
		50
	2.5	20
		40
		50
40	1.5	20
		40
		50
	2.0	20
		40
		50
	2.5	20
		40
		50
100	1.5	20
		40
		50
	2.0	20
		40
		50
	2.5	20
		40
		50
150	1.5	20
		40
		50
	2.0	20
		40
		50
	2.5	20
		40
		50

sized AgNPs and three different densities of LA combinations were tested on five Shiga toxigenic *E. coli* serotypes.

The combination of AgNPs and lactic acid inhibited bacterial viability in a concentration-dependent manner. Due to the heterogeneous distribution of colony counts, all results were converted to logarithmic values. The size of the AgNPs, the density of the lactic acid, and the application temperatures used in the combined solutions for bacterial decontamination did not cause a significant difference between *E. coli* serogroups (P>0.05) (Table 2-4). It has been indicated that for an antimicrobial agent to be considered adequate, a 3-$\log_{10}$ CFU/ml (99.9% bactericidal effect) decrease in bacterial colonies must be achieved in in-vitro conditions (Glueck et al., 2017; Ryskova et al., 2013). In this context, when we observed our data sets 20, 100, and 150 nm AgNPs in 2% and 2.5% LA density at 40 °C and 50 °C have a decrease of 3 $\log_{10}$ CFU/ml and more (Fig. 1-3); the expected decrease in LA density of 2% and 2.5% was observed at 50°C application temperature for 40 nm sized AgNP. It was figured out that the antimicrobial effect seen in combinations containing NPs of the same size increased as the lactic acid concentration and application temperature increased (P<0.05).

It was determined that the highest antimicrobial effect was seen at 150 nm AgNP in each combination. Moreover, the antimicrobial activity increases as the application temperature increases (Table 2, Fig. 1-3). It was also observed that the highest effect in all combinations was seen at 50 °C (4.20 to 4.79 $\log_{10}$ CFU/ml reduction) (P<0.05). The highest antimicrobial activity was in the combination of 150 nm in 2.5% lactic acid at 50 °C ambient temperature (mean reduction of 4.79 $\log_{10}$ CFU/ml) (P<0.05)

Table 2: Reduction of bacterial cells exposed to Ag-NPs-LA combination at different temperatures (n=160)

Temperature	Temperature	Mean Difference	Sig.
20°C	40°C	1.041*	0.000
	50°C	1.746*	0.000
40°C	20°C	-1.041*	0.000
	50°C	0.705*	0.000
50°C	20°C	-1.746*	0.000
	40°C	-0.705*	0.000

*: The mean difference is significant at the 0.05 level

Table 3: Reduction of bacterial cells exposed to Ag-NPs-LA combination at different AgNP size (n=90)

	AgNP size	Mean Difference	Sig.
Control	20 nm	2.877*	0.000
	40 nm	2.219*	0.000
	100 nm	2.629*	0.000
	150 nm	3.020*	0.000
20 nm	Control	-0.287*	0.000
	40 nm	-0.659*	0.000
	100 nm	-0.248*	0.000
	150 nm	0.143*	0.000
40 nm	Control	-2.219*	0.000
	20 nm	0.659*	0.000
	100 nm	0.410*	0.000
	150 nm	0.802*	0.000
100 nm	Control	-2.629*	0.000
	20 nm	0.248*	0.000
	40 nm	-0.410*	0.000
	150 nm	0.391*	0.000
150 nm	Control	-3.020*	0.000
	20 nm	-0.143*	0.000
	40 nm	-0.802*	0.000
	100 nm	-0.391*	0.000

*:The mean difference is significant at the 0.05 level

DISCUSSION

Microbial resistance is one of the most severe issues caused by chemical antimicrobial agents (Kim et al., 2007). As a result, the use of natural organic acids such as lactic acid has grown in popularity in recent years. However, some lactic acid-resistant *E. coli* serotypes have been discovered in recent studies (Gyawali & Ibrahim 2012, Yu et al., 2021). Furthermore, STEC is recognized as a major pathogen responsible for numerous outbreaks (CDC, 2018). Their ability to resist acid is identified as one of the critical factors jeopardizing food security (Yu et al., 2021). It is crucial to design studies to combat this risk, which may have a negative impact on public health. According to Klabunde and Richards (2009), reactive metal oxide nanoparticles have excellent bactericidal effects, so it is fascinating to investigate the use of other inorganic nanoparticles as antibacterial materials. In this regard, we aimed to develop an experimental model with AgNP-added LA combinations to provide a practical antimicrobial effect with lactic acid in lower concentrations. In our study, individual strains of the four non-O157 STEC serogroups (O26, O103, O111, O145) and O157 were used as test microorganisms. *E. coli*, a Gram-negative bacterium,

Table 4: Reduction of bacterial cells exposed to Ag-NPs-LA combination at different AgNP size (n=90)

LA conc. (w/w)	LA conc. (w/w)	Mean Difference	Sig.
Control	1.5	1.884*	0.000
	2	2.714*	0.000
	2.5	3.461*	0.000
1.5	Control	-1.884*	0.000
	2	0.831*	0.000
	2.5	1.577*	0.000
2	Control	-2.714*	0.000
	1.5	-0.831*	0.000
	2.5	0.747*	0.000
2.5	Control	-3.461*	0.000
	1.5	-1.577*	0.000
	2	-0.747*	0.000

*.The mean difference is significant at the 0.05 level

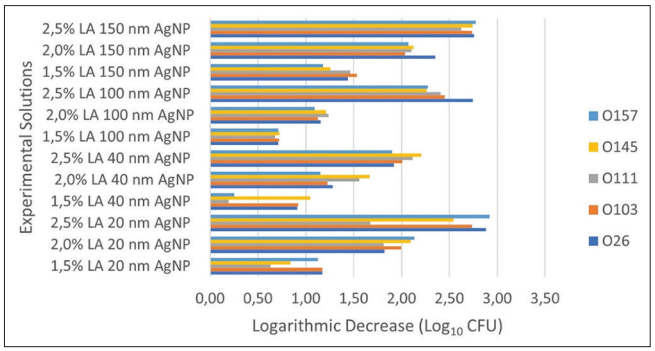

Fig 1. The logarithmic reduction of STEC at 20°C.

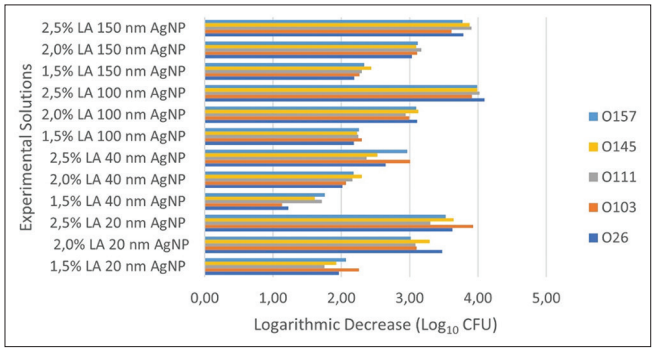

Fig 2. The logarithmic reduction of STEC at 40°C.

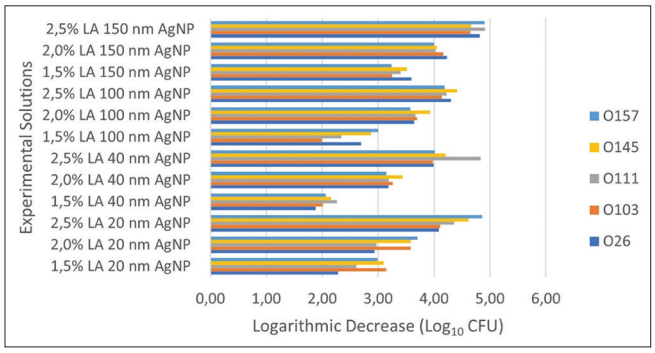

Fig 3. The logarithmic reduction of STEC at 50°C.

is more sensitive to AgNPs than Gram-positive bacteria, as it contains a thinner layer of peptidoglycan in its cell membrane (Dakal et al., 2016). There is no diversity in the cell wall structure and thickness between *E. coli* serotypes. As a result, no significant difference was found in any of our STEC colonies. We have not encountered a study with a similar structure before. However, some researchers reported that the antimicrobial resistance phenotypes corresponded well to the presence of specific genes (Pan et al., 2021). Moreover, *E. coli* O157 and O26 presented similar gene expression profiles in acid stress (Yu et al., 2021). In contrast to our results, Beier et al. (2016) reported that the six serogroups of non O157 STEC responded differently to the antimicrobial agents.

According to the mechanism described by Abbaszadegan et al. (2015), AgNPs are thought to adhere to the cell wall and membranes of our test bacteria due to their positive charge in our applications. The positive charge provides electrostatic attraction between AgNPs and the negatively charged cell membrane of bacteria, thus facilitating the attachment of AgNPs to cell membranes (Dakal et al., 2016). Morphological changes become evident upon such interaction and can be characterized by shrinkage of the cytoplasm and membrane detachment, eventually leading to rupture of the cell wall (Nalwade and Jadhav, 2013). Besides electrostatic attraction, the interaction of AgNPs with the sulfur-containing proteins present in the cell wall causes irreversible changes in the cell wall structure, resulting in its disruption (Ghosh et al., 2012).

Silver's risk of causing acute toxicity in humans is extremely low. LD50 >2000 mg/kg, according to EC Directive 2004/73/EC (Height, 2011). Chronic toxicity is proportional to the cumulative effect and ranges between 70 and 1500 mg silver/kg body weight (Hadrup et al., 2018). Various studies have conducted that antibacterial feature of the AgNPs are mightly influenced by their colloidal state, concentration, size and shape (Bhattacharya and Mukherjee, 2008; Nateghi and Hajimirzababa, 2014; Pal et al., 2007; Rai et al., 2012; Raza et al., 2016). AgNPs in colloidal form, i.e., suspended nano-sized silver particles, have shown enhanced antimicrobial potential over AgNPs alone in several studies (Lkhagvajav et al., 2011; Panacek et al., 2006; Sondi and Salopek-Sondi, 2004). The colloidal state of AgNPs is an essential attribute for their antimicrobial activity. On the contrary, AgNPs in the liquid system have only limited applications as bacteriocidal agents because of their low colloidal stability (Kumar et al., 2014; Shi et al., 2014). Studies on growth inhibitions of Gram-negative *E. coli*, *S. typhii* and Gram-positive *S. aureus*, *P. aeruginosa*, *V. cholera* in the presence of AgNPs have put forth that Gram-negative bacteria was inhibited at lower AgNPs concentrations than Gram-positive. However, both classes of bacteria display complete growth inhibition at >75 ppm AgNPs concentrations (Kim et al., 2007). Gomaa (2017) remarked in his experimental results that 50 ppm AgNPs could completely inhibit the growth of bacterial cells and destroy the permeability of bacterial membranes and depress the activity of some membranous enzymes, which cause bacteria to die eventually. Therefore, 1.5%, 2% and 2.5% lactic acid solutions containing 200 ppm AgNP were used in our study.

To be effective, nanoparticles should be no larger than 50 nm in size, but it is also known that larger sizes also affect bacteria. Silver nanoparticles with sizes ranging from 10 to 15 nm have improved stability, biocompatibility, and antimicrobial activity (Yacaman et al. 2001). According to some studies, the antibacterial effect of AgNPs against *S. aureus* and *K. pneumoniae* is enhanced when smaller

diameter (30nm) nanoparticles are used (Collins et al., 2010). AgNPs' antibacterial effect is primarily due to their smaller particle size, which appears to have better Gram-negative bacteria penetrating ability (Morones et al., 2005). Bacteriostatic and bactericidal effects of AgNPs 5-10 nm in size against *S. aureus*, MSSA, and MRSA (Ansari et al., 2011). AgNPs are frequently found in large aggregates with a low surface area that ranges in size from 70 nm to 400 nm and can act intra- or extracellularly depending on their nature and size. AgNPs with a size of 10–15 nm embedded in a support matrix is more effective because their small size allows them to easily penetrate bacterial cells and eventually damage them by creating pores on their walls. (Espinosa-Cristobal et al., 2009; Lemire et al., 2013; Li et al., 2013; Wahab et al., 2021). An et al. (2015) and Wahab et al. (2021), on the other hand, emphasize that AgNPs have demonstrated good antimicrobial activity due to the unique physicochemical properties of Ag, as well as the large specific surface area, which provides microbes with a more exposed surface, resulting in higher antimicrobial efficiency. Gliga et al. (2014), on the contrary, emphasize that AgNPs smaller than 10 nm can enter human cells and exhibit toxic properties. In light of this, we chose AgNPs with diameters of 20, 40, 100, and 150 nm for our study. One of the properties that affect other physicochemical properties of nanoparticles is their shape (Burda et al., 2005). Shape-dependent interactions of AgNPs with bacteria, fungi, and viruses (Galdiero et al., 2011; Panacek et al., 2009; Raza et al., 2016; Tamayo et al., 2014; Wu et al., 2014;). Compared to spherical or rod-shaped AgNPs, truncated triangular shaped AgNPs exhibit superior antibacterial activity (Chen and Carroll, 2002; Pal et al., 2007). AgNPs with the same surface area but different shapes exhibit differential bactericidal activity, which can be attributed to differences in practical surface areas and active facets of AgNPs. (Elechiguerra et al., 2005). The most excellent effect was observed in our study at 20 and 150 nm. While the effect is expected to decrease as the NP size increases, the data obtained at 150 nm shows the opposite. This is thought to be due to the fact that 150 nm AgNP is in the form of nanopowder.

Heat applications in mild degree are applied massively in industrial food processing schemes especially plant food (Barry-Ryan, 2012). It is known that the increase in the temperature leads decrease in the number of bacteria (Chipley, 1993; Venkitanarayanan et al., 1999). Huang and Chen (2011) applied decontamination processes to spinach contaminated with *E. coli* O157:H7 at 1% and 2% lactic acid at 20°C, 40°C and 50°C and reported that increasing the temperature of the decontamination solution also increased the antimicrobial effect. Another study reported that the sanitation efficacy of acidified sodium chlorite coupled with mild heat (50°C) significantly increased the reduction of *E. coli* O157:H7 compared with room temperature and 4 C (Inatsu et al., 2005). These results are consistent with the findings reported by Akbaş and Ölmez (2007), who found that increasing the concentration of organic acid species from 0.5% to 1% at room temperature did not further decrease the number of *E. coli* in lettuce.

In our study, we applied three different temperatures (20°C, 40°C, 50°C) on tested organisms at different LA-AgNPs combinations, statistically significant reduction of *E. coli* O157:H7 and non-O157 STECs occurred. Gradual increase in heat treatment has been shown to reduce our experimental STEC loads successfully. Indeed, we observed that mild heat degree could increase the antibacterial efficacy of disinfectants (Inatsu et al., 2005).

CONCLUSION

Our findings demonstrate that Ag nanoparticles combined with organic acids using the low-cost reduction method described here promise novel antimicrobial agents. Our findings show that the bactericidal properties of silver nanoparticles vary with size. Improving antimicrobial efficacy with silver nanoparticles and lactic acid may be an appealing and cost-effective approach to addressing the problem of antimicrobial resistance in Gram-negative bacteria. The high safety limits of AgNPs are thought to allow for their use by interacting synergistically with traditional antibacterial agents. Lactic acid and AgNp and heat applications can be proposed for the meat industry as a preventative measure to trigger a decrease in STEC borne infections. This study was undertaken in-vitro, where the effect of the decontaminants and heat was analyzed directly on STEC. However, more studies should be undertaken for the antimicrobial and customer acceptance of this conceptual approach on meat and meat-related environment.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial o financial relationships that could be construed as a potential conflict of interest.

Authors' contributions

Serkan Kemal BUYUKUNAL: Data analysis, writing - review and editing Karlo MURATOGLU: Data analysis, Writing - review and editing Ahmet KOLUMAN: Experiment design; Experiment performer, Reviewing

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