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Author	Hag Ju Lee[1], Yeseul Heo[1], Hye-Jin Kim[1], Ki Ho Baek[2], Dong-Gyun Yim[1], Anand Kumar Sethukali[1], Dongbin Park[1], and Cheorun Jo[1,3,*]
Affiliation	[1]Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, South Korea [2]Department of Nano-Bio Convergence, Korea Institute of Materials Science, Changwon 51508, Republic of Korea [3]Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang 25354, Korea
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ORCID (All authors must have ORCID) **https://orcid.org**	Hag Ju Lee: 0000-0003-2906-7666 Yeseul Heo: 0000-0001-8161-778X Hye-Jin Kim: 0000-0002-9384-6720 Ki Ho Baek: 0000-0002-5438-9547 Dong-Gyun Yim: 0000-0003-0368-2847 Anand Kumar Sethukali: 0000-0003-0817-6396 Dongbin Park: 0000-0003-4979-6049 Cheorun Jo: 0000-0003-2109-3798
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CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Cheorun Jo
Email address – this is where your proofs will be sent	cheorun@snu.ac.kr
Secondary Email address	
Postal address	Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea
Cell phone number	+82-10-3727-6923
Office phone number	+82-2-880-4820
Fax number	+82-2-873-2271

Bactericidal Effect of Combination of Atmospheric Pressure Plasma and Nisin on Meat Products Inoculated with *Escherichia coli* O157:H7

Abstract

This study was conducted to investigate the bactericidal effect of nisin (Nisin) only, atmospheric pressure plasma (APP) only, and a combination of APP and nisin (APP + Nisin) on beef jerky and sliced ham inoculated with *Escherichia coli* O157:H7, gram-negative bacteria. The bactericidal effect against *E. coli* O157:H7 and *Listeria monocytogenes* was confirmed using a nisin solution at a concentration of 0-100 ppm, and APP + Nisin was tested on beef jerky and sliced ham using 100 ppm nisin. Beef jerky and sliced ham were treated with APP for 5 min and 9 min, respectively. In the bacterial solution, 100 ppm nisin out of 0-100 ppm nisin exhibited the highest bactericidal activity against *L. monocytogenes* (gram-positive bacteria) ($p<0.05$); however, it did not exhibit bactericidal effects against *E. coli* O157:H7 (gram-negative bacteria). The APP + Nisin exhibited a 100% reduction rate in both *E. coli* O157:H7 and *L. monocytogenes* compared to the control group, and was more effective than the Nisin. The APP + Nisin decreased the number of colonies formed by 0.80 and 1.96 Log CFU/g for beef jerky and sliced ham, respectively, compared to the control, and exhibited a higher bactericidal effect compared to the Nisin ($p<0.05$). These results demonstrate the synergistic bactericidal effect of APP and nisin, providing a possible method to improve the limitations of nisin against gram-negative bacteria. In addition, this technology has the potential to be applied to various meats and meat products to control surface microorganisms.

Keywords: atmospheric pressure plasma, nisin, gram-negative bacteria, meat products

Introduction

Meat consumption per capita and total meat consumption are increasing worldwide (Baek et al., 2020). Meat and meat products are a good environment for the growth of microorganisms (Heredia and Garcia, 2018), and can be easily contaminated with food-borne pathogens during their manufacture, storage, and distribution (Yoo et al., 2021). Contamination of meats can cause diseases such as food poisoning, which is caused by the ingestion of pathogens and microbial toxins. The symptoms of food poisoning include abdominal pain, diarrhea, vomiting, and fever; in severe cases, it can even cause death (Gourama, 2020). Therefore, to consume meat products safely, food safety must be ensured, for which technology to effectively control microorganisms is required.

Thermal treatment is one of the representative technologies for inactivating foodborne pathogens and ensuring food safety. However, it degrades the nutritional and sensory qualities of food (Heo et al., 2021). Therefore, there is a need for technology that can efficiently control pathogenic bacteria and minimize the negative effects on the nutritional and sensory quality of meat products. Non-thermal treatment, unlike thermal technology, can minimize the nutritional and sensory quality degradation of food (Jadhav et al., 2021). Non-thermal technologies include physical techniques, such as ultrasonication, plasma technology, irradiation, pulsed electric field, and high-pressure processing (Osae et al., 2020; Jadhav et al., 2021), as well as chemical methods, such as the use of chlorine-based sanitizers, organic acids, and bacteriocins (Demirok et al., 2013; Min et al., 2007; Mani- López et al., 2012).

Among these technologies, nisin is a peptide produced by a specific strain of *Lactococcus lactis*, which has been designated generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). It exhibits microbial inactivation properties, and is the most commonly used bacteriocin (Zhao et al., 2020). Nisin causes leakage of intracellular bacterial components,

leading to the death of bacterial cells (Tong et al., 2014). It exhibits high antibacterial activity against gram-positive bacteria. However, the lipopolysaccharide layer in the outer membrane of gram-negative bacteria confers resistance to nisin, limiting its efficacy against gram-negative bacteria (Liang et al., 2020). This limitation can be overcome by combining it with other technologies that can destroy the outer membrane (Wang et al., 2018). Some studies have been conducted to improve the antibacterial activity of nisin against gram-negative bacteria by damaging the integrity of the outer membrane in combination with an antibacterial agent (Zhao et al., 2020) or physical technology, such as electric field, high-pressure processing, ultrasound, and plasma (Gallo et al., 2007; Novickij et al., 2018; Mok et al., 2020; López-Pedemonte et al., 2003; Li et al., 2016; Pokhrel et al., 2019; Liao et al., 2018; Costello et al., 2021; Ukuku et al., 2019).

Cold atmospheric pressure plasma (APP) is a non-thermal sterilization technology that use ionized gas under quasi-neutral conditions (Lee et al., 2011). According to Yong et al (2015), in can be seen that plasma can effectively inactivate *Escherichia coli*, *Salmonella* Typhimurium, and *Listeria monocytogenes.* APP generates various UV photons, electrons, positive and negative ions, atomic species, and free radicals (Kim et al., 2020). In particular, reactive species such as superoxide anions, hydroxyl radicals, nitric oxide, and ozone generated through plasma (Yong et al., 2015) cause cell membrane damage via physical and chemical changes, such as lipid peroxidation of cell membranes, which ultimately induces cell leakage and lead to cell death (Zhao et al., 2022). In addition, owing to structural differences, such as the peptidoglycan layer of gram-positive and gram-negative bacteria, APP exhibits a greater bactericidal effect against gram-negative bacteria than gram-positive bacteria (Yoo et al., 2021). Therefore, the bactericidal effect of APP seems to overcome the problems related to the resistance of gram-

negative bacteria to nisin. In addition, there are few studies on improving the bactericidal effect of the combination of plasma and nisin treatment (Costello et al., 2021; Ukuku et al., 2019). However, this phenomenon needs to be investigated in more detail.

Therefore, in this study, combined treatment with APP and nisin was performed to determine their bactericidal effect on meat products. To achieve this, the effect of APP and nisin combination on the reduction of *E. coli* O157:H7 and *L. monocytogenes* was evaluated. The APP and nisin combination was then applied beef jerky and sliced ham inoculated with *E. coli* O157:H7 to confirm its bactericidal effect.

Materials and methods

Encapsulated APP system

The encapsulated atmospheric pressure dielectric barrier discharge plasma described by Kang et al. (2022) was used. Briefly, the plasma device was fabricated by attaching copper electrodes and a polytetrafluoroethylene sheet to a rectangular plastic container (137×104×53 mm) and a lid. The sample (bacterial solution or inoculated meat product) was placed in a glass dish in the plasma device, and discharge was performed at 2.2 kHz and 8.4 kV using atmospheric air.

Bacterial strains and culture preparation

E. coli O157:H7 (NCCP 15739) and *L. monocytogenes* (ATCC 19111) were obtained from the National Culture Collection for Pathogens (Cheonju, Korea) and Korean Culture Center of Microorganisms (Seoul, Korea), respectively. Single colonies of *E. coli* O157:H7 and *L.*

monocytogenes were transferred into 25 mL tryptic soy broth (TSB) and TSB containing yeast extract (YE), respectively, and cultured twice at 37°C for 24 and 18 h using orbital agitation at 120 rpm. Then, each broth was transferred to a 50 mL centrifuge tube and centrifuged at 4,001×g at 4°C for 10 min. The supernatant was discarded and cell pellet was completely diluted with 0.85% NaCl to adjust the initial concentration of each bacterial strain from 10^7 to 10^8 CFU/mL by measuring the optical density at 600 nm (OD_{600} = 0.2).

Microbial analysis

To quantify the number of bacteria in samples, serial dilutions were performed using 0.85% NaCl. After mixing reacting the bacterial solution with the treatment solution, 1 mL of the solution was transferred to a glass tube containing 9 mL of 0.85% NaCl, followed by serial dilution. The appropriately diluted solution was spread on tryptic soy agar (TSA) and TSA + YE to count *Escherichia coli* O157:H7 and *L. monocytogenes*, respectively. The media was incubated at 37°C for 48 h. The number of colonies is expressed as Log CFU/mL or g.

Effect of nisin on the bacterial solution

A stock solution (2,500 ppm) of nisin (N5764-5G, Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving 125 mg of nisin in 50 mL of 20 mM hydrochloric acid (7647-01-0, Duksan Pure Chem. Co. Ltd, Korea). The stock solution was diluted with deionized water (DDW) to prepare 25, 50, and 100 ppm nisin solutions. Then, 2.7 mL of each concentration of nisin solution and 0.3 mL of the bacterial solution were placed in a centrifuge tube. The centrifuge tube was vortexed for 5 s and incubated for 30 min at room temperature (25 ± 2°C). For control, 2.7 mL DDW and 0.3 mL of the bacterial solution were used. In all treatment

groups, the mixture of 2.7 mL treatment solution and 0.3 mL bacterial solution was considered as the 10^0 state. Microbial analysis was performed after dilution with 0.85% NaCl to an appropriate concentration.

Synergistic effect of APP and nisin on the bacterial solution

The scheme for preparing nisin (Nisin) only, APP only, and combination of APP and nisin (APP + Nisin) is shown in Fig. 1A. Control and Nisin were prepared using a bacterial solution (0.3 mL) with DDW (2.7 mL) and 100 ppm nisin solution (2.7 mL), respectively. For APP, DDW (2.7 mL) was added to the APP-treated bacterial solution (0.3 mL), which was discharged for 5 min. To determine the synergistic effect of APP + Nisin, APP was treated to the bacterial solution (0.3 mL), and 100 ppm nisin solution (2.7 mL) was added. All treated solutions were transferred to a 50 mL centrifuge tube, vortexed for 5 s, and incubated for 30 min at room temperature (25 ± 2°C) to match the reaction time with the Nisin. The solution in the centrifuge tube was regarded as 10^0 state. Microbial analysis was performed as described above after dilution with 0.85% NaCl to an appropriate concentration.

Effect of APP and nisin combination treatment on *E. coli* O157:H7 inoculated beef jerky and sliced ham

Beef jerky and sliced ham were purchased from a local market (Seoul, Korea). All samples were exposed to UV light for 30 min to remove surface microorganisms prior to cutting the samples and inoculating them with *E. coli* O157:H7. Beef jerky and sliced ham were cut into 5 g (35×35 mm, n=2) and 3 g (40×40 mm, n=3) pieces, respectively, and 100 μL and 50 μL of *E. coli* O157:H7 (OD_{600} = 0.20), respectively, were spot-inoculated on the surface. The

inoculated samples were then dried at room temperature (25 ± 2°C) for 30 min. The scheme of preparing the Nisin, APP, and APP + Nisin for inoculated beef jerky and sliced ham is shown in Fig. 1B. The Nisin involved dipping the samples in nisin solution (100 ppm) for 30 min. For the APP, beef jerky and sliced ham were treated with APP for 5 and 9 min, respectively, and immersed in 45 and 27 mL of DDW, respectively. They were then vortexed for 5 s and allowed to react for 30 min at room temperature (25 ± 2°C). For APP + Nisin, APP was directly applied to the beef jerky and sliced ham for 5 min and 9 min, respectively. Both samples were then dipped in 45 and 27 mL of nisin solution (100 ppm), respectively, vortexed for 5 s, and incubated at room temperature (25 ± 2°C) for 30 min. After completion of the reaction, the beef jerky and sliced ham were transferred to a centrifuge tube containing 0.85% NaCl and vortexed for 2 min to detach the bacterial strain present in the sample, which was considered as the 10^0 state. Microbial analysis was performed as described above.

Statistical analysis

All the experiments were performed in triplicate, except for those involving beef jerky, which was replicated. All data were assessed using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) with statistical significance set at $P<0.05$. Statistical analysis was conducted using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

Results and discussion

Effect of nisin on reduction of *E. coli* O157:H7 and *L. monocytogenes*

Fig. 2A shows the bactericidal effect of nisin on *E. coli* O157:H7 and *L. monocytogenes*.

The initial count of *E. coli* O157:H7 was 7.11 Log CFU/mL. The number of viable *E. coli* O157:H7 cells at 25, 50, and 100 ppm nisin decreased by 0.04, 0.04, and 0.01 Log CFU/mL, respectively, compared with that at 0 ppm nisin. However, this difference was not statistically significant. This suggests that nisin concentration below 100 ppm has no bactericidal effect on *E. coli* O157:H7.

The initial count of *L. monocytogenes* was 7.66 Log CFU/mL. The number of viable *L. monocytogenes* cells at 50 and 100 ppm nisin decreased by 0.09, and 0.24 Log CFU/mL, respectively, compared to that at 0 ppm nisin ($p<0.05$). In addition, the 100 ppm treatment had the highest bactericidal effect among the nisin concentrations tested ($p<0.05$). When compared the result of the bactericidal effect of nisin at 100 ppm on *E. coli* O157:H7 and *L. monocytogenes*, there was a higher reduction rate in *L. monocytogenes* than in *E. coli* O157:H7 (3.13% vs. 0.14%). This result confirmed that nisin has a lower bactericidal effect on gram-negative bacteria (*E. coli* O157:H7) than gam-positive bacteria (*L. monocytogenes*). Unlike gram-positive bacteria, gram-negative bacteria have an outer membrane composed of phospholipids, proteins, and lipopolysaccharides (Abee et al., 1995). These outer membranes exhibited impermeability to nisin. As nisin is greater than 3 kDa, it is unable to react the cytoplasmic membrane (Stiles et al., 1991; Abee et al., 1995). Therefore, to improve the bactericidal effect of nisin on *E. coli* O157:H7, the APP + Nisin was evaluated. Based on the bactericidal effect of nisin on the bacterial solution, a concentration of 100 ppm was selected for the APP + Nisin.

Synergistic effect of APP and nisin combination against *E. coli* O157:H7 and *L. monocytogenes*

196 The synergistic effect of APP + Nisin against *E. coli* O157:H7 and *L. monocytogenes* was
197 evaluated (Fig. 2B). The Nisin exhibited the lowest reduction of *E. coli* O157:H7 among all
198 treatments ($p<0.05$), and there was no difference compared to the control. No viable cells were
199 detected in *E. coli* O157:H7 cells treated with APP. Furthermore, no viable *E. coli* O157:H7
200 cells in the APP + Nisin were detected.
201 Similar to the result for *E. coli* O157:H7, Nisin exhibited the lowest bactericidal effect
202 among all the treatment groups against *L. monocytogenes* ($p<0.05$). No viable *L.*
203 *monocytogenes* cells were detected after treatment with either APP or APP + Nisin.
204 In this study, APP treatment (APP and APP + Nisin) exhibited significantly higher
205 bactericidal effects than Nisin on *E. coli* O157:H7 and *L. monocytogenes* ($p<0.05$). However,
206 the bactericidal effect of APP was too strong to evaluate the synergistic effects of APP and nisin.
207 Unlike the bactericidal effect of Nisin on *E. coli* O157:H7 and *L. monocytogenes*, APP
208 exhibited the same bactericidal effect on both pathogens. This is because during APP, the outer
209 membrane of *E. coli* O157:H7 is damaged by reactive species capable of destroying the DNA,
210 proteins, lipids, and cell membranes of bacterial cells (Kim et al., 2013), resulting in increased
211 permeability of the lipopolysaccharide layer, which would enhance the activity of nisin against
212 bacterial cells. Similarly, according to Yoo et al. (2021), APP of *E. coli* O157:H7 for 4 min
213 reduced viable cells by 22.31% compared to the control group. Furthermore, *E. coli* O157:H7
214 and *L. monocytogenes* exhibited a 100% reduction in cell viability compared with the control
215 group when treated with APP for 90 s and 10 min, respectively (Yong et al., 2015).
216
217 **Bactericidal effect of APP and nisin combination treatment against inoculated beef jerky**
218 **and sliced ham**

219 The effect of the APP + Nisin on beef jerky and sliced ham was assessed by inoculation
220 with *E. coli* O157:H7 (Fig. 3). The Nisin, APP, and APP + Nisin decreased the number of viable
221 cells of *E. coli* O157:H7 in beef jerky by 0.19, 0.38, and 0.80 Log CFU/mL with a reduction
222 rate of 3.37, 6.75, and 14.21%, respectively, compared to the control. Nisin and APP treatment
223 in beef jerky did not exhibit significant differences when compared with the control. In contrast,
224 the APP + Nisin exhibited the highest bactericidal effect among the treatments ($p<0.05$).
225 Similarly, Nisin did not decrease the number of *E. coli* O157:H7 in sliced ham. However,
226 APP and the APP + Nisin induced a decrease in *E. coli* O157:H7 in sliced ham at 1.34 and 1.96
227 Log CFU/g with a reduction rate of 21.72 and 31.77%, respectively, compared to the control.
228 Both the APP and the APP + Nisin exhibited significantly higher bactericidal effects than the
229 control and Nisin; in particular, the APP + Nisin exhibited a higher bactericidal effect than the
230 APP ($p<0.05$).
231 The APP + Nisin exhibited synergistic bactericidal effects in both beef jerky and sliced
232 ham inoculated with *E. coli* O157:H7. However, the bactericidal effect of APP and the APP +
233 Nisin on *E. coli* O157:H7 in meat products was lower than that in bacterial solutions. This is
234 because changes in the physicochemical properties of plasma occurred during APP treatment
235 for organic matters, such as beef jerky and sliced ham. Specifically, the various reactive species
236 generated via APP could react with organic matter, which would reduce the concentration of
237 reactive species capable of responding to bacterial cells. In addition, during the reaction with
238 organic matters, pH may increase and oxidation-reduction potential may decrease, which may
239 also affect the bactericidal effect of APP (Baek et al., 2020; Xiang et al., 2019). For this reason,
240 the degree of damage that APP can induce on the outer membrane of *E. coli* O157:H7 may be
241 reduced, which may be the cause of the lower bactericidal effects of meat products compared

to bacterial cells.

In addition, the degree of reduction rate of the APP + Nisin against *E. coli* O157:H7 on beef jerky and sliced ham were 14.21% and 31.77%, respectively. Differences in appearance, such as surface shape and thickness of the samples, can affect the degree of destruction of the bacterial cell membrane by APP. An irregular surface of a sample can create a physical barrier to protect the bacterial strain, which require more energy and time for agents to exert their antibacterial effect (Fernandez et al., 2013). In other words, compared to sliced ham, beef jerky with its irregular surface, makes it more difficult for the APP + Nisin to exert its bactericidal effect, compared to that in solutions on *E. coli* O157:H7. This could be responsible for the lower bactericidal effect of APP on beef jerky.

Conclusion

The APP + Nisin exhibited a synergistic bactericidal effect against *E. coli* O157:H7 (gram-negative bacteria). In addition, their bactericidal effect was demonstrated in beef jerky and sliced ham inoculated with *E. coli* O157:H7. This shows that the APP + Nisin has the potential to efficiently control microorganisms on the surfaces of meat and meat products. Further research is needed to establish the optimal conditions for the APP + Nisin applicable for various meat products.

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361

362 **Figure captions**

363

364 **Fig. 1.** Schematic diagram of bactericidal test treatment for bacterial solution (A) and
365 inoculated meat products (B). DDW, deionized water; APP, cold atmospheric pressure plasma.

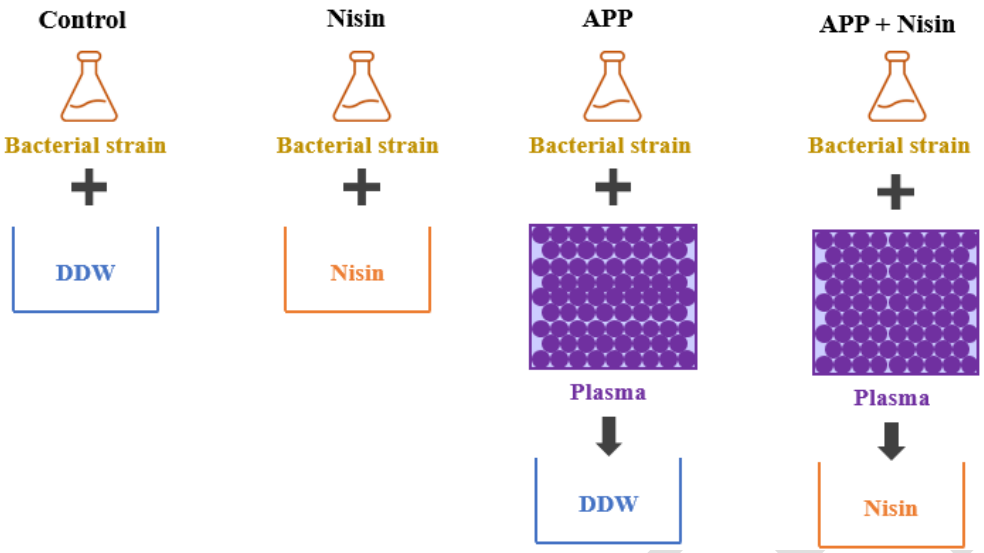
366

367 **Fig. 2.** Bactericidal effect of nisin by concentration (A) and the Nisin, APP, and APP + Nisin
368 treatment (B) on the bacterial solution. Nisin, 100 ppm nisin-only treatment; APP, cold
369 atmospheric pressure plasma-only treatment; APP + Nisin, combination of APP and nisin
370 treatment. Error bars represent standard deviation. $^{A-C}$ Different letters indicate a significant
371 difference ($P<0.05$) among the treatment.

372

373 **Fig. 3.** Bactericidal effect of Nisin, APP, and APP + Nisin treatment on beef jerky (A) and
374 sliced ham (B) inoculated with *E. coli* O157:H7. Nisin, 100 ppm nisin-only treatment; APP,
375 cold atmospheric pressure plasma-only treatment; APP + Nisin, combination of APP and nisin
376 treatment. Error bars represent standard deviation. $^{A-C}$ Different letters indicate a significant
377 difference ($P<0.05$) among the treatment.

(A)



(B)

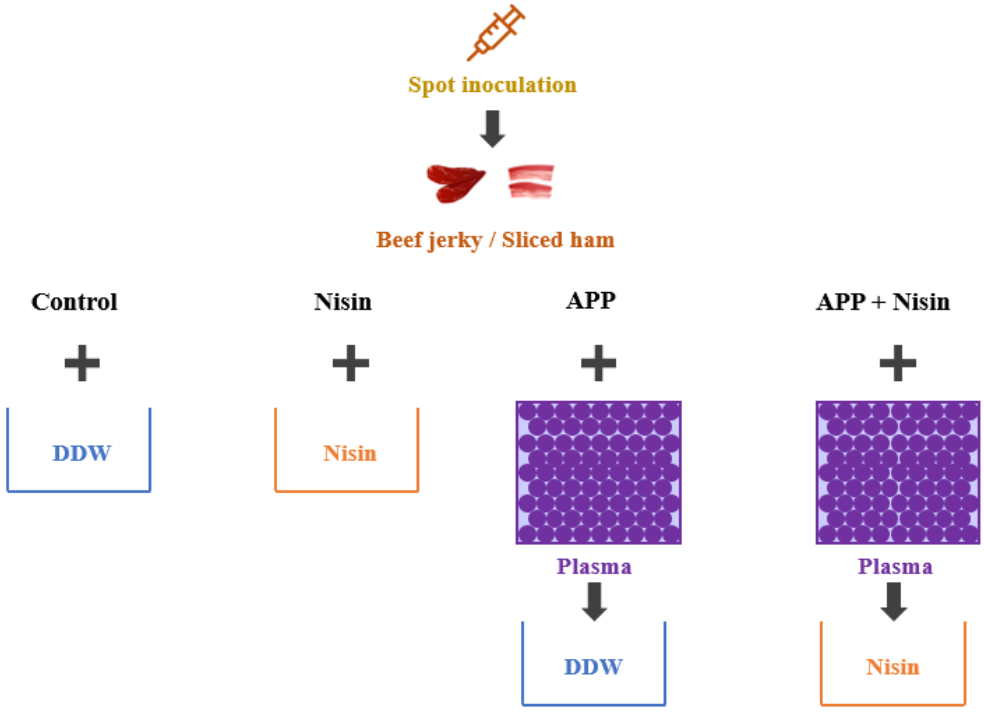
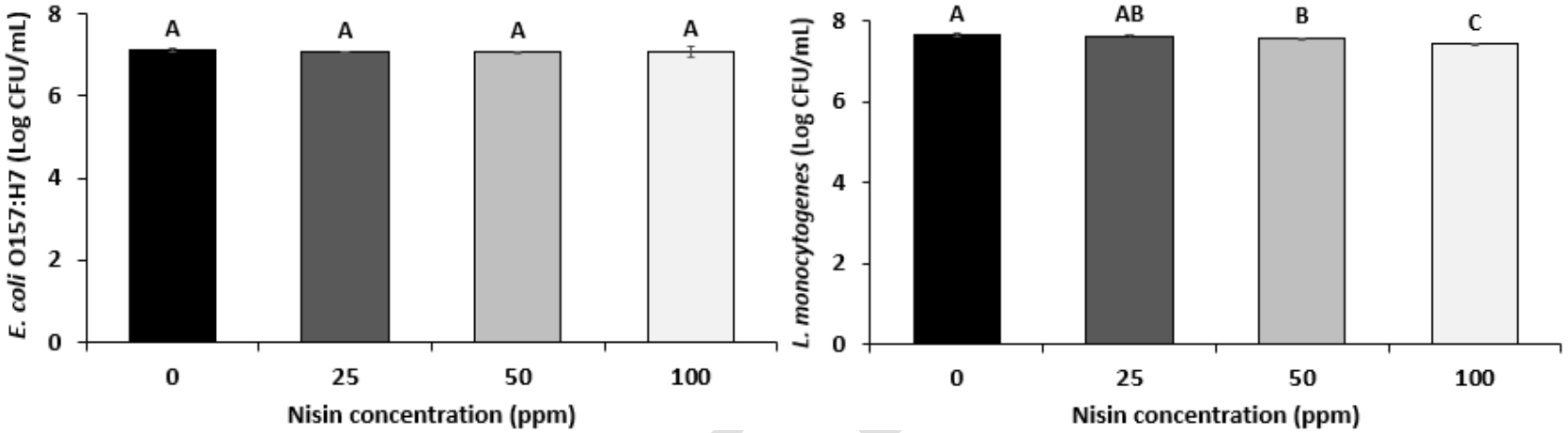


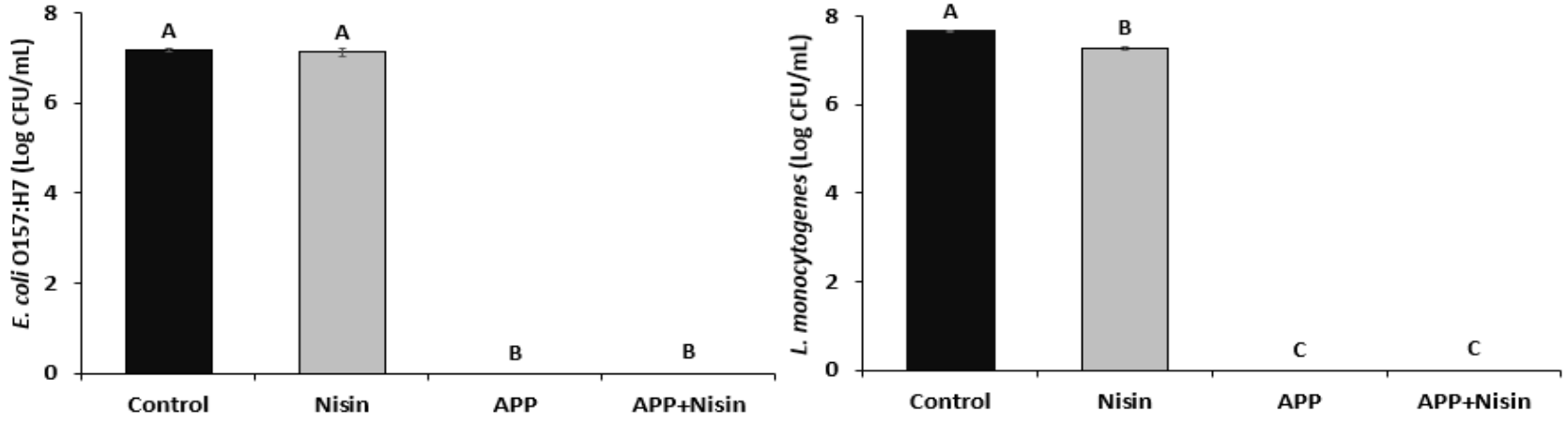
Fig. 1. Schematic diagram of bactericidal test treatment for bacterial solution (A) and inoculated meat products (B). DDW, deionized water; APP, cold atmospheric pressure plasma.

384 **(A)**



385

386 **(B)**



387

388 **Fig. 2.** Bactericidal effect of nisin by concentration (A) and the Nisin, APP, and APP + Nisin treatment (B) on the bacterial solution. Nisin, 100
389 ppm nisin-only treatment; APP, cold atmospheric pressure plasma-only treatment; APP + Nisin, combination of APP and nisin treatment. Error
390 bars represent standard deviation. $^{A-C}$ Different letters indicate a significant difference ($P<0.05$) among the treatment.

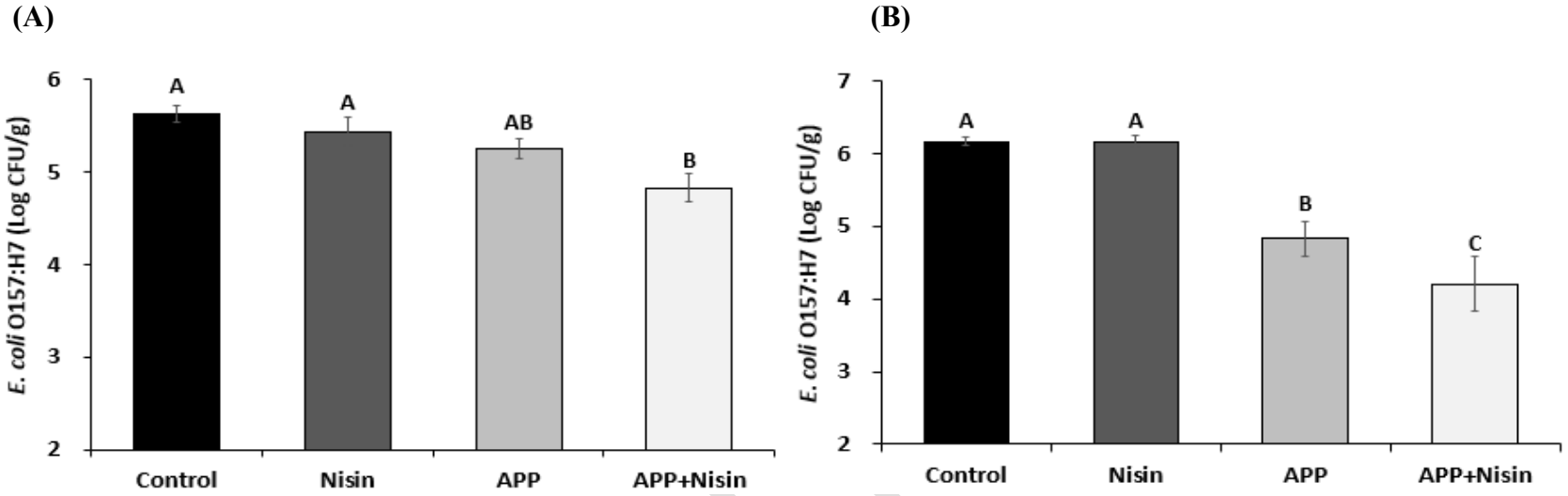


Fig. 3. Bactericidal effect of Nisin, APP, and APP + Nisin treatment on beef jerky (A) and sliced ham (B) inoculated with *E. coli* O157:H7. Nisin, 100 ppm nisin-only treatment; APP, cold atmospheric pressure plasma-only treatment; APP + Nisin, combination of APP and nisin treatment. Error bars represent standard deviation. $^{A\text{-}C}$ Different letters indicate a significant difference ($P<0.05$) among the treatment.