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ORIGINAL ARTICLE

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Biodegradable Antimicrobial Films Incorporated with Silver Nanoparticles Inhibit the Growth of Multiple Drug-resistant *Staphylococcus aureus* Experimentally Inoculated in Chicken Fillets

Omnia A.M. Ahmed · Fathy A. Khalafalla · Fatma H.M. Ali · Abdelrahim H.A. Hassan*

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Department of Food Safety and Technology, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62511, Egypt

Correspondence

Abdelrahim H.A. Hassan, Department of Food Safety and Technology, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62511, Egypt

Email: abdelrahim@vet.bsu.edu.eg

Abstract

Antimicrobial food packaging was developed in response to the growing demand for longer fresh food shelf life and protection against foodborne infections. Therefore, the current study was conducted to investigate the antimicrobial effect of homemade biodegradable antimicrobial films incorporated with silver nanoparticles (Ag-NPs) against *Staphylococcus aureus* (*S. aureus*) experimentally inoculated in chicken breast. Chicken breast slices (5 × 5 cm) were experimentally inoculated with a cocktail of three *S. aureus* strains at a concentration of about 6.6 $\log_{10}$ CFU/cm^2 and wrapped in homemade biodegradable antimicrobial films. Following that, the residual bacterial counts in wrapped chicken breast fillet slices were monitored for up to 10 days in a refrigerator (4 °C). It was found that at the end of the chilling period the films with biosynthesized Ag-NPs (1mM), biosynthesized Ag-NPs (2mM), chemically synthesized Ag-NPs (1mM), and chemically synthesized Ag-NPs (2mM) reduce the *S. aureus* counts by about 4.66, 5.24, 4.79, and 5.43 $\log_{10}$ CFU/cm^2, respectively when compared with control films. Biodegradable antimicrobial films also prolonged the shelf-life of samples by approximately 4 days when compared to control samples.

Keywords

Antimicrobial Films, Biosynthesized Nanoparticles, Chicken, Shelf-life, *Staphylococcus aureus*

1. Introduction

The application of different barriers to control the growth of foodborne pathogens in high risky foods could provide an increased safety margins during long-term chilling storage of both raw and ready-to-eat (RTE) poultry and their products. One of the new methods to reduce microbial growth and increase the shelf-life of foods is the use of antimicrobial food packaging **(Dirks et al., 2012).** Poultry as a food has high nutritive value; cheap and widespread consumed it is the second more consumed meat around the world by a percent of 35%. According to OECD/Food and Agriculture Organization of the United Nations 2016 chicken meat consumption has risen dramatically in many nations in recent years, making it one of the most popular foods. It is easily contaminated with food borne pathogens such as *S. aureus* which have many dangers to the consumer's health. So, it is very important to apply different methods to reduce its microbial contamination and make it safe to the consumer. Additionally, the herbal extracts spraying on the food surface has a weak effect and is rapidly evaporated. Therefore, it is a goal to find a method keep antimicrobials on the food items surface as long as possible that could be a solution for that issue. Edible films made of natural biopolymers (proteins and polysaccharides) have attracted considerable attention in recent years due to their biodegradability and potentials to reduce serious environmental problems associated with non-biodegradable petroleum-based plastic materials **(Cazón et al., 2017).** Food packaging alternatives such as edible films have been offered as a way to improve the quality and safety of food products. This technique protects foods from dehydration and serves as a gas barrier between the food and the environment. Furthermore, edible films can act as carriers

for active ingredients including antimicrobials, antioxidants, and texture enhancers **(Krochta et al., 1994).** The demand for natural antimicrobials has been increased due to health hazards associated with synthetic antimicrobials **(Sadiq et al., 2015)** so many herbs which have antimicrobial effect could be used in food production to enhance color, odor, taste and extend the shelf life of food. Microbes are inhibited from growing due to the controlled release of encapsulated bioactive substances which could be incorporated in antimicrobial packaging **(Ahmed et al., 2018).** To manage food-borne infections, several tactics have been employed; however, the development of active food packaging materials not only ensures food safety but also extends the shelf life of perishable food products **(Espitia et al., 2016).** In addition to attempting to prevent foodborne pathogens from causing public health issues and extending the shelf life of goods also the world has focused on reducing plastic waste in order to protect the environment. As a result, natural biocompatible and edible films and coatings made of polysaccharides, proteins, or lipids with addition of natural or chemical antimicrobials have sparked a lot of attention in recent years. Furthermore, consumers prefer natural food packaging materials also due to increasing the antimicrobial resistance to different antimicrobials attentions. Starch is the most widespread carbohydrate reserve in plants. As a result, it is a biodegradable natural resource that is abundantly easy to handle, produced at low cost, and possesses thermoplastic qualities **(Babu et al., 2013),** so it can be easily used as a base for biodegradable packages.

Fresh or dried *Origanum marjorana* leaves, as well as their essential oil, are widely utilized as food ingredient, herbal tea, flavoring, coloring, nutritious, and natural preservatives in the food sector **(Holley and Patel, 2005).** Silver metal has been recognized as a very effective antibacterial agent, capable of killing several types of microbes that cause various infectious diseases and has been used by mankind for nearly 7000 years **(Chernousova and Epple, 2013).** Because of their particular activity against a wide range of bacteria and the bacterial resistance to routinely used antibiotics, silver nanoparticles have become an important topic among researchers **(Hutchison, 2008).**

A range of approaches and technologies, including physical, chemical, and biological procedures, have evolved for the successful synthesis of silver nanoparticles. On an economic basis, physical and chemical processes are more cost-effective, but biological methods are less hazardous to the environment **(Hulkoti and Taranath, 2017).** The capability of plant metabolites to act as reducing and capping agents, as well as contribute to the aggregation of metal ions into nanoparticles, is essential **(Makarov et al., 2014)** due to these facts, plants such as *Origanum marjorana* can be used in biosynthesis of metal nanoparticles.

According to this, the goal of this study was to produce silver nanoparticles using both chemical and biological methods using *Origanum marjorana* extract as a reducing agent. Furthermore, both types of nanoparticles were separately incorporated with different concentrations in starch-based biodegradable food films. The antibacterial activity of the produced films with two different nanoparticle concentrations was tested against foodborne pathogens inoculated in chicken breast fillets samples during chilling period. The sensory characteristics and acceptability of poultry samples packed in handmade films containing various antimicrobials were also investigated.

2. Materials and Methods

2.1. Bacterial Strains

Three different multiple drug-resistant coagulase positive *S. aureus* strains, isolated from minced meat and beef burger samples tested for antibiotic sensitivity, identified by **Saleh et al., (2021),** were used for this study. Frozen (-80˚C) *S. aureus* strains in glycerol were inoculated into tryptic soy broth and incubated at 37˚C for 18 hrs, then streak-plated onto tryptic soy agar plates at 37˚C for 18 hrs. then the stock culture on tryptic soy agar slopes kept on refrigerator for further using. All bacterial culture media used in this study were obtained from Oxoid (Hampshire, UK), unless otherwise is mentioned.

2.2. Chemical Synthesis and Biosynthesis of Silver Nanoparticles (Ag NPs)

The chemical synthesis method described by **Li et al. (2010)** was used. Furthermore, the biosynthesis of silver nanoparticles using *Origanum marjorana* extract was made according to the method reported by **Hamelian et al. (2018).**

2.3. Biodegradable Antimicrobial Films Synthesis

The films were made according to the method described by **Hassan and Cutter (2020)** with modifications as following. Firstly, corn starch (10–12g), gelatin (1–3g), xanthan gum (0.05–0.3g), and glycerol (3–5mL) were dissolved in 100 mL of sterile distilled water to the film mixture. Starch was progressively dissolved in distilled water with stirring on hot plate stirrer. Then the mixture was warmed, and gelatin added with stirring then the xanthan gum with heating. Finally, glycerol was added to the mixture. The mixture was sterilized then being cooled to 55˚C. The antimicrobial solutions were sterilized before being mixed with the solution to make different films. Biodegradable control films were created using starch-based solution without antimicrobials. The mixture was then spread and allowed for dryness. All films were kept aseptically at 25˚C.

2.4. Challenge Study

Fresh raw chicken breast fillets samples were acquired from a local supermarket, transported at 4˚C to Food Safety and Technology laboratory, and sliced into thin slices ($5\times5cm^2$) under complete aseptic conditions. The slices were sterilized under UV light t for 15 min on each side to decrease the bacterial contamination **(Cutter and Siragusa, 1994; Hassan and Cutter, 2020).** One mL of *S. aureus* pathotype

cocktail (8 log_{10} CFU/mL) was spread over each side of the slice aseptically to reach a concentration of 6.6 log_{10} CFU/cm^2. Before the other side inoculation, the inoculated side was kept stable for 20 min. for bacterial attachment. Control negative slices were that had not inoculated.

Both inoculated and control chicken breast samples were wrapped in biodegradable control and antimicrobial films. The samples were then wrapped aerobically in polyethylene bags aseptically and refrigerated at 4˚C for up to 10 days before examined.

On days zero, 2, 4, 6, 8, and 10 of refrigerated storage, pathogen populations were evaluated in inoculated and un-inoculated chicken breast fillets samples. The remaining bacterial counts were tested in triplicates for each sample. Each 5×5cm sample was withdrawn off the film aseptically, placed to a sterile homogenization flask containing 50mL BPW, and homogenized for 2 minutes at each time point.

The resultant homogenate was diluted tenfold in buffered peptone water, and the dilutions were spread-plated onto Baired Parker's plates to count the residual *S. aureus.* After performing duplicate counts, the Baired Parker's plates were incubated for 24 hrs. at 37˚C. The inoculated bacterial pathotype's typical colonies were manually enumerated and transformed log_{10} CFU/cm^2. Whenever colonies similar to inoculated bacterial pathotypes appeared on the un-inoculated sample, they were subtracted from the bacterial counts on inoculated samples.

2.5. Sensory Evaluation and Shelf-life of Wrapped Chicken Breast Fillets

Sensory evaluation to detect color, taste, texture and odor was applied to the control and wrapped un-inoculated chicken samples in triplicates according to the method of **Neumann et al. (1983).**

2.6. Statistical Analysis

In the challenge experiment, after the conversion of bacterial counts to log_{10} CFU/cm^2, means and standard errors were calculated. The remaining counts in biodegradable antimicrobial films were subtracted from the remaining counts in control films to determine log reductions. A one-way ANOVA test was used to determine significant differences and comparisons between means (Minitab 18 statistical software). If $P<0.05$, the means were judged substantially different.

3. Results and Discussion

3.1. The Effect of Biodegradable Antimicrobial Films on Experimentally Inoculated *S. aureus* in Chicken Fillets

Biodegradable antimicrobial films containing 1mM and 2mM chemically synthesized silver nanoparticles or 1mM and 2mM biosynthesized silver nanoparticles, as well as control films, were used to wrap chicken breast slices inoculated with *S. aureus* pathotype cocktail at an approximate concentration of 6.6 log_{10} CFU/cm^2 and chilled stored at 4˚C up to 10 days.

The remaining bacterial counts of the inoculated *S. aureus* pathotype cocktail are presented in **Table (1),** and the log reductions (log_{10} CFU/cm^2) are shown in **Table (2).** Chicken breast slices wrapped with control films showed a continuing increase in *S. aureus* counts, which progressed from 6.72 to reach 7.74 log_{10} CFU/cm^2 at the end of storage (day 10). Whereas the aerobically packaged chicken breast slices with no wrappings showed a similar rate of increase in the bacterial count to that of control films, as they reported 7.84 log_{10} CFU/cm^2 at day 10 of refrigerated storage **(Table, 1).** This increase in *S. aureus* counts in aerobically packaged chicken without film wrapping and chicken slices wrapped in starch-based control films without any antimicrobial agent was anticipated, as **Yoksan and Chirachanchai (2010)** found that starch-based films have no antimicrobial activity when compared with chitosan-based ones.

Table (1). Remaining bacterial loads (log_{10} CFU/cm^2) of *Staphylococcus aureus* strain cocktail, experimentally inoculated onto raw chicken breast slices, wrapped in biodegradable control or antimicrobial films under chilling storage at 4°C up to 10 days.

Wrapping films	Zero day (after 4h.)	Day 2	Day 4	Day 6	Day 8	Day 10
Packaged without films	6.79 ± 0.76^{a}	6.99 ± 0.52^{a}	7.3 ± 0.95^{a}	7.6 ± 0.76^{a}	7.74 ± 0.76^{a}	7.84 ± 0.82^{a}
Control film	6.72 ± 0.52^{a}	6.77 ± 0.76^{a}	6.9 ± 0.76^{a}	7.04 ± 0.52^{ab}	7.6 ± 0.95^{a}	7.74 ± 0.52^{a}
Films with chemically synthesized Ag-NPs (1mM)	5.07 ± 0.76^{a}	5 ± 0.76^{a}	4.77 ± 0.52^{a}	3.9 ± 0.76^{bc}	3.3 ± 0.52^{b}	2.95 ± 0.52^{b}
Films with chemically synthesized Ag-NPs (2mM)	4.85 ± 0.64^{a}	4.60 ± 0.76^{a}	4.3 ± 0.76^{a}	3 ± 0.52^{c}	2.6 ± 0.52^{b}	2.31 ± 0.52^{b}
Films with biosynthesized Ag-NPs (1mM)	5.14 ± 0.64^{a}	5.04 ± 0.64^{a}	4.9 ± 0.76^{a}	4 ± 0.76^{bc}	3.6 ± 0.52^{b}	3.08 ± 0.76^{b}
Films with biosynthesized Ag-NPs (2mM)	4.95 ± 0.76^{a}	4.84 ± 0.76^{a}	4.6 ± 0.76^{a}	3.69 ± 0.5^{bc}	3 ± 0.52^{b}	2.5 ± 0.46^{b}

Different small letters (a, b and c) superscript within column indicates significance difference between means at $p < 0.05$

On one hand, chicken breast slices wrapped with films containing chemically synthesized Ag-NPs showed significant decreases in the counts after 4 h of chilling as the remaining *S. aureus* counts reached 5.07 and 4.85 $\log_{10}$ CFU/cm^2 in the case of 1mM and 2mM concentrations, respectively. While the final counts at the end of storage period (day 10) were 2.95 and 2.31 $\log_{10}$ CFU/cm^2, respectively **(Table 2).** The log reductions in *S. aureus* cocktail counts by films containing chemically synthesized Ag-NPs 1mM and 2mM were 4.79 and 5.43 $\log_{10}$ CFU/cm^2, respectively, at the end of the chilling period (day 10) when compared with control films **(Table, 2).**

On the other hand, chicken breast slices wrapped with films containing biosynthesized Ag-NPs showed reduction in the bacterial count bacterial after 4 hrs of refrigerated storage to 5.14 and 4.95 $\log_{10}$ CFU/cm^2 by films containing biosynthesized Ag-NPs 1 mM and 2 mM, respectively with continuous reduction to reach 3.08 and 2.5 $\log_{10}$ CFU/cm^2 by at the end of storage **(Table, 1).** The $\log_{10}$ reductions day 10 were 4.66 and 5.24 $\log_{10}$ CFU/cm^2 by the films containing biosynthesized Ag-NPs 1mM and 2mM concentrations, respectively with no significant difference between both types of films **(Table, 2).**

Table (2). Reductions of *Staphylococcus aureus* strain cocktail ($\log_{10}$ CFU/cm^2) experimentally inoculated onto raw chicken breast slices, wrapped in biodegradable antimicrobial films incorporated with silver nanoparticles during chilling storage (4 °C) up to 10 days.

Wrapping films	Zero day (after 4h.)	Day 2	Day 4	Day 6	Day 8	Day 10
Films with chemically synthesized Ag-NPs (1mM)	**1.65 ± -0.24**	**1.77 ± 0**	**2.13 ± 0.12**	**3.14 ± -0.12**	**4.3 ± 0.43**	**4.79 ± 0**
Films with chemically synthesized Ag-NPs (2mM)	**1.87 ± -0.12**	**2.17 ± 0**	**2.6 ± 0**	**4.04 ± -0.12**	**5 ± 0.43**	**5.43 ± 0**
Films with biosynthesized Ag-NPs (1mM)	**1.58 ± -0.12**	**1.73 ± 0**	**2 ± 0**	**3.04 ± -0.12**	**4 ± 0.43**	**4.66 ± -0.12**
Films with biosynthesized Ag-NPs (2mM)	**1.77 ± -0.24**	**1.93 ± -0.12**	**2.3 ± 0**	**3.35 ± 0**	**4.6 ± 0.43**	**5.24 ± 0.06**

These homemade starch-based biodegradable antimicrobial films incorporated with chemically or biosynthesized synthesized Ag-NPs at 1mM or 2mM concentrations achieved >4.5-log reduction in pathogenic *S. aureus* cocktail, which translates to >99.99% reduction in the bacterial population, which is considered a significant antimicrobial effect is achieved by these films. Additionally, low-density polyethylene films incorporated with silver nanoparticles performed by **Brito et al. (2020)** show an inhibitory effect against *S. aureus*. Also, **Raigond et al. (2019)** recorded the antimicrobial effect of potato starch-based nano-composite films incorporated with clove oil against *S. aureus.*

Starch-based biodegradable antimicrobial films containing Ag-NPs reported in this study proved a higher antimicrobial activity when compared with the pullulan-based composite antimicrobial films incorporated with 2.5% lauric arginate used for ready-to-eat turkey breast slices reported by **Hassan and Cutter (2020),** as they reported that pullulan-based composite antimicrobial films incorporated with 2.5% lauric arginate reduced count of *S. aureus* cocktail by approximately 3.43 $\log_{10}$ CFU/cm^2 after 28 days of storage.

3.2. Sensory Evaluation of Chicken Breast Fillets Samples

Sensory characteristics of chicken breast samples variously affected by the application of all types of films. At the first days of chilling storage the biodegradable antimicrobial films enhanced the color of the samples and give them tenderness but after 4 days of chilling storage, the color of the samples began to be changed to yellowish coloration.

The overall acceptability of all sample groups declined with storage however, this reduction was more significant in control films and unwrapped chicken breast samples than antimicrobial films. This could be ascribed to the fact that the fresh chicken meats are easily damaged due to enzymatic, chemical and microbial reactions **(Yuliani et al., 2019).** Unwrapped chicken samples received a 10.41 score on day 4 of chilling, on day 6, this group was not evaluated due to apparent signs of spoilage. Although chicken samples wrapped in control films declined nearly to the acceptability limit (7.2) stated by **Neumann et al. (1983)** by day 6 of chilling, on day 8, they displayed apparent signs of deterioration, so they were discarded as well. On the other hand, wrapped chicken samples with biodegradable antimicrobial films revealed acceptable sensory attributes up to day 8 of refrigerated storage, yet they were found deteriorated on day 10 and thus were not further assessed. These results indicate 4 days extension in the shelf stability of chicken when compared with unwrapped aerobically packaged samples, while only a 2-day shelf-life extension was achieved by the control films **(Table, 3).**

Table (3). The sensory acceptability of chicken breast samples wrapped in homemade biodegradable control or antimicrobial films incorporated with silver nanoparticles during chilling storage (4°C).

Wrapping films	Zero day (after 4 h.)	Day 2	Day 4	Day 6	Day 8	Day 10
Packaged without films	**17.91 ± 0.09^{b}**	**17.56 ± 0.05^{c}**	**10.14 ± 0.11^{c}**	NA	NA	NA
Control film	**18.48 ± 0.05^{a}**	**18.41 ± 0.06^{a}**	**16.04 ± 0.04^{b}**	**10 ±0.15^{c}**	NA	NA
Films with chemically synthesized Ag-NPs (1mM)	**18.48 ± 0.1^{a}**	**18.26 ± 0.14^{ab}**	**16.01 ± 0.11^{b}**	**11.57±0.11^{b}**	**7.31 ± 0.16^{b}**	NA
Films with chemically synthesized Ag-NPs (2mM)	**18.5 ± 0.04^{a}**	**18.26 ± 0.09^{ab}**	**15.86 ± 0.07^{b}**	**11.31± 0.05^{b}**	**7.55 ± 0.17^{b}**	NA
Films with biosynthesized Ag-NPs (1mM)	**18.5 ± 0.07^{a}**	**18 ± 0.14^{b}**	**15.88 ± 0.09^{b}**	**13.09 ± 0.29^{a}**	**8.59 ± 0.13^{a}**	NA
Films with biosynthesized Ag-NPs (2mM)	**18.5 ± 0.07^{a}**	**18.24 ± 0.06^{ab}**	**16.88 ± 0.02^{a}**	**13.14 ± 0.05^{a}**	**8.79 ± 0.18^{a}**	NA

Different small letters (a, b and c) superscript within column indicates significance difference between means at $p < 0.05$

Starch-gelatin thermo processed films containing lauric arginate (LAE) reported by **Moreno et al. (2018)** were found to increase the shelf life of chicken breast fillets. It was observed by **Alizadeh-Sani et al. (2020)** that the shelf life of lamb meat samples packed with active packaging made from nanostructured biopolymer matrix incorporated with TiO2 nanoparticles and rosemary oil was around 12–15 days whereas the control sample was only around 6 days.

Also, antimicrobial film based on agar/konjac glucomannan loaded with 2% carvacrol examined by **Peng et al. (2022)** can be a promising environmentally friendly antimicrobial packing material that inhibit *S. aureus* and extend the shelf life of chicken breast samples.

4. Conclusion

Homemade starch-based biodegradable films incorporated with silver nanoparticles (either chemically synthesized or biosynthesized) presented a significant reduction in the cocktail of MDR coagulase positive *S. aureus* strains experimentally inoculated in chicken breast at the end of the storage period. Biodegradable antimicrobial films showed a 4-day extension in the shelf stability of uninoculated samples when compared with control films.

5. Authors Contributions

All authors contributed equally to study design methodology, interpretation of results and preparing of the manuscript.

6. Conflict of Interest

The authors declare no conflict of interest.

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