RESEARCH ARTICLE

Polyethylene and Polyamide Covers Containing Silver Nanoparticles in Reduction of the Mince Microbial Load

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ABSTRACT

Objective(s): The use of new technologies, which can be realized as nanotechnology, is a novel approach in this sector. Covers made of silver nanoparticles effectively prevent and reduce invasion of microorganisms, compared with other conventional covers. This study was conducted to determine the effects of nanosilver covers in comparison with other conventional covers.

Materials and methods: In order to measure the effect of antibacterial nano-covers, in comparison with other commonly used covers in packing food products, the direct contact of covers with food (in this study, mince mixture of sheep- calf) was used as control. Furthermore, the sample was contaminated with standard strains of gram-negative and gram-positive bacteria in specified time periods (zero, 24, 48 and 72 hours) was performed.

Findings: Despite the large number of Staphylococcus *aureus* colonies in meat tested as normal flora, except for one case, no other positive Staphylococcus *aureus* coagulase bacteria were either found or reported by confirmatory tests; however, the absence of even a colony of positive staphylococci coagulase, despite the manual infection of 2 sections from 7 sections, raised the possibility of the domination of Escherichia *coli* bacteria and prevention of the growth of Staphylococcus *aureus* bacteria in the broth.

Conclusion: Nano-silver cover has been identified as the most efficient cover in reducing the microbial load and increasing the shelf life of mince.

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INTRODUCTION

In this century, the world population is on the rise and healthy food production is one of the major concerns in the food industry. Therefore, today's tendency to minimizing the process of food production, facilitating the long term storage of food, and preventing food-borne outbreaks has led to the development of methods that impede the growth of microorganisms and the use of antimicrobials in packed food products. It is worth mentioning that antimicrobial substances are utilized in foods that are heat-sterilized or possess a self-controlling immune system with the aim of preventing their spoilage due to the secondary contamination during packing, distribution or unpacking. Various antimicrobial substances are used in food packing and each of these substances has their own properties and mechanism of action. These substances will be selected and utilized based on factors such as type of food, type and growth rate of microorganisms, antimicrobial activity and spread, antimicrobial chemical composition and other factors. Researchers examined the effect of the antimicrobial mixture of nanocomposite low silver films and low-density polyethylene as well as modification of atmosphere on the shelf life of packed chicken breast fillets[1]. The findings indicated a significant difference ($p<0.05$) between chicken breast fillet shelf life and oxidation of the control film. Furthermore, the results showed that low density polyethylene films, containing silver nanoparticles, might be used as antimicrobial

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packaging for food. The data were analyzed using Tukey's test and one-way analysis of variance by SPSS. In this study, samples packed with 4000 ppm nano-particle covers, based on titanium dioxide, indicated a significant level at p< .05 even when samples of sheep-calf mince were contaminated by Escherichia *coli* and Staphylococcus *aureus* bacteria in a controlled condition [2].

The use of wrapping (film) is growing in food packing industry. Substances used for packing foods have a number of disadvantages that limit their use. Non-biodegradability in the environment, low resistance and low strength as well as oxygen permeability that results in the early spoilage of food are among the issues that led the scientists to develop new technologies for tackling these problems. Patiño *et al*. (2014) investigated the production of polyamide composite covers containing silver nanoparticles for packing sausages. The results revealed that the cover is effective in inhibiting the growth of Salmonella *typhimurium* (at the significance level of p<0.05) and reduces the transfer of oxygen from the cover. The statistical analysis was performed by Duncan multi range test (ANOVA). Silver nanoparticle covers inhibited the growth of pathogenic bacteria at the significance level of p<0.05 due to the antibacterial properties of this type of cover in comparison with other covers[3].

The effect of a polyamide film releasing lactic acid bacteria on the growth of Escherichia *coli*, Enterobacteriaceae and total aerobic bacteria in beef packed in vacuum and found that the number of bacteria had been reduced using this method[4]. The use of covers containing silver nanoparticles, a better contact with microorganism was made due to the increase in the surface to volume ratio and a large number of metal atoms per surface area. And these covers might be used as an effective antimicrobial agent against pathogenic bacteria, viruses and other microorganisms. This property of silver nanoparticle covers increase the shelf life of the mince [5].

The microbial (biological) degradation of low density polyethylene were examined [6]. They also applied various analysis techniques in vitro condition and dealt with some of the issues related to low-density polyethylene such as fungal activity in LDPE, biological benefits and challenges of LDPE, microorganisms involved in the degradation of LDPE, etc. Major concerns were reported for LDPE covers. Some of other research, shows that even normally (without controlled contamination) packed with polyethylene and polyamide covers, the total count of bacteria was increased after a certain period of time. However, the samples (controlled contamination) packed with silver nanoparticle covers revealed a significant decrease in contamination after 48 hours (p<0.05)[7].

Cruz-Romero *et al*. (2013) investigated the antimicrobial activity of chitosan, organic acids and nano-sized solvents for potential use in smart active antimicrobial packing and potential applications for food products. The results indicated that chitosan, with average molecular weight (MMW), benzoic acid, and sorbic acid nano-sized solvents indicated significantly (p <0.05) higher antimicrobial properties than their non-nano equivalents. [8].

During the investigation of the effects of physically manipulated packaging materials on the quality and safety of meat products, it was found that innovative measures such as active packaging –nanotechnologies and so on- have certain problems that should be considered and solved in the future. In addition, consumers' expectations and safety issues during the packaging process and storage of meat products should be considered. Given the importance of food safety and the research conducted, it has to be emphasized that meat covers should be selected so that they not only maintain the quality of products, reduce microbial load and increase their shelf life, but also address the concerns about how to prevent product waste.

Researchers studied the effect of active packing with citrus juice on fat oxidation and the quality of cooked turkey meat. The results indicated that oxidation is influential on the fat of the cooked turkey meat as significant differences were observed in their sensory properties when being kept in refrigerator[9]. This finding highlights the anti-bacterial properties of covers containing 4000 ppm silver nanoparticles which further increase the shelf life of the products [10]. The effects of antimicrobial property of nanocomposite low silver films, low-density polyethylene and modified atmosphere were examined, considering the shelf life of packed chicken breast fillets. The findings indicated a significant difference (p< 0.05) between chicken breast fillet shelf life and oxidation of the control film. Low density polyethylene films, containing silver nanoparticles, can be used as antimicrobial packages for food. In this research, common packaging available in the market and Polyethylene and Polyamide Covers Containing

Silver Nanoparticles are examined in Reduction of the Mince Microbial Load.

MATERIALS AND METHODS

Materials

(Escherichia *coli* –code of 1399 (ATCC 25992)) and gram-positive bacteria (Staphylococcus *aureus* –code of 1431 (ATCC 25923)) provided by the Industrial Bacteria and Fungi Collection Center of Iran.

Silver nanofilm having purity 99.5% by trace metals was purchased from Sigma-Aldrich. To compare the toxicity of nano-silver with their ionic form, a stock solution of silver nitrate (AgNO3) (Sigma-Aldrich) was prepared in deionised water (Milli-Q, Millipore).

Methods

The effect of nano-silver cover, in comparison with other conventional covers (e.g., polyethylene and polyamide), on the microbial effects of mince was investigated in two microorganisms which are usually considered in mince. The types investigated were Escherichia *coli* and Staphylococcus *aureus*, the maximum allowed amounts of which are presented in Table 1. Furthermore, the present study sought to uncover the effect of antimicrobial nano-silver cover in packed mince in lowering the microbial load of microorganisms mentioned above that, in turn, greatly influences the final product health.

The mince (mixture of sheep-calf) in a one-kilogram package, was purchased from a randomly selected shop in region 2 of Tehran. Afterward, in order to measure the effect of anti-bacterial nano-covers and comparing them with other conventional covers used in packing food products, the method of direct contact with the food products as control method as well as infecting the same samples with standard strains of gram-positive and gram-negative bacteria (Pure strains of "industrial bacteria and fungi collection center of Iran") was used and then these two samples were compared at defined time intervals (after 0, 24, 28 and 72 hours). Samples sent to the laboratory was quite homogenized and divided into two equal parts. Half of this sample was used as the control and the second half was manually mixed, contaminated and homogenized with 1 ml of suspension containing approximately 10^6 CFU/ml of standard stains of Escherichia *coli* and Staphylococcus *aureus* bacteria, as stated above.

Test method

A. Search method

First, the presence or absence of Escherichia *coli* was examined. If positive (the presence of Escherichia *coli* in the sample), counting method could be used to estimate the probable number of microorganisms per gram. A certain amount of sample or initial suspension was inoculated in the selected enriching liquid broth (Lauryl sulfate broth). Afterwards, the inoculated broth was incubated at 37 °C for 48 hours and its amount of gas was measured after 24 and 48 hours. If turbidity or gas was observed in the tube, it would be inoculated in the tube containing liquid EC broth. The inoculated EC tube was incubated at 44 ° C for up to 48 hours; afterwards, its amount of gas was measured after 24 and 48 hours. If the gas was observed in the tube, it would be inoculated in the indole-free peptone water tube, then the tube would be incubated at 44 ° C for up to 48 hours and the production of indole would be checked. With regard to the presence of Escherichia *coli* in a certain amount of weight or volume, if tubes caused turbidity or gas in the selected broth; the confirmed EC broth produced gas; and the peptone water produced indole at 44 ° C, they were all considered as affirmative results [12, 13].

B. Counting method

1. Three tubes from the selected enriched broth were inoculated with double concentration and a certain amount of the initial suspension.

2. Three tubes from the selected enriched broth were inoculated with a typical concentration and a certain amount of the initial suspension; then in an identical condition, three other tubes from the selected enriched broth with typical concentrations were inoculated with a certain amount of decimal dilutions. The addition of other decimal dilutions

Table1. Maximum allowed number of colonies per pack of minced meat

Product type	Experiment type	Maximum allowed
Fresh or frozen mince	Total count of microorganisms	$5*10^5$ in gram
	Staphylococcus *coagulase* +	$5*10^2$ in gram
	Escherichia *coli*	5*10 gram

to the selective broth with typical concentration continued until ensuring that the tubes with the last dilution produced negative results.

3. The tube was incubated at 37 ° C for up to 48 hours and the amount of gas production was investigated after 24 and 48 hours.

4. EC broth was incubated with each tube of double concentration that had produced turbidity or gas and also with each tube of typical concentration that had produced gas. The results of the analysis are presented in tables.

Data analysis

The findings of the present study were analyzed based on a simple random plan using SPSS version 21 and Kruskal-Wallis test was performed to estimate the significance of findings.

RESULTS

The results of the analysis of total bacterial count microbial tests

One gram of the sample was carefully weighed under sterile conditions and after homogenization, transferred to a test tube containing 9 ml of sterile Ringer's solution and was well mixed. Then the required dilutions were prepared and cultivated in plate count agar broth (pour plate mixed cultivation). The prepared plates, after closing the broth, were incubated upside down in groups of less than 6 at proper distance from each other, on the roof and walls of the incubator, at 30°C for 72 hours. After the incubation, plates with two successive dilutions which contained at least 15 and at most 300 colonies were selected, all of the colonies in the plates were counted, the results were calculated using the standard formula (5272 standard), and the number of microorganisms were reported based on two significant values (value between 1.1 and 9.9) multiplied by an appropriate power of 1 (sampling was done based on the Iranian National Standard No. 690; samples, experiments and dilutions were prepared based on Iran national standards numbered 1-8923 and 2-8932; and the broth was prepared and sterilized according to the manufacturer's instructions, stated in its brochure).

Search method positive Staphylococcus aureus coagulase count based on the national standard 6806-1

One gram of the sample was carefully weighed in sterile conditions. After homogenization, it was transferred to a test tube containing 9 ml of sterile Ringer's solution and was well mixed. Subsequent to preparing the requisite dilutions, 0.1 ml of the initial suspension and each prepared dilution were transferred to two sterile plates containing Brad Parker agar broth (which was previously prepared and the emulsion of egg yolk and potassium tellurite had been added to it) using a sterile pipette or a sampler. The dilutions were quickly spread on the surface of the plates using a glass rod. Plates were placed in vitro for 15 minutes (25 °C) for the liquid to be absorbed in the broth. They were placed at 37 °C for 24 hours; then incubated for 48 hours and at the end of each incubation, the plates were investigated for marking the colonies. The marked colonies could be seen as dark or light gray, convex and with the diameter of 1 to 1.5 mm (after 24 hours of incubation) or 1.5 to 2.5 mm (after 48 hours of incubation) with a transparent halo; a sedimentary ring also might be clung to the colony which was quite distinguishable. To confirm a number of selected specified and unspecified colonies (commonly 5 of each), they were cultivated in the Brain Heart Infusion broth and after 24 hours of incubation in 25 ° C, confirmatory coagulate test (clot formation), using rabbit plasma, was conducted according to the manufacturer's instructions.

The results and the estimated number of positive Staphylococcus *aureus* coagulase were

Table 2. Comparing mean and standard deviation logarithm of 7 treatments (mentioned packages) of total bacteria count in four hours (Zero, 24, 48 and 72), PE (Polyethylene), PA (Polyamide), NS (Nano-silver), PEC (Polyethylene Contaminated), PAC (Polyamide Contaminated), NSC (Nano-silver Contaminated)

Samples \ Times	Zero	Time 24H	Time 48H	Time 72H
Control	5.71±0.18^{a}	5.86±0.31^{a}	5.97±0.18^{a}	6.07±0.5^{a}
PE	5.76±0/18^{b}	5.06±0.76^{b}	5.34±0.76^{b}	5.12±0.18^{b}
PA	5.68±0.30^{a}	5.01±0.76^{b}	5.64±0.30^{ab}	5.67±0.23^{c}
NS	5.69±0.63^{a}	4.68±3/18^{c}	4/20±4/31^{c}	3.34±0.2^{d}
PEC	6.04±0.5^{c}	5.99±0.18^{d}	5.82±0.18^{ab}	5.88±0.76^{ae}
PAC	6.06±0.76^{c}	6.00±0.84^{ad}	5.91±0.18^{ab}	5.97±0.18^{af}
NSC	6.06±0.76^{c}	5.91 ±0.4^{a}	5.53±0.30^{b}	5.26±0.18^{b}

obtained using the formula (Standard 6806) and the results were reported as the number in each gram of sample (sampling was done according to the Iranian National Standard No. 690; the samples, experiments and dilutions were prepared based on Iran national standards numbered 1-8923 and 2-8932; and the broth was prepared and sterilized according to the manufacturer's instructions, stated in its brochure)[5, 11]. The overall results of the comparison of polyethylene, polyamide and silver nanoparticle covers in total count test of microorganisms are presented in Table 2.

By examining the total count of microorganisms and comparing them with each other, it was found that at the initial time (zero hour), the contaminated sample covered with nano-silver nano-particles, polyethylene and polyamide covers significantly differed from the control group ($p < .05$) which was in line with the enhancement of the bacteria growth in meat (according to Tables 1, 2 and 3).

Finally (after 72 hours), the final results showed the reduction in the bacteria growth rate in the samples of polyethylene, polyamide and silver nanoparticles, compared with the control sample ($p < .05$). The fewest number of bacteria was observed in silver nanoparticles as compared with other groups. This finding was due to the antibacterial property of the silver nanoparticles covers that prevented the growth of pathogenic bacteria.

Due to the controlled inoculation with bacterial strains, samples of contaminated polyethylene, contaminated polyamide and contaminated silver nanoparticles, compared with the control sample, showed an increase in bacteria growth. No significant difference was observed between contaminated polyethylene and polyamide samples with the control group considering the bacteria growth rate ($p < .05$). However, the contaminated sample of silver nanoparticles, due to the use of silver nanoparticles with anti-bacterial properties, showed a slight decrease in the bacterial growth, compared with the control sample as well as the contaminated polyethylene and polyamide samples ($p < .05$).

Comparing results of the four time periods on the total bacteria count

According to Table 2 and Fig. 2, the total count indicated significant differences at different time periods ($p < .05$). As illustrated in Fig. 2, significant differences were observed among zero, 24, 48 and 72 hours ($p < .05$). However, no significant difference was observed between 24 and 48 hours considering the total bacteria count ($p < .05$).

The results of the analysis of microbial tests for Escherichia coli

The results of comparing polyethylene, polyamide and silver nanoparticle covers for total count of microorganisms are presented in Table 3.

As indicated in Table 3, the increase in the microbial load of Escherichia *coli* strain in samples of contaminated polyethylene, contaminated polyamide and contaminated silver nanoparticles was observed, compared with the control sample. The difference between the contaminated sample and the control group was significant ($p < 0.05$). This finding is because of the controlled inoculation of the sample (mince) with the bacteria strain.

Given that P=0.05, we conclude that the degree of significant of the total count variable differs in repeated measures. In other words, passage of time significantly influences the total count. Looking at the graphs of each group, we are able to see whether the difference is due to the decrease or increase in the total count. Considering Table 3 and Fig. 2, the differences in Escherichia *coli* bacteria count is significant in different times ($p<0.05$). To put it more specifically, Fig. 1 indicates significant differences in zero, 24, 48 and 72 hours ($p < 0.05$).

Table 3. Comparing mean and standard deviation logarithm of 7 treatments (mentioned packages) of total bacteria count in four hours (Zero, 24, 48 and 72) PE (Polyethylene), PA (Polyamide), NS (Nano-silver), PEC (Polyethylene Contaminated), PAC (Polyamide Contaminated), NSC (Nano-silver Contaminated)

Samples \ Times	0 h	24 h	48 h	72 h
Control	1 ± -0.23^{a}	2.04 ± 0.31^{a}	4.36 ± 0.1^{a}	5.06 ± 0.40^{a}
PE	0.97 ± -0.23^{a}	0.86 ± 0.31^{a}	1.25 ± 0.4^{ab}	1.97 ± 0.67^{ab}
PA	0.88 ± -0.23^{a}	1 ± 0.5^{a}	1.23 ± 0.63^{ab}	$2/06\pm0/88^{ab}$
NS	1 ± 0.30^{a}	0.84 ± 0.4^{a}	1.19 ± -0.23^{b}	1.93 ± 0.40^{b}
PEC	4.75 ± 0.55^{b}	4.92 ± 0.78^{b}	6.31 ± 0.76^{c}	$6/55\pm0/42^{abc}$
PAC	$5.06\pm4/06^{c}$	4.70 ± 0.63^{b}	6.49 ± 0.03^{d}	6.63 ± 0.06^{c}
NSC	5.04 ± 0.84^{c}	4.49 ± 0.93^{c}	2.27 ± 1.42^{c}	2.05 ± 0.76^{ab}

The results of the test for Staphylococcus aureus bacteria

Despite counting a large number of Staphylococcus *aureus* colonies as normal flora in the meat tested, except for one case (the control sample (not contaminated) between two layers of disks made from polyethylene cover), no other cases of positive Staphylococcus *aureus* coagulase growth was either observed or recorded by confirmatory tests. However, the absence of the growth of even a colony of positive staphylococci coagulase, despite manual contamination, (the manually contaminated sample between two layers of a polyethylene covered disk; the manually contaminated sample between two layers of a polyamide covered disk; and the manually contaminated sample between two layers of a silver nanoparticle covered disk), raise the possibility of Escherichia *coli* bacteria dominance in the broth and prevention of the growth of Staphylococcus *aureus* bacteria. The hypothesis was tested by the standard method of Spots & Lawn Method and no growth sign of positive Staphylococcus coagulase (mixed cultivation with plate count agar broth in the second layer) was observed in the plate containing Escherichia *coli* cultivated in MacConkey agar broth. However, in the suspension containing positive Staphylococcus *aureus* coagulase in agar count broth plate, the turbidity caused by bacteria growth observed after 24 hours or the lack of growth might be attributed to inappropriate bacteria used.

DISCUSSION AND CONCLUSION

After preparation of the above samples, in the time intervals of zero, 24, 48 and 72 hours, microbial tests were performed on them, with three iterations, and the results were recorded. Despite counting a large number of colonies of Staphylococcus as normal flora in meat tested, except for one case in one section, no growth sign of positive Staphylococcus *aureus* coagulase bacteria was either observed or reported by confirmatory tests. However, the absence of the growth of even a colony of positive staphylococcus coagulase,

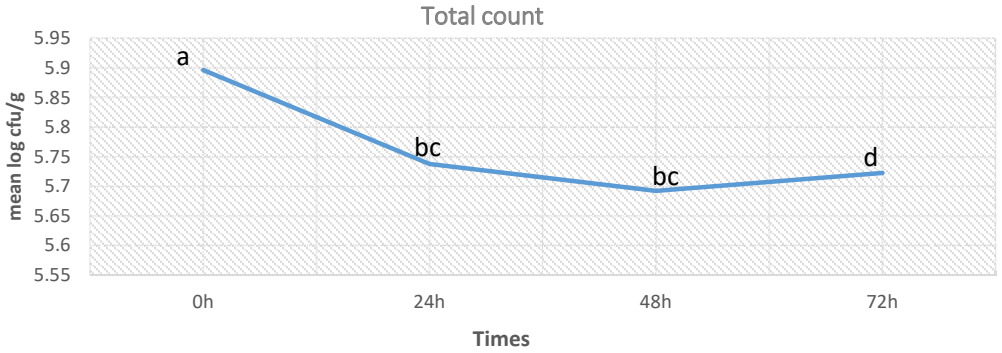

Fig. 1. Comparing mean logarithms of total bacteria count in four hours (zero, 24, 48, 72)

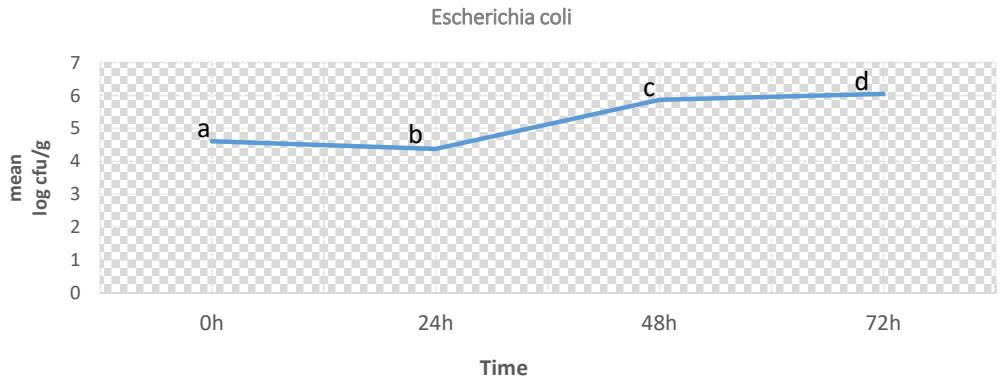

Fig. 2. Comparing mean logarithms of total Escherichia *coli* bacteria count in four hours of zero, 24, 48 and 72

despite manual contamination of two sections from seven sections, offer the possibility of Escherichia *coli* bacteria dominance in the broth and the prevention of the growth of Staphylococcus *aureus* bacteria. The hypothesis was tested by the standard method of Spots & Lawn Method and no growth sign of positive Staphylococcus coagulase (mixed cultivation with PCA broth in the second layer) was observed in the plate containing Escherichia *coli* cultivated in MacConkey agar broth. However, in the suspension containing positive Staphylococcus *aureus* coagulase in PCA broth, the turbidity caused by bacteria growth was observed after 24 hours. The lack of growth might be attributed to the inappropriate bacteria used. Due to the high surface to volume ratio and the large number of metal atoms, silver Nano-particles have a better contact with microorganisms and work as effective antimicrobial substance against bacteria, viruses and other microorganisms. Therefore, we might be able to produce covers that increase the shelf life and reduce the microbial load of meat. Considering food safety, the necessity of using covers for mince, which in addition to maintaining the quality of this type of products, reduce the microbial load, increase their shelf life and reduce the concerns about how to prevent product waste, is further emphasized by the present study [7, 14, 15].

The synergistic antimicrobial effect of low silver nanocomposite films and low density polyethylene under modified atmosphere on the maintenance of packed chicken breast fillets was previously studied by Azlin-hasim *et al* (2015) [1]. The results showed the significance increase of chicken breast fillet maintenance ($p < 0/05$) compared to the amount of oxidation in control film. Therefore, the achieved results suggested that the low density polyethylene films containing silver nanoparticles can be used as antimicrobial food packages. According to researches the common used packaging in the market are polyethylene and polyamide which is suitable for perishable products like mincemeat but silver nanoparticles covers with anti-bacterial activity can favorably increase the maintenance period of the product. The application of polyamide composite cover containing nano-silver as sausage packaging reviewed by Patino *et al* (2014)[3], in which showed the suggested cover can significantly inhibit the growth of bacteria salmonella *typhimurium* and oxygen transfer through the cover ($P<0/05$). In the present study, covers containing silver nanoparticles prohibited the growth of pathogenic bacteria related to prior antibacterial characteristics of cover compared to other types. In the previous study done by Smulders *et al* [4], using the effect of lactic acid releasing polyamide film on Escherichia *coli*, Enterobacteriaceae, demonstrated the decrease of bacterial growth in preservation of packed vacuum beef with this method. Considering present studies, covers containing silver nanoparticles could be implemented as potential antimicrobial agent against pathogen bacteria, viruses, and other microorganisms because of providing higher ratio surface to volume which makes better possibility for metal atoms to contact with microorganisms. Hence, this effect of silver nanoparticle covers leads to prolong the preservation period of mincemeat products.

After the preparation of above samples, microbial profile tests performed within period of 24, 48, 72 hours and results were reported of three independent experiments. Although several numbers of staphylococcal colonies as normal flora in meat samples, except of a band case it was observed neither the growth of coagulase positive staphylococcus *aureus* nor reported by confirmatory tests. The Lack of growth of any coagulase positive Staphylococcus aureous colony despite of manual contamination of two bands out of seven would imply the theory of surmounting of Escherichia *coli* in medium in order to prohibit the growth of Staphylococuus aureous. PCA demonstrated turbidity of bacterial growth after 24 hours. The lack of growth could be resulted from nonsuitable bacteria used in the experiment.

With the view to the food safety, the necessity to use of special covers for mincemeat in order to maintain the quality, decrease the microbial load and extend the preservation period also concerns regarding not to waste of product were promoting factors to accomplish current project.

The overall results achieved from this project implicated the better efficacy of the covers included silver nanoparticles (4000ppm) in compared to polyethyleneand polyamide covers. In the experiment with covers included silver nanoparticles rather the common types the growth of Escherichia *coli* remarkably decreased in the control samples, furthermore the growth of staphylococcus aureous decreased completely. The covers included silver nanoparticles have been recognized as the most efficient cover to eliminate microbial load of mincemeat and preservation

time can be extended from 2 days to 1-2 weeks in refrigerated temperature but the achieved results of the experiments using other types of covers for samples and control were not statistically significant ($P<0.05$) and results were not consequently reported.

CONFLICT OF INTEREST

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

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