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STABILITY OF ANTIMICROBIAL ACTIVITY OF PULLULAN EDIBLE FILMS INCORPORATED WITH NANOPARTICLES AND ESSENTIAL OILS AND THEIR IMPACT ON TURKEY DELI MEAT QUALITY

Khalaf, H.H.; Sharoba, A.M.; El-Tanahi, H.H. and Morsy, M.K. *

[1]***Department of Food Science, Faculty of Agriculture, Benha University, Egypt***

***Corresponding author:Mohamed Khairy Morsy, Food Science Department, Faculty of Agriculture, Benha University, Qaluobia, Egypt.**
Tel.: +2 012 23831825; fax: +2 013 2467786.
E-mail address: mohamed.abdelhafez@fagr.bu.edu.eg,
drkhairy3000@yahoo.com

ABSTRACT

Edible films containing antimicrobials are gaining importance as potential treatment to extend product shelf life and reduce risk of pathogens. Antimicrobial activities of pullulan films incorporated silver nanoparticles (Ag NPs) 100 nm, zinc oxide nanoparticles (ZnO NPs) 110 nm, oregano oil (OR) 2% and rosemary oil (RO) 2%, against *Listeria monocytogenes* and *Staphylococcus aureus,* were evaluated during the preparation and storage at 4, 25, 37, and 55°C up to 49 days in plate overlay assays (*in vitro*). Moreover, the prior pullulan films were applied directly on turkey deli meat were inoculated by *L.monocytogenes* and *S. aureus* and stored at 4 °C for two weeks. The results from this study demonstrate that Ag NPs and OR edible films more active than ZnO NPs and RO, respectively. The optimum conditions for storage pullulan edible films incorporated nanoparticles (NPs) and/or essential oils (EOs) were 4 and 25°C. Ag NPs, ZnO NPs, OR, and RO based films were exhibited significant effects as antibacterial activity against pathogens. These results revealed that (NPs) and/or (EOs) have a good potential to be incorporated into pullulan to make an antimicrobial edible film or coating for various food applications.

Keywords: nanotechnology, antibacterial activity, oregano, rosemary, silver nanoparticles, zinc oxide nanoparticles, pullulan, edible film, physical properties, storage stability.

INTRODUCTION

In recent years, antimicrobial packaging has attracted much attention from the food industry to the increase in consumer demand for minimally processed and preservative-free products. Use of antimicrobial substances based on nanoparticles and essential oils are of great importance and can control the microbial population and target specific microorganisms to provide higher safety and quality products (Appendini and Hotchkis 2002). These packaging technologies could play a role in extending shelf-life of foods and reduce the risk from pathogens. Antimicrobial packaging is a promising form of active food packaging, in particular for meat products. Since microbial contamination of these foods occurs primarily on the surface, due to post-processing handling, attempts have been made to improve safety and to delay spoilage by use of antibacterial sprays or dips (Quintavalla and Vicini 2002).

Active packaging is an innovative concept in which the package, the product, and the environment interact to prolong the shelf-life, or to enhance safety or sensory properties, while maintaining the quality of the product (Marcos *et al*. 2013). These biodegradable polymeric films offer alternative packaging options with no contribution to the environmental pollution while being obtained from renewable sources with low cost (Tharanathan 2003; Lu *et al*. 2005). The antimicrobial properties of silver nanoparticles (Ag NPs), for example, have been increasingly exploited in consumer products such as food packaging, deodorants and bandages (Chen and Schluesener 2008; Kumari *et al.* 2010) as antimicrobial agents in food contact material (Suppakul *et al*. 2003; Damm *et al.* 2008). It has been reported that some antimicrobial agents may affect the physical properties, processability or machinability of the packaging material. As several recent reports, ZnO nanoparticles (ZnO NPs) have demonstrated fair antimicrobial activity (Yadav *et al*. 2006) and it has been shown on the basis of preliminary growth analysis, that ZnO nanoparticles have higher antibacterial effects on microorganism like *S. aureus* than any other metal oxide nanoparticles (Jones *et al*. 2007; Nafchi *et al*. 2013). Also, essential oils from herbs and spices have been known to exhibit antimicrobial properties. Numerous of essential oils have shown antimicrobial activity in vitro against different food-borne pathogens such as *E. coli, C. jejuni, S. enterica,* and *L. monocytogenes* (Friedman *et al*. 2002; Olasupo *et al*. 2003). Extracts of clove and thymol were effective against *S.* Typhimurium and *E. coli* O157:H7 on fresh lettuce and fruits (Kim *et al*. 2011; Gniewosz and Synowiec 2011).

Pullulan is an extracellular polysaccharide produced by the fungal organism, *Aureobasidium pullulans*. (Diab *et al.* 2001; Cheng *et al*. 2011) showed that the resulting films are colorless, tasteless, resistant to oil and exhibit very low oxygen permeabilities and appropriate barrier properties. This microbial polysaccharide has not been largely exploited as a packaging material, except in some applications to prevent rancidity. (Kandemir *et al.* 2005) demonstrated that pullulan film exhibited antimicrobial activity against *E. coli* by introducing partly purified lysozyme to a solution of pullulan. Trinetta *et al.* (2010) demonstrated the effectiveness of sakacin A-containing pullulan film to control the growth of *L. monocytogenes* and the applicability of active pullulan films in delivering a bacteriocin directly to a food surface.

Properties of edible films and coatings generally vary with storage time due to the intrinsic instability of their raw materials. These variations could affect their functionality on foods; therefore, stable properties of films are generally required (Oses *et al*. 2009). When films or coatings are exposed to certain environmental conditions, antimicrobial activity, physical and chemical changes may take place during storage. Chemical changes such as oxidation of sulfhydryl groups were reported to cause the degradation of protein polymeric chains in wheat gluten film (Micard *et al*. 2000). It is also reported that physical changes such as the migration of low molecular weight plasticizers cause lower flexibility in whey protein films (Anker *et al*. 2001).

The objective of this work was to study of physical properties of pullulan films.

Also, the effects of storage temperature on antimicrobial activity of nanoparticles and essential oils-containing pullulan edible films against pathogenic microorganisms were investigated. Moreover, the effectiveness of pullulan films to control the growth of the pathogen in turkey deli meat was evaluated.

MATERIALS AND METHODS

Bacterial strains:

Two bacterial strains used in the present study were *Staphylococcus aureus* (11988 American Type Culture Collection [ATCC]; Manassas, VA, USA), and *Listeria monocytogenes* (ATCC 94229). The strains were maintained on Tryptic Soy Agar (Difco Laboratories, Spark, MD) plates at 37 °C for 24 h and stored at 4°C. Cultures were propagated twice in Tryptic Soy Broth (Difco Laboratories) and incubated for 16 h at 37°C before use in subsequent experiments.

Antimicrobials:

Food grade essential oils (EOs) of oregano (*Origanum minutiflorum)* and rosemary (*Rosmarinus officinalis* L.) were purchased from (Sigma Aldrich Company, USA). Aqueous silver nanoparticles dispersion (Ag NPs) with diameter 100 nm, and zinc oxide nanoparticles dispersion (ZnO NPs) with diameter 110 nm, were purchased from (Sigma Aldrich Company, USA).

Pullulan film formation:

Pullulan (Pul) was supplied by Hayashibara Company (Okayama, Japan). Glycerin (Gly) was obtained from the VWR Company (Batavia IL, USA); xanthan gum (Xa) was purchased from TCI America (Portland OR, USA), and locust bean (Lb) from CP Kelco (Lille Skensved, Denmark).

Pullulan (5% w/v) was dissolved in distilled water with a temperature of 80°C ± 1°C. Glycerol was added (1.5% w/v), xanthan gum and locust bean were added (0.1% w/v) for each and the solution was mixed using a magnetic stirrer. The solution was autoclaved at 121ºC for 15 min. Then, the solution was cooled to room temperature. Rosemary and oregano oil were added in concentration 2% (w/v), as well as Ag NPs and ZnO NPs were added with different size. Earlier, rosemary and oregano oil were dissolved in 2 ml of 95% ethanol. After the components of the film had been mixed, the solutions were poured into plastic Petri dishes. The films were dried overnight in a laminar flow cabinet under a flow of sterile air, at a temperature of 25°C and relative humidity of 45 ± 5%.

Sample collection and preparation:

Ready to eat (RTE) from turkey, was purchased from local market in Copenhagen City, Denmark. Samples were transported to the laboratory within 15 min., packed in separate insulated polystyrene boxes with ice. The meat was cut into 0.7 mm thickness of slices, and cut into sections (5 cm x 5 cm). The prior slices were treated with ultraviolet light (UV) for 15 min. to reduce background microflora (Cutter and Siragusa 1994), and inoculated aseptically with overnight and diluted cultures of *S. aureus* and *L.*

monocytogenes, to obtain approximately 6 $\log_{10}$ CFU/cm^2 on the surface. After inoculation, the samples were kept at room temperature for 20 min. to allow for cell attachment. Inoculated meat surfaces were covered with pullulan films (5 cm x 5 cm) containing either the essential oils or nanoparticles. Inoculated control samples were covered with pullulan film without any compounds. All samples were transferred individually into a standard vacuum-packaging bag (Ultravac Solutions LLC, Kansas City, Mo, USA) and vacuum sealed using a vacuum packaging machine (Ultravac 250 vacuum packaging machine; St. Louis, MO, USA). Vacuum packaged samples were held at 4°C for 2 weeks. Modified Oxford Agar (MOX, Difco) for *L. monocytogenes*, and Baird–Parker agar (BP, Difco) for *S. aureus* were used to determine the number of remaining cells. Resulting colonies were counted after 1-2 days of incubation at 37°C, populations were converted to $\log_{10}$ (Trinetta *et al*. 2010).

Storage conditions of pullulan films:

Once the films had been formed, they were cut in rectangular strips of 25.4mm in width and 75 mm length and were stored at different temperature (4, 25, 37, and 55°C). Films containing nanoparticles and essential oils were stored under the same conditions during 7 weeks in order to study the evolution of antimicrobial activity against *S. aureus* and *L. monocytogenes*. Samples were analyzed at 0, 7, 14, 21, 28, 35, 42 and 49 days of storage.

Antimicrobial activity of pullulan films:

Antimicrobial activity test on films was carried out using the agar diffusion method according to (**Chen *et al.* 1996).** The zone of inhibition assay on solid media was used for determination of the antimicrobial effects of films against *S. aureus* and *L.monocytogenes*. The edible films were cut into 6-mm-diameter disks and then placed on Mueller Hinton agar (Merk, Darmstadt, Germany) plates, which had been previously seeded with 0.2 ml of inoculums containing approximately 1x10^6 CFU/ml. The plates were then incubated at 37°C for 24 h. Then, the plates were examined for “zone of inhibition” of the film discs. The contact area was used to evaluate growth inhibition underneath the film disk in direct contact with target microorganisms in the agar. The area of the whole zone was calculated and then subtracted from the film disk area, and this difference area was reported as a zone of inhibition (Fernandez-Pan *et al*. 2012).

Physical properties of films:

Film thickness:

The thickness of each film sample was measured using a 0–25 mm manual micrometer (Mitutoyo Corporation, Kanagawa, Japan) with an accuracy of 0.001 mm. Measurements were taken at 3 different points on each film sample and the average values were represented as the film thickness.

Transparency test:

The transparency of the film samples was measured according to the method described by Al-Hassan and Norziah (2012). A rectangular piece cut from each film sample was directly placed in a spectrophotometer cell. The

absorbance was measured at 550 nm using digital UV/Vis-and Vis-Unicam Spectrophotometer (model 9423 Automatic Scanning Spectrophotometer). The measurements were carried out in triplicates for each film sample and the average values were calculated. The transparencies of the various films were calculated by dividing the absorbance by thickness (mm).

Film solubility:

The solubility of film samples in water was measured by the method of Ghanbarzadeh *et al*. (2010) with some modifications. Film samples were cut into 3 × 2 cm pieces and dried at 100 °C for 12 h. The dried film samples were dissolved in distilled water (20 ml) under gentle agitation using a magnetic stirrer at room temperature. After the 3 min dissolution, the solution was taken out and dried in the oven at 105 °C until the weight became stable. Before and after dissolution, the accurate weights of the dried film sample pieces were measured, and the weight difference was considered as soluble solids. The water solubility of film was calculated as a percentage based on the initial weight of the film. All the experiments were performed in triplicate.

Oxygen permeability:

Oxygen transmission rates through the pullulan films were determined, using an optical measuring system Optech™ Platinum O_2 sensor device and disposable O_2 sensor stickers from Mocon (Minneapolis, USA). The optical O_2 sensor works on the principle of a phosphorescent dye that is incorporated in a polystyrene polymer membrane as adherent agent and for environmental protection. The Optech device uses LED technology to take measurement in 10 s. For O_2 measurements, the instrument need to be brought in an optical contact with the sensor (5–10 mm distance) to produce an O_2 reading (% of O_2, compensated for temperature and pressure variation). The OxyDot-indicators were placed in the headspace of the samples using transparent adhesive. Windows-based software was used for the recording of measured parameters (oxygen concentration) and data storage (Molinaro *et al.* 2013)

Statistical Analysis:

The statistical analysis was carried out using ANOVA with one factor under significance level of 0.05 for the obtained results using SPSS and data were treated as a complete randomization design according to (Steel *et al.* 1997). The experiments were performed in triplicate, using three samples per treatment (Siragusa *et al.* 1999). Multiple comparisons were carried out applying LSD test.

RESULTS AND DISCUSSION

Appearance of pullulan films:

Pullulan, excellent film-former, which has been used as an edible film with various flavors, herb extracts and spices (Diab *et al.* 2001). Its additional advantages are colorless, tasteless, odorless, transparent, flexible, highly impermeable to oil, heat-sealable, and with good oxygen barrier properties (Gounga *et al.* 2008). Aqueous solutions of pullulan may be used to form transparent, colorless and glossy films with relatively low oxygen permeability (Gounga *et al.* 2008). Pullulan can form homogeneous films, which is an important property for its use as a coating material in the food industry. However, there are very few reports in the literature regarding the application of pullulan edible films (Goksungur *et al.* 2005).

Figure 1 shows images of the surface of pullulan films with and without the addition of essential oils (EOs) and nanoparticles (NPs). The pullulan film produced without any additives was completely transparent, smooth, and glossy. No changes were observed in the films containing oregano 2%, rosemary 2%, ZnO (NPs) 110 nm and Ag (NPs) 100 nm, compared with the control film. The addition of essential oils to the film resulted in intensive odor of oregano and rosemary. These results are in agreement with those reported by **Gniewosz and Synowiec (2011)**

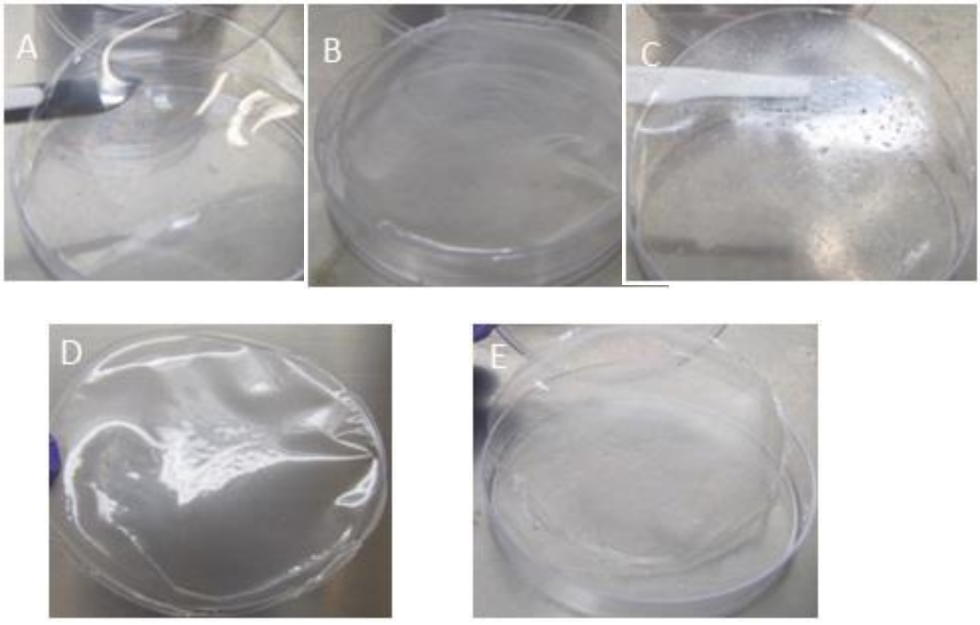

Figure (1) Images of the surfaces of the pullulan films: (A) without the addition; (B) with the addition of 2% oregano; (C) with the addition of 2% rosemary; (D) with Ag (NPs) 100 nm; and (E) with ZnO (NPs) 110 nm.

Physical properties of pullulan films:

Thickness:

Thickness one of the most important parameters, related to transparency, water vapor permeability, and mechanical properties of the films. The thickness of the pure pullulan film, and pullulan incorporated with essential oils and nanoparticles are shown in Table (1). Changes in film thickness were observed with the addition of essential oil into the pullulan film-forming solutions. There are high thicknesses in pullulan-containing essential oil compared with control. However, no significant differences between films incorporated nanoparticles with control. Attributed an increase of thickness in pullulan essential oil to properties of oil with polysaccharides, while the nanoparticles film were decreased due small molecular and matched with polysaccharide structure. These results are generally in agreement with those of Maizura *et al.* (2007).

Transparency:

Transparency is an important index in terms of general appearance and consumer acceptance. The transparency of the various films is summarized in Table (1). Pullulan edible film without essential oil incorporation appeared clear and transparent. Addition of oregano and rosemary essential oil affected the appearance of edible film in both color and transparency. The color tended to yellowish as indicated by the increase of optical density. However, addition of nanoparticles hasn't affected the appearance of edible film, so there were no significant differences. These results are in agreement with those reported by Pranoto (2005) and Pinto (2013).

Water solubility:

Film solubility is an important factor that determines biodegradability of films when used as packaging wrap (Gnanasambandam *et al.* 1997). Table (1) shows that percent water solubility was higher for pure pullulan film (100%). Incorporation of both rosemary and oregano essential oils in pullulan film decreased the solubility ranged from 99.15 to 99.18%, respectively. Also, the pullulan film incorporated Ag and ZnO nanoparticles decreased the solubility ranged from 98.65 to 98.94%, respectively. Attributed of decreased of water solubility to essential oils and nanoparticles scarce of soluble in water. These results are in agreement with those reported by Pranoto (2005) and Rojas-Graü (2006). Although a lower solubility of edible films is required during storage, a high solubility of edible film will be advantageous during cooking food products coated with edible films (Laohakunjit and Noomhorm 2004).

Oxygen permeability:

Data in Table (1) show the percentage of oxygen permeability in pure pullulan film and film's incorporation of essential oils and nanoparticles. Indicated that films containing essential oils and nanoparticles exhibit relatively poor oxygen barrier properties. Oil chemical nature plays a major role in the barrier properties of edible films. Similar results were found by Rojas-Graü *et al.* (2006) who showed lower oxygen permeability was

observed in films that contained oregano, lemon grass and cinnamon oils than in those that contained its antibacterial compounds carvacrol, citral and cinnamaldehyde, respectively**.**

Table (1):Physical properties of pullulan edible films incorporated with essential oils and nanoparticles.

Properties	Antimicrobial pullulan films*				
	Control	Oregano oil 2%	Rosemary oil 2%	Ag (NPs) 100 nm	ZnO (NPs) 110 nm
Thickness (mm)	0.108 ±0.001	0.112 ±0.001	0.111 ±0.001	0.108 ±0.001	0.109 ±0.001
Transparency	0.768 ±0.01	0.857 ±0.02	0.892 ±0.01	0.809 ±0.02	0.789 ±0.02
Water soluble (%)	100 ±0	99.18 ±0.02	99.15 ±0.02	98.65 ±0.27	98.94 ±0.28
Oxygen permeability (%)	21.35 ±0.06	18.3 ±0.16	19.2 ±0.03	19.84 ±0.03	18.86 ±0.34

*** Mean of triplicate determinations ±SE**.

Effect of storage temperature on antimicrobial activity of pullulan films:

The stability of pullulan films incorporated essential oils and nanoparticles at (4, 25, 37 and 55°C) against *S. aureus* and *L. monocytogenes* during seven weeks was investigated. As shown in Fig. (2 and 3), it could be observed that inhibition zone of pullulan film incorporated oregano oil was 32 mm against *S. aureus* in time zero, while after seven weeks of storage were, 30.33, 28, 0 and 0 mm at 4, 25, 37 and 55°C, respectively. The inhibition zone of pullulan incorporated oregano oil in time zero is 29.33 mm against *L. monocytogenes,* while after seven weeks were, 27.83, 25.5, 0 and 0 mm at 4, 25, 37 and 55°C, respectively. Pullulan films incorporated oregano oil did not change during storage at 4 and 25°C, against *S. aureus* and *L. monocytogenes* while, there are changes during storage at 37 and 55°C. The antimicrobial effect of oregano oil is attributed to the relatively high concentration of caracole and p-cymene compounds, which causes depletes the intracellular ATP pool, changes the membrane potential, and increases the permeability of the cytoplasmic membrane to potassium ions and proteins (Ultee *et al*. 2002). Low temperature kept the carvacrol compound from evaporating than high temperature. These obtained results are in general agreement with previously those reported by Seydim and Sarikus (2006); Du *et al*. (2009) and Zhang *et al*. (2009).

Results in Fig. (4 and 5) show that inhibition zone of pullulan film incorporated rosemary oil was 18.67 mm against *S. aureus* in time zero, while after seven weeks were 17.17, 13.5, 0 and 0 mm at 4, 25, 37 and 55°C, respectively. The inhibition zone of pullulan incorporated rosemary oil in time zero is 22.17 mm against *L. monocytogenes,* while after seven weeks were19.83, 17.17, 0 and 0 mm at 4, 25, 37 and 55°C, respectively. Pullulan film incorporated rosemary oil was more effective against *S. aureus* and *L.monocytogenes* when stored at 4 and 25°C. However, the effectiveness during stored at 37 and 55°C. The antimicrobial effect of rosemary oil is due to

cineole and α-pinene compounds, which causes thickening and disruption of the cell wall together with increased roughness and lack of cytoplasm (Nowak *et al*. 2012).

The low temperature maintain of the major effective compounds rosemary oil (cineole and α-pinene) from evaporating when compared to high temperature. These results are in harmony with those previously reported by Abdollahi *et al*. (2012). Gram-positive bacteria are found to be more sensitive to the essential oils effects (Burt 2004). Fig. (6 and 7) illustrate that the effect of storage temperature on antimicrobial activity of pullulan edible film incorporated silver nanoparticles 100 nm against *S. aureus* and *L. monocytogenes*.

The inhibition zone diameters yielded by pullulan based edible film disks with silver nanoparticles 100 nm was 30.33 mm against *S.aureus* in time zero, while after seven weeks of storage were 28.67, 26, 15.5 and 0 mm at 4, 25, 37 and 55°C, respectively. Also, observed that inhibition zone is 22.17 mm against *L. monocytogenes* in time zero, while after seven weeks were19.83, 17, 13.83 and 0 mm at 4, 25, 37 and 55°C, respectively. The pullulan films incorporated silver nanoparticles 100 nm was stable during storage at 4 and 25 °C as antibacterial, against *S. aureus* and *L. monocytogenes*, but, not stabile during storage at 37 and 55°C. The low temperature kept silver nanoparticles than high temperature. These obtained results in general agreement with those previously reported by Fayaz *et al*. (2009).

As shown in Fig. (8 and 9) the inhibition zone diameters for zinc oxide nanoparticles against *S. aureus* in time zero was 18.33 mm, while after seven weeks were 16.83, 13.5, 9.17 and 0 mm at 4, 25, 37 and 55°C, respectively. As same time the inhibition zone of zinc oxide nanoparticles against *L. monocytogenes* in time zero was 17.67 mm, while after seven weeks were 15.67, 12.5, 0 and 0 mm at 4, 25, 37 and 55°C, respectively. The pullulan films incorporated zinc oxide nanoparticles 110 nm more effective during storage at 4 and 25°C, against *S. aureus* and *L. monocytogenes*. However, this films effectiveness during storage at 37 and 55°C. The low temperature kept zinc oxide nanoparticles than high temperature. These obtained results in general agreement with those previously reported with Bajpai *et al*. (2012).

From above mentioned results, it could be concluded that optimum conditions for storage pullulan edible films incorporated essential oils (EOs) and/or nanoparticles (NPs), in order to work as active packaging were 4 and 25°C.

Inhibition of pathogenic microorganisms in turkey deli meat by essential oils and nanoparticles-containing pullulan films:

In the last phase of this study, pullulan films containing oregano 2%, rosemary 2%, Ag (NPs) 100 nm and ZnO (NPs) 110 nm were evaluated for their long-term antimicrobial effectiveness against *L. monocytogens* and *S. aureus* when applied in turkey deli meat, under vacuum packaged, and stored at 4 °C for up to 2 weeks. Data in Table (2) present effect of pullulan films without and with the addition of different types of antimicrobials against *L.*

monocytogens on turkey deli meat. Therefore, observed that progressively grew (1 $\log_{10}$ CFU/g) in samples treated pullulan film (control) but continually during the 2 weeks of refrigerated vacuum packaged storage. Conversely, the samples packaged with essential oils and nanoparticles containing pullulan films, and demonstrated a decrease of approximately 1 log unit in populations of *L. monocytogens* after 24 h. This 1 log reduction observed between control and treated samples remained constant until the end of the challenge study. As shown in Table (3) *S. aureus* was sensitive organism tested in these experiments, population reductions on turkey deli meat surfaces which treated with the essential oils and nanoparticles incroprated in pullulan films were approximately 2-3 $\log_{10}$ CFU/g, as compared with the control.

Table (2):Effect of antimicrobial pullulan films on *Listeria monocytogenes* in turkey deli meat.

Additives of pullulan films	Storage periods (days)				
	Zero	1	2	7	14
Control	6.01±0.04*^{aA}	6.05±0.02^{cA}	6.23±0.14^{cA}	7.69±0.02^{bB}	7.53±0.03^{cB}
Rosemary 2%	6.25±0.32^{aE}	2.93±0.03^{aD}	2.03±0.13^{aB}	2.4±0.07^{aC}	1.06±0.53^{bA}
Oregano 2%	6±0.02^{aD}	3.62±0.22^{bC}	2.56±0.05^{bB}	2.68±0.03^{aB}	1.68±0.21^{bA}
Ag (NPs) 100 nm	5.98±0.02^{aD}	3.03±0.05^{aC}	2.65±0.06^{bB}	2.36±0.07^{aB}	0.43±0.43^{aA}
ZnO (NPs) 110 nm	5.95±0.01^{aE}	2.8±0.03^{aD}	2.23±0.16^{aB}	2.54±0.02^{aC}	0.53±0.53^{aA}

*** Mean of triplicate determinations ±SE.**

Data in the table are log number of bacteria

Mean values in the same column (as a small letter) or row (as a capital letter) with the same letter are not significant different at 0.05 level.

Table (3): Effect of antimicrobial pullulan films on *Staphylococcus aureus* in turkey deli meat.

Additives of pullulan films	Storage periods (days)				
	Zero	1	2	7	14
Control	5.99±0.02*^{aA}	6.18±0.14^{cA}	6.39±0.15^{bA}	6.38±0.29^{cA}	6.76±0.04^{cB}
Rosemary 2%	6.25±0.36^{bD}	2.54±0.05^{bC}	2±0.13^{aB}	2.63±0.02^{bA}	0.43±0.43^{aA}
Oregano 2%	5.98±0.02^{aD}	2.68±0.07^{aC}	2.05±0.15^{aB}	2.68±0.04^{bA}	1.03±0.51^{bA}
Ag (NPs) 100 nm	5.91±0.01^{aD}	1.91±0.08^{aC}	1.58±0.07^{aB}	1.59±0.06^{aB}	0±0^{aA}
ZnO (NPs) 110 nm	5.94±0.01^{aE}	2.07±0.12^{bC}	1.56±0.14^{aB}	2.4±0.05^{bD}	0.49±0.49^{aA}

*** Mean of triplicate determinations ±SE.**
Data in the table are log number of bacteria
Mean values in the same column (as a small letter) or row (as a capital letter) with the same letter are not significant different at 0.05 level.

These results clearly demonstrate that essential oils (EOs) and nanoparticles (NPs) can migrate from the biopolymer into the food and inhibit the pathogen over 2 weeks of refrigerated storage. The use of packaging films containing antimicrobials appears to be a promising delivery method of the antimicrobial compound, even if the release of the active agent is limited to the surface of the foods (Quintavalle and Vicini 2002). Oussalah *et al*. (2006) stated that alginate and milk protein films containing 1.0% oregano oil were effective against *E. coli* O157:H7 *S.Typhimurium, L. monocytogens and S. aureus* inoculated on beef. Rhim *et al*. (2006) found that chitosan-based nanocomposite films incorporates sliver nanoparticles were effective against foodborne pathogens. Moreover, a pullulan packaging system requires less amount of antimicrobial, exhibits longer antimicrobial activity and may permit controlled migration of the molecule from film to the food matrix. This approach not only allows for initial inhibition of undesirable microorganisms, but also allows for release and residual activity over time, especially during transportation, storage and distribution. It is important to note that direct addition of antimicrobials could result in some loss of activity, because of cross-reactions with food components, such as lipids or proteins (Quintavalle and Vicini 2002**).**

These experiments clearly demonstrated the antimicrobial activity of the pullulan film containing Ag (NPs), ZnO (NPs), oregano and rosemary essential oils, its effect against pathogen microorganisms associated with raw and processed meats.

CONCLUSION

The results of the present study concluded that Ag (NPs), ZnO (NPs), rosemary and oregano containing pullulan films were inactivated pathogen microorganisms especially *L. monocytogens* and *S. aureus* that contribute to meat spoilage. The antimicrobial activity of Ag NPs and oregano essential oil in pullulan edible films against both pathogens was significantly greater than the activities of ZnO NPs and rosemary oil, respectively. There was no effect on the physical properties of the edible film formed as observed. The optimum condition for storage pullulan edible films incorporated (NPs) and/or (EOs), in order to work as active packaging was 4 and 25°C. Antibacterial pullulan

edible film incorporating NPs and EOs provides a novel way to enhance the safety and shelf-life in food systems. Therefore, are promising and have good potential in many food applications.

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ثبات النشاط المضاد الميكروبى لأغلفة البوليولان المحتويه على جزيئات النانو والزيوت العطرية وتأثيرها على جودة لحم الرومى المعد للأستهلاك

حسن حسن خلف، أشرف مهدى شروبه، حسن حسن الطناحى، محمد خيرى عبد الحافظ مرسى

قسم علوم الأغذية- كلية الزراعة- جامعة بنها- مصر [١]

تعتبر الأغلفة القابله للأكل المحتويه على مضادات بكتيريه، ذات أهمية بارزة فى إطالة صلاحية المنتجات الغذائية وتقليل مخاطرالبكتريا الممرضه والملوثه لأسطح الأغذية. تضمن هذا البحث دراسة وتقييم النشاط المضاد للبكتريا، والثبات التخزينى، لأغلفة البوليولان المحتوية على الزيوت العطرية وجزيئات النانو. النشاط المضاد للبكتريا لأغلفة البوليولان المحتوية على زيت أوريجانو (٢%)، زيت روزمارى (٢%)، جزيئات الفضة النانويه (١٠٠ نانو متر)، جزيئات أوكسيد الزنك النانو (١١٠ نانو متر) ضد بكتريا ***Staphylococcus aureus*** **and** ***Listeria monocytogenes*** تم تقييمه أثناء التجهيز والتخزين على درجات حراره مختلفه (٤، ٢٥، ٣٧، ٥٥ م°) لمدة ٤٩ يوم (فى المختبر). كما تضمنت الدراسة تأثير الاغلفة الحيوية على إطالة فترة صلاحية منتج الرومى المخزن بالتبريد على ٤ م°. أظهرت النتائج المتحصل عليها أن أغلفة البوليولان المتضمنه زيت أوريجانو (٢%) وجزيئات الفضة النانويه (١٠٠ نانو متر) أكثر فاعليه وتأثيراً بصوره معنويه ضد كلا الميكروبين مقارنة بزيت روزمارى (٢%) و جزيئات أوكسيد الزنك النانو (١١٠ نانو متر) على التوالى. بينت الدراسه أيضاً أن الظروف المثلى للمحافظة على ثبات الأغلفه المحتويه على مضادات بكتيريه بصوره نشطة هو (٤ و ٢٥ م°). كما أشارت النتائج أيضاً على مدى كفاءة الأغلفة الحيوية فى إطالة العمر التخزينى لمنتج الرومى وتقليل الحمل الميكروبى. وبناء على النتائج المتحصل عليها نوصى بإستخدم وتطبيق الأغلفه المحتويه الزيوت العطرية وجزيئات النانو فى مختلف الأغذية.

قام بتحكيم البحث

أ.د / أحمد عبد العزيز الرفاعى	**كلية الزراعة – جامعة المنصورة**
أ.د / حمدى عبد اللطيف المنسى	**كلية الزراعة – جامعة بنها**

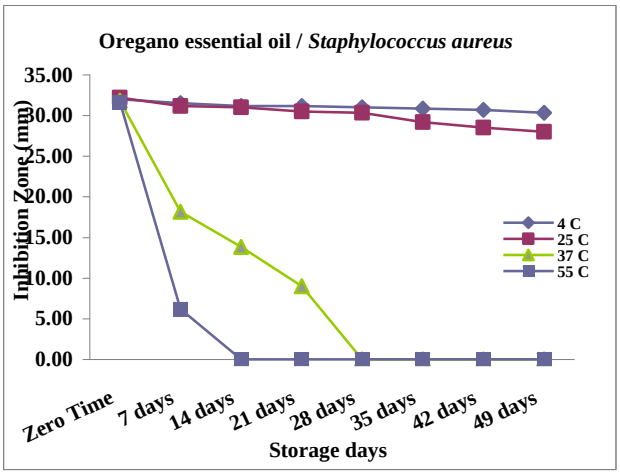

Figure (2): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated oregano essential oil against *Staphylococcus aureus.*

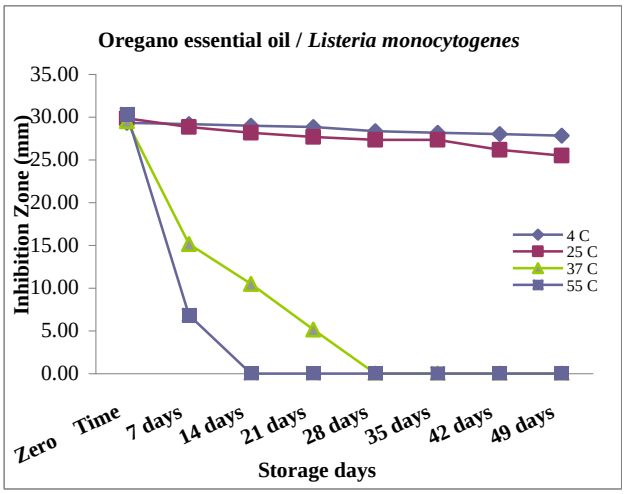

Figure (3): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated oregano essential oil against *Listeria monocytogenes.*

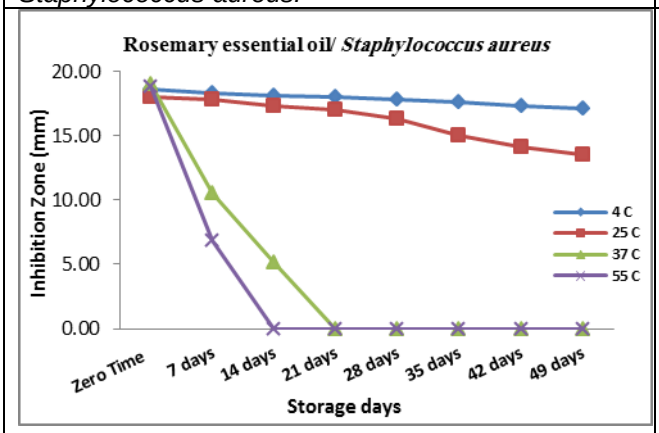

Figure (4): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated rosemary essential oil against *Staphylococcus aureus.*

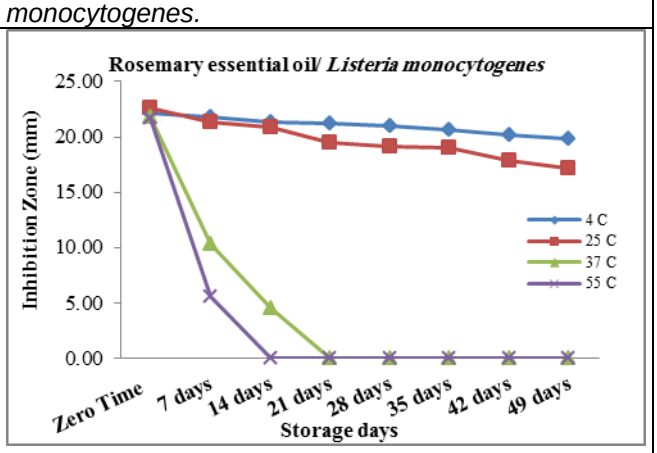

Figure (5): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated rosemary essential oil against *Listeria monocytogenes*.

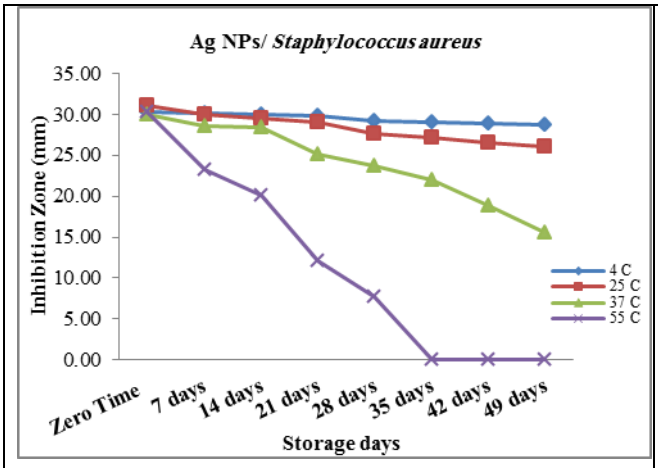

Figure (6): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated silver nanoparticles 100 nm against *Staphylococcus aureus.*

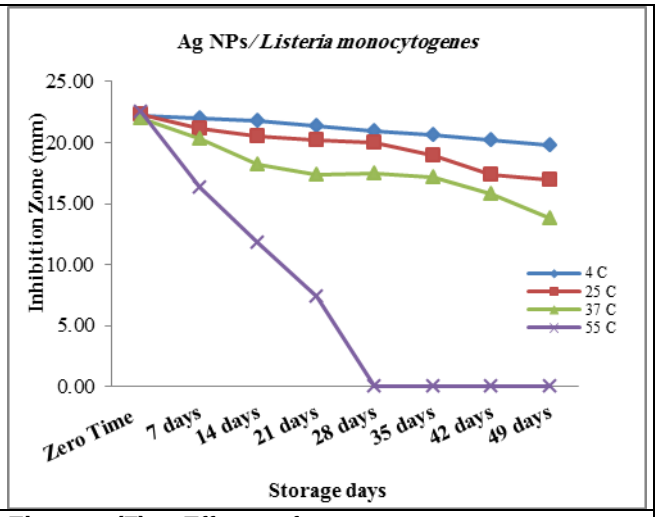

Figure (7): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated silver nanoparticles 100 nm against *Listeria monocytogenes.*

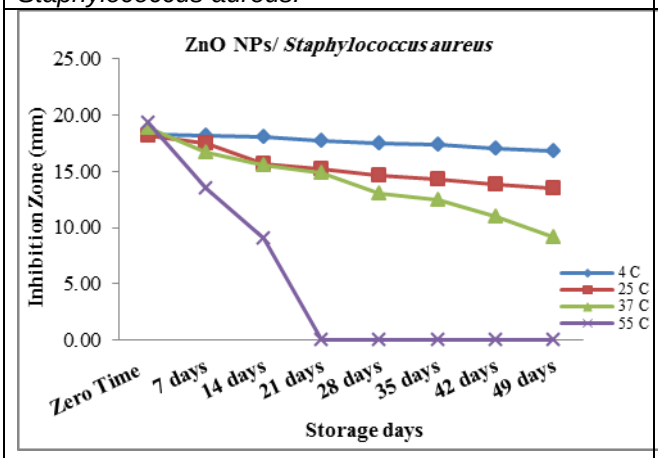

Figure (8): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated zinc oxide nanoparticles 110 nm against *Staphylococcus aureus.*

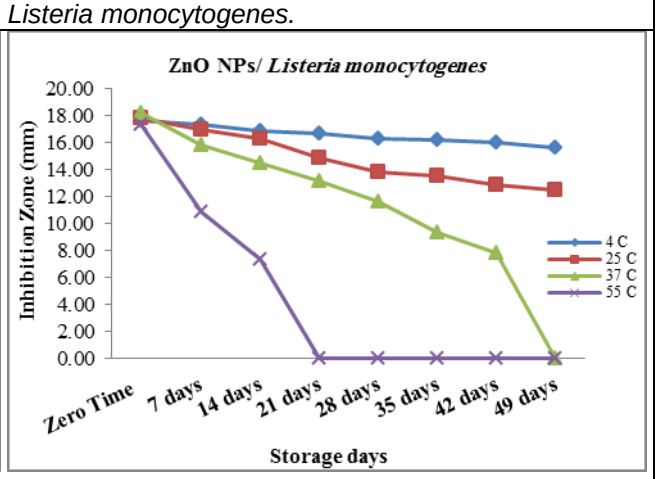

Figure (9): Effect of storage temperature on antimicrobial activity of pullulan edible film incorporated zinc oxide nanoparticles 110 nm against *Listeria monocytogenes*.