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Effect of Chitosan-Nanoparticles on the shelf life of chilled chicken meat and decontamination of *Staphylococcus aureus* and *Salmonella typhimurium*

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Abstract

The antibacterial effect of chitosan nanoparticle (CsNp) with different concentrations (0.5, 1% and 2%) which were prepared according to ionic gelatin method on the shelf life and the sensory parameters (odor, color, texture, taste and overall acceptance) of chicken meat were done during chilling storage. Also two experiments were done to investigate the effect of chitosan nanoparticles as antibacterial agent on chicken meat contaminated with some food poisoning microorganisms (*Staphylococcus aureus* and *Salmonella typhmurium*) during chilling storage. Microbial analysis showed that coating with different concentrations of chitosan nanoparticle (CsNp) (0.5, 1 % and 2%) has a significant reducing effect on growth of bacteria during 12 days at 4ºC and extending the shelf life of chicken meat for 4-6 days more than uncoated samples. All contaminated chicken meat samples treatmented with different concentrations were completely free from *Staphylococcus aureus and Salmonella typhmurium* at a zero-time and throughout the chilling storage. The present study cleared that the coating of chicken meat with chitosan nanoparticles (CsNp) may be a promising technology for the control of undesirable microbial and sensorial changes in poultry industry.

Keywords: *Chitosan Nanoparticles, Salmonella Typhimurium, Staph. aureus, Antimicrobial activities and chicken meat*

Introduction

Poultry meat is an ideal medium for bacterial growth because of its high moisture content, richness in nitrogenous compounds (essential amino acids), good source of minerals, vitamins and other growth factors. Furthermore, its pH is favorable for the growth of microorganisms. The water activity (a_w) of poultry meat ranged from 0.98 to 0.99 depending on how long the meat has been stored in dry air. The pH of chicken breast muscle is 5.7 to 5.9, while that of leg muscle is 6.4 to 6.7. Both poultry muscle and skin are excellent substrates for supporting the growth of a wide variety of microorganisms **(ICMSF, 2005).**

Handling, processing and storage are some of the factors that affecting the microbial status of chilled ready to eat foods (**Akbar and Anal 2014).** Therefore, poultry meat is often found contaminated with potentially pathogenic microorganisms such as *Salmonella*, *Campylobacter*, *Staph. aureus, E. coli* and *Listeria*. Microbial contamination and lipid oxidation are the principal problems causing potential public health issues and deterioration in nutritional, texture, and sensory quality of meat and meat products. Additionally, consumers prefer to use food commodities which are free from harmful pathogenic microorganism and synthetic preservatives with minimal processing **(Jayasena and Jo**, **2013).**

Hence, global food industry is under rising pressure to meet consumers demand for safe, healthy and fresh food, along with a challenge

to meet updated strict food safety regulations and focuses on finding modern methods and technologies to increase the shelf life of fillet. Conversely, consumers' demand for healthy meals free from chemical preservatives, have been increased in respect to the past **(Giatrakou and Savvaidis, 2012).**

Recently, a wide type of polysaccharide- based nanoparticles has been used for encapsulation of natural preservative with biological functions. One of them is chitosan (CS). Chitosan is cationic carbohydrate biopolymers obtained by chitin deacetylation from the exoskeleton shrimps (**Tømmeraas *et al*., 2002**). There are many preparation methods for CsNp include emulsion, precipitation, reverse micelles, ionic gelation, molecular self-assembly and template polymerization (**Shi *et al*., 2011**). Chitosan nanoparticles have many biomedical applications as deliver drugs, proteins, DNA, and antigens; as antibacterial and antifungal acting against bacteria and fungi to increase shelf life by preserving the food and maintaining food quality (**Papadimitriou *et al*., 2008 and Aider, 2009**).

Chitosan has unique features including biocompatibility, biodegradability, and low toxicity **(Jang and Lee, 2008).** Also, it has antimicrobial activity and ability to form protective films (**Cuero, 1999 and Jeon *et al*., 2002),** texturizing (**Benjakul *et al.,* 2003),** binding action **(No *et al.,* 2000)** and its antioxidant property **(Kamil *et al.,* 2002),** chitosan nanoparticles (CsNp) has been widely considered as a versatile polymer in development of micro and nano-encapsulation system as a wall material.

The main goal of the present study was to prepare and characterize of new size chitosan nanoparticles, followed by investigate the preservative effect of different concentrations of chitosan nanoparticles solutions on the shelf-life of chicken meat and its antimicrobial effect on *Staph.aureus* and *Salmonella typhimurium* during chilling storage.

Materials and Methods

Chitosan (CS) in powder form with 93% degree of deacetylation (DDA), was obtained from oxford Lab. Chem. Food grade sodium tripolyphosphate (99.5% purity) was obtained from El-Gomhoria for chemicals Co., Egypt. Glacial acetic acid was purchased from Sigma Aldrich (0.5% and 1%).

Preparation of Chitosan Nanoparticles:

Nanoparticles were produced based on ionic gelation of sodium tripolyphosphate (TPP) and chitosan (**Calvo *et al*., 1997**). Nanoparticles were spontaneously obtained upon the addition of 0.5%, 1% and 2% chitosan acidic solutions (0.5% and 1% acetic acid) respectively, to solutions of TPP aqueous basic solution (0.7mg / ml); the ratio of TTP to chitosan was 1:3 under magnetic stirring at room temperature for 1hr. Therefore we have six concentrations to evaluate.

Characterization of CsNp: was done through Fourier transmittance Infrared FT/IR-6100 Spectrometer, High-resolution transmission electron microscopy (**HRTEM**) imaging JEM 2100F transmission electron microscope with accelerating voltage 200 kV. and Zetasizer-Nano ZS instrument (Malvern Instruments, Worcestershire, UK).

Microbiological analysis

Part (1): Studying the effect of different concentration of **chitosan nanoparticles on shelf -life of chicken meat samples**

Preparation of coated chicken meat samples for Shelf-life study according to **Ojagh *et al*. (2010).** Chicken fillets without skin was obtained from local manufacture shops and transported aseptically under refrigeration to the laboratory without delay. Upon arrival, chicken fillets were divided into 4 groups. The first was the untreated control group. From the second to the fourth groups each group were dipped separately for five minutes into solutions of different concentration of chitosan nanoparticles 0.5%, 1% and 2% in a ratio of 1:2 (w/v) fillets to chitosan nanoparticles solutions. All treated groups and control were stored at re-

frigerator shelf at 4°C and investigated for sensory attributes, and bacteriological load at zero, 3rd, 6th, 9th, and 12th day to study the effect of treatments on the shelf life.

Bacterial procedures:

According to **Tajik *et al.* (2015);** the control and coated chicken meat samples were examined for the determination of the aerobic plate count (APC) using plate count agar (PCA) incubated at 35 °C for 48 h. All counts were expressed as log10 CFU/g and performed in duplicate.

Sensory evaluation:

The sensory parameters of the control and treated chicken meat samples different concentration of chitosan nanoparticles 0.5%, 1% and 2% was evaluated at 0, 3, 6, 9 and 12 days of refrigeration storage by a six trained sensory panelist. Panelists were selected from qualified analysts of Food Hygiene Department) at Animal Health Research Institute (AHRI). Chicken samples were used to evaluate color, odor, and overall acceptability attributes of raw sample, as well as taste of cooked samples in a small frying pan for 10 min. The panelists scored the sensory attributes by using 5-point descriptive scale and had to fill in a questionnaire in which 1 was the worst (unacceptable) and 5 was the best (**Pesavento *et al.,* 2015**). The samples that presented mean scores lower than 3 were considered unacceptable. From the 6th day of experiments, only raw samples with TBC lower than 10^5 CFU/g were used for the taste sensory evaluation of cooked chicken meat.

Part (2):

Experiments for studying the effect of chitosan nanoparticles on contaminated chicken meat samples with some food poisoning strains:

Preparation of inoculated cultures:

Staph. aureus NCTC 7447/ ATCC® 6538P and *S. Typhimurium* NCTC 12023/ATCC® 14028 (were obtained from Becton Dickinson, France) . The strains were activated at Food Hygiene Department -Animal Health Research Institute- Dokki, Giza, Egypt according to **Edward (2012)**: as follow two successive passes in 9 ml of Tryptic Soy Broth (TSB) (Oxoid) and incubated at 37°C for 18 h. For each individual strain, 1 ml of the stock inoculums was added to 100 ml of TSB and incubated with shaking at 37°C for 18 - 24 h to reach a final concentration of approximately 10^8 CFU/ml (determined by plating serial dilutions on Baird Parker agar and XLD, Oxoid). One ml of these inoculums was added to 99 ml of sterile saline to give final concentration of approximately 10^6 CFU/ml, to be used in the dipping solution.

Determination of the initial count of inoculated chicken meat samples:

Two groups (four samples of skinless chicken fillet each), the 1st group was dipped in the inoculated *Staph. aureus* solution for 1 min and 2nd group was dipped in the inoculated *S. Typhimurium* solution for 1 min; then left to dry in laminar air flow for 20 min and packaged into polyethylene bags. Twenty-five grams of the inoculated fillet was stomached with 225 ml of peptone water and serially diluted to be counted for determination of the initial count of the inoculated microorganisms on selective media for each strain (BP, for *Staph. aureus and* XLD, for *S. Typhimurium*) in duplicate before treatment.

Determination the effect chitosan nanoparticles on contaminated chicken meat samples with *Staph. aureus* and *Salmonella typhimurium* strains:

After that the two previously inoculated groups (four samples of skinless chicken fillet each), were divided into the control sample without coating and the other three samples of inoculated group were dipped in the over mentioned chitosan nanoparticles solutions (0.5%, 1% and 2%) for 5 minutes, then left to dry in the laminar flow for 15 minutes and stored in the refrigerator at 4°C. Each group was periodically examined for the count of the inoculated strains (as in determination of initial count) at zero, 3rd, 6th, 9th, and 12th day of chill-

ing storage.

Statistical analysis

using Analysis of Variance (ANOVA) with further separation of significantly different means using Duncan's Multiple Range test using **SPSS (2012)**.Significant differences were reported at ($P<0.05$).

Results and Discussion

Particle size, morphology and size Distribution: The method of preparation and effect of various factors on size and shape of nanoparticles. The size and morphology of the nanoparticles are mainly determined by HRTEM which CsNp size had 26.98 nm 0.5% acetic acid while, 1% concentration had 28.82nm with a narrow size distribution (polydispersity index: 0.903,0.341, respectively) which indicated that greater homogeneity can be realized (**Fig 1a,b**). The chitosan nanoparticles are nanosphere shape with no aggregation. **Abdou *et al*., (2012)** found that chitosan / TPP (with the same ratio 1:1) has average particle size of 10 nm due to complexation of positively charged chitosan with negatively charged (TPP). **Ardila *et al*. (2017)** found that CsNp nanospheres show uniform spherical shape which displayed higher antibacterial activity than CS solution with particles size 178 nm, in average. Chitosan-TPP nanoparticles size was 175 ± 9 nm and their surface charge was +25 ± 4 mV explained by **Jahromi *et al*. (2014).** The antimicrobial activity of nanoparticles increased with decreasing particle size that reported by **Zhang *et al.* (2007)**.

Zeta potential: The zeta potential is an indicator to stable and unstable suspensions is generally taken at either +30 or −30 mV. Particles normally stable when zeta potentials more positive than +30 mV or more negative than −30 mV, (**Perera and Rajapakse, 2014).** The zeta potential results for the present study; CsNp had a +30.1 mV of concentration 0.5% acetic acid while, 1% concentration had+ 46.1 mV measured at pH 5 (**Fig 2**). Whilst **Ardila *et al*., 2017** found that CsNp nanospheres, zeta potential +53.3 (higher stability) and +14.8 mV when measured at pH 5.8 and 8.0 Therefore, as the pH increases, charged amino groups promoting the stability of CsNp nanosphere to decrease. Similar zeta potential values with values between +51kV and +35 to +55 kV were prepared CsNp by ionic gelation (**Qi *et al.*, 2004** and **Yien *et al.*, 2012**), respectively.

Chemical interaction: (Fig.3): Fourier Transform Infrared spectroscopy analysis is distinctive molecular fingerprint and detection of functional groups in pure compounds and mixtures and for compound comparison. (FT-IR) spectra of CsNp explain the interaction between chitosan chains molecular and TPP which compared with TPP and chitosan. A broad peak between 3350 and 3270 $cm^{-1}$was attributed to a combination of stretching modes of O H and N H bonds in chitosan matrix. In the sample of chitosan nanoparticles, this band becomes wider and shifts to lower wave numbers, indicating an enhancement of the hydrogen bond interactions **(Yu *et al*., 1999 and Dudhani and Kosaraju, 2010).** In addition, the 1523cm^{-1}peak of the NH_2 bending vibration of chitosan samples shifted to 1533 cm^{-}in the NPs. A similar result has been observed in literature on chitosan-TPP NPs **(Xu and Du, 2003)**.

FTIR spectra of chitosan and chitosan-TPP nanoparticles have three characterization peaks (1,080 $^{cm-1}$ of ν (C O C), 3.432 $cm^{-1}$of ν (OH), and 1.647 cm^{-1} of ν (NH) existed in the spectrum of purified chitosan. In comparison with chitosan, a different spectrum was observed for chitosan-TPP nanoparticles. In chitosan-TPP nanoparticles, a new sharp peak appeared at 1,632 cm^{-1}, and the 1,647cm^{-1} peak of – NH 2 bending vibration shifted to 1,519 cm^{-1}. It can be supposed that the phosphoric groups of TPP were linked with ammonium groups of chitosan in nanoparticles which had been reported by **Wu *et al*.** (**2005**).

Microbiological analysis

Part (1) studying the shelf life of chicken meat samples:

The skin of poultry carcasses and cuts is directly in-contact with air and equipment surfaces, therefore it is easily contaminated. In fresh meat, bacteria are present on the surface rather than in the meat (**Luber *et al.,* 2009).** However, in processed products such as those which have been marinated, bacteria can migrate into the muscles (**Warsow *et al.,* 2008)**. Even new rubber fingers can host bacteria and be a source of contamination for carcasses (**Arnold, *2*009)**. Cross contamination between carcasses or cuts may occur by direct contact or through contact with contaminated surfaces (**Veluz *et al.,* 2012**), or during the subsequent processing steps (deboning, cutting, mincing, and mixing) **(Álvarez-Astorga *et al*., 2002)**. Therefore, the development of alternative food grade potent antibiofilm agents is urgently required by the food industry for the prevention of biofilms.

The present results of bacteriological counts of chicken meat samples during 12 days of refrigerated storage were shown in Fig (4). The bacteria determined on PCA medium were dominant from the beginning and during the storage of chicken meat samples at refrigerated temperature. The averages of initial aerobic plate counts (APC) in all samples were in the range of 4.02–4.42 Log_{10} CFU/g, indicative of acceptable chicken total bacterial counts. APC increased with time until exceeded the value of 5 log CFU/g_{10} on sixth day for the control samples which considered exceeded the permissible APC limit for chicken meat as defined by (**EOS, 2005).** While the samples treated with chitosan nanoparticles (0.5 %, 1%, and 2%) showed pronounced reduction of bacterial counts by (1.5, 2 and 2.5 Log_{10} CFU/g, respectively) as compared with control samples at the end of storage time. It is clear that chitosan nanoparticles had a great influence on the reduction of APC, extending the microbiological shelf life of chicken meat by 4-5 days. The obtained results were in agreement with the results published by **Darmadji and Izumimto (1994**) who described the effectiveness of chitosan on storage stability of minced beef. Solutions of chitosan at 0.5-1.0% were able to inhibit the growth of spoilage bacteria on red meat after 10 days of storage at 4°C. These results were in accordance with those obtained by **Jeon, *et al.* (2002)** who found that the log of psychotropic bacterial count in fish coated by chitosan in day 12 was less than 6, while this value was obtained in day 6 for uncoated samples. The most realistic hypothesis (mechanism of the antimicrobial activity) is that chitosan nanoparticles is able to change cell permeability due to interactions between the positive charges of its molecules and the negative charges of the bacterial cell membranes **(No *et al.,* 2007 and Friedman and Juneja, 2010).** Generally, chicken samples coated with different concentration of chitosan nanoparticles solutions had lower TBC than those coated with chitosan solutions during chilling storage. This may be due to chitosan nanoparticles had higher antimicrobial effect than chitosan alone.

Sensory evaluation:

Sensory evaluation was done during chilling storage period showed a clear distinguishing variaton between the control and treated samples with chitosan nanoparticles. The summary of overall acceptance are stated in (Fig.5) showed that control chicken samples, preserved without any treatment, reached the lowest value, assumed unacceptable color ,odor and over all acceptance at the day six of storage, that undesirable parameters were mainly related to microbial spoilage. While treated samples with chitosan nanoparticles showed a significantly reduction of the microbial growth which improved the shelf life and sensory parameters as it began to decline from the tenth day of refrigeration storage. **Kanatt *et al.* (2008**) indicated that chitosan coating had no undesirable influence on meat products**. Fan *et al.* (2009)** also suggested that chitosan coating

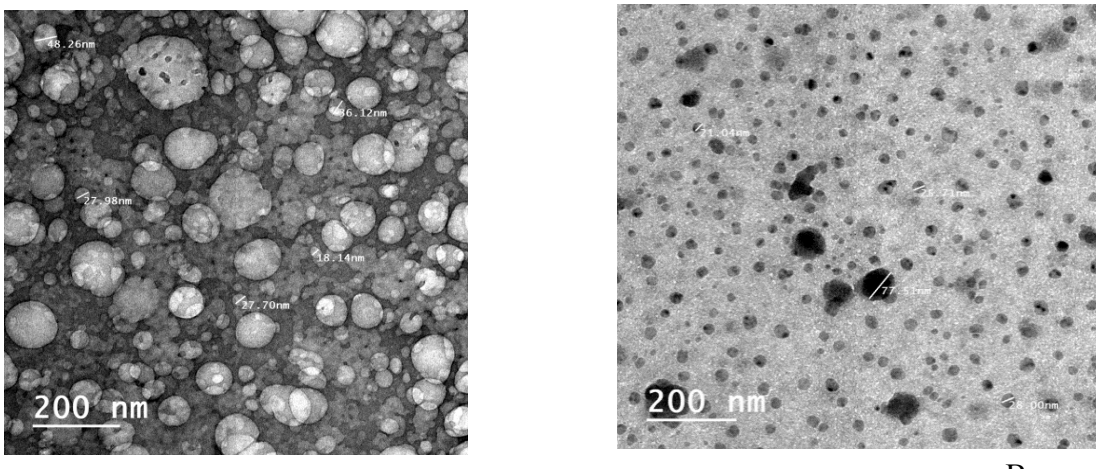
has positive effects and prolong the shelf life of fish meat.

*Sensory evaluation of the treated chicken samples (0.5%, 1% and2% CsNp) with 1% Glacial acetic acid showed extreme undesirable effect on overall sensory parameters (unacceptable color, odor and overall).

Part (2):

Decontamination effect of chitosan nanoparticles on *Staph. aureus* and *S. typhimurium*

In the present study, dipping *Staph. aureus* contaminated chicken meat samples for 30s in a solution containing 0.5% ,1%, 2% chitosan nanoparticles was able to significantly reduce the recovery of *Staph. aureus* cfu/g at a zero-time in all trials, as well as the same effect for dipping *Salmonella typhmurium* contaminated chicken meat samples. All chicken meat samples treatment with different concentrations were completely free from *Staph. aureus* and *Salmonella typhmurium* at a zero-time and throughout the chilling storage. Nowadays, the presence of spoilage bacteria in food products is an important economic problem. Therefore, an inexpensive and safe treatment to prevent spoilage is needed. Chitosan nanoparticles have been shown to be an effective antimicrobial, especially antibacterial. From the beginning with 0.5% chitosan nanoparticles was effective in reducing Gram negative bacteria (spoilage bacteria) to undetectable levels that which confirmed by many studies **(Helander *et al.*, 2001; Yi *et al.*, 2003 and Xue *et al.*, 2006).** The antibacterial activity of chitosan under acidic environment may be result from its polyatomic structure due to the prolongation of $-NH_2$ on the C-2 position of the D-glucosamine repeat unit. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, either by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells. Chitosan also inhibits the microbial growth by the creation of essential metals and nutrients, spore components, as well as the penetration of the nuclei of the microorganisms, which leads to the interference with protein synthesis by binding with DNA. Furthermore, chitosan coatings act as an oxygen barrier and thus inhibit the growth of aerobic bacteria. **(Shahidi *et al,* 1999 and Devlieghere *et al.,* 2004)** Generally, chicken meat coated with different concentrations of chitosan nanoparticles coatings had lower bacterial load.



A B

Figure (1). HRTEM of chitosan nanoparticles CsNp (A) 0.5% acetic acid (B)1% acetic acid, A and B showed nanosphere shape, no aggregation and size between 26.98 -28.8 nm.(Central lab. in NRC)

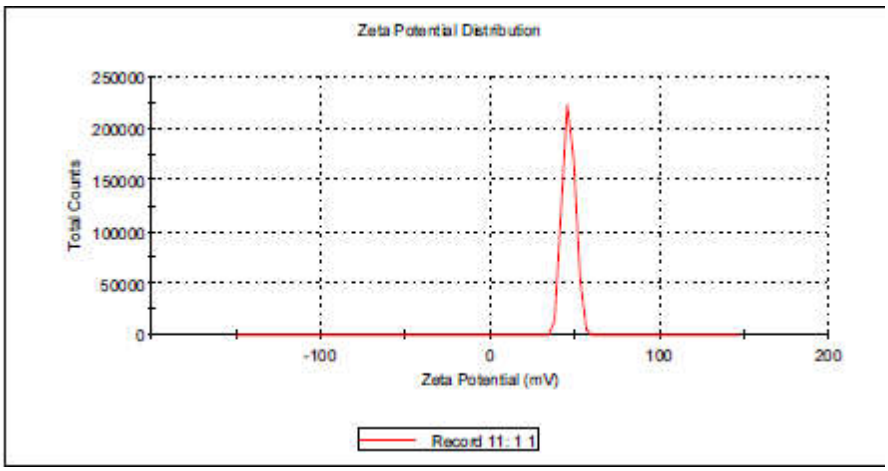
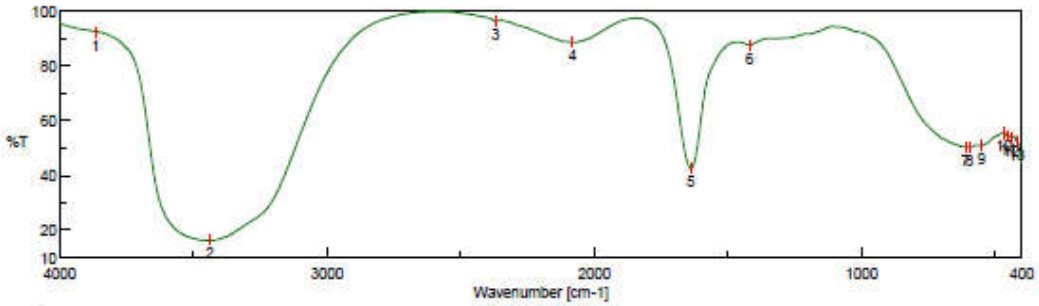


Figure (2). Zeta potential and Particle size distribution of chitosan nanoparticles CsNp showed +30.1mV indicator to stable nanoparticle. (Central lab. in NRC)



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3864.65	92.3414	2	3436.53	16.1948	3	2368.16	96.2271	4	2080.62	88.5831
5	1638.23	42.4803	6	1418.39	87.527	7	612.288	50.3957	8	594.932	50.353
9	551.542	50.7519	10	467.653	55.3515	11	451.261	54.0993	12	439.69	53.7551
13	419.442	52.3311									

Figure (3). FTIR of chitosan nanoparticles CsNp. (Central lab. in NRC)

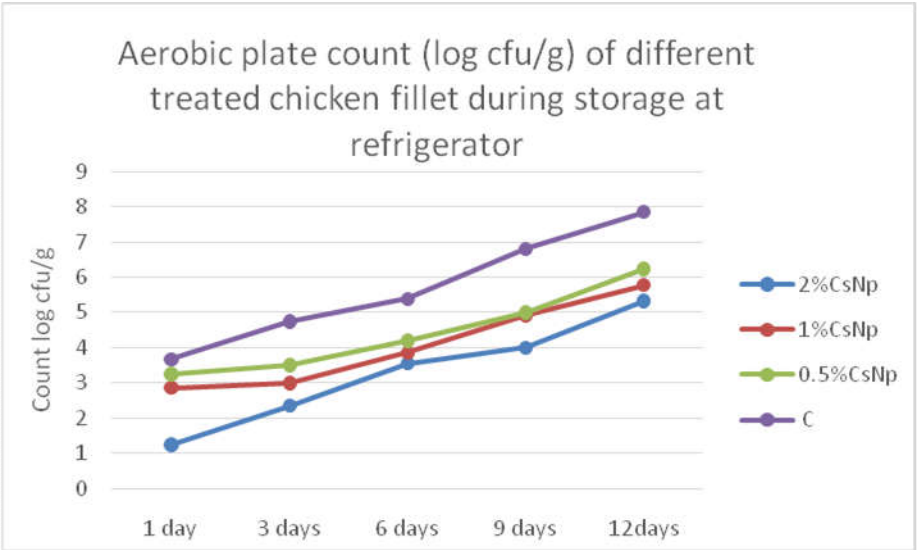


Figure (4). Aerobic plate count (log cfu/g) of different treated chicken Samples with (CsNp) 0.5% glacial acetic acid during storage at refrigerator

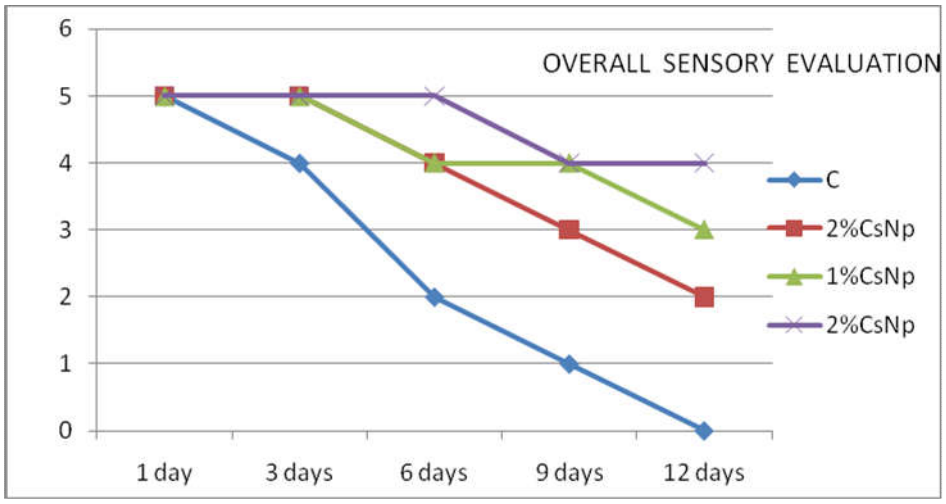


Figure (5). Overall sensory scores of different treated chicken samples during chilling storage.
*CsNp solution in 1% Glacial acetic acid did not evaluated due to the extreme undesirable effect on sensory parameters from zero day

Conclusion

Chitosan nanoparticle (CsNp) solution in 0.5% Glacial acetic acid exhibited several distinct advantages during chilling storage of chicken meat .It improved its shelf life and minimized the initial bacterial load and could maintain or even increase its antimicrobial activity until the end of chilling storage. Also there was a significant antimicrobial activity of 0.5%, 1%, and 2% chitosan nanoparticles for eradicating food-borne pathogen such as *Staph. aureus* and *Salmonella typhimurium* with acceptable sensory quality of chicken meat, while using (CsNp) solution in 1% Glacial acetic acid showed an extreme undesirable effect on sensory parameters from zero day.
On the basis of the obtained results food industries would benefit from the use of nanotechnology in order to improve the microbial safety and sensory quality of poultry.

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