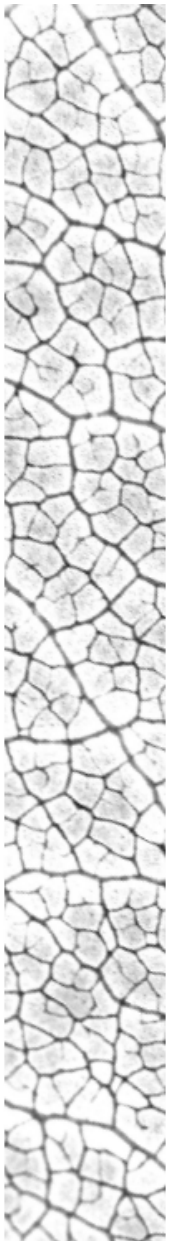

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Assay of chitosan complex as edible film through inhibited abilities against some microbes caused food poisoning

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Abstract
This study was aimed to prepare the thinly edible film complex of Chitosan (1.5%) - sorbic acid (1%) to determine of inhibitory efficacy against the test-causing microbes and the effect of their use in the preservation of soft cheese and chicken breast samples at 4 ºC. The results showed that the minimum inhibitory concentration of the chitosan complex was between 1.0 to 1.5 mg /5 ml of the dietary medium and that the addition of sorbic acid supporting substances increased the sensitivity of the test bacteria to each of the complex at 0.75 to 1.0 mg respectively. The inhibitory viability of the packaging complex against *E.coli, P.flourescens*, *Staph.aureus*, *B.cerus* and *A.parasiticus*, which cause food poisoning, increased with the concentration of 0.5, 1.0 and 2.0 mg for the diameter of the inhibition zone at 11-14 and 19-23 and between 24-26 mm respectively. The conservation of packaging was complicated at 4° C in maintaining the weight of the soft cheese samples encapsulated for the 15th day and the chicken meat samples at the 12th compared to six days in control group samples. The results also indicated that the packaging of cheese samples by the chitosan complex was significantly ($p<0.05$) increased in the number of contaminated microbial species with increased storage time but was significantly less than the increase in these samples of the non-coated cheese samples. The total microbial counts were in storage days 3, 9, 12 and 15 at 0.1, 0.1, 1, 1, 2, and 2 (log cfu/ g) respectively, compared with the total numbers of microbes in the unpaired samples of 0.1, 4, 7, 9, 10, 12 (Log cfu/ g) respectively. It was similar to the types of bacteria, lactic acid, colorectal and total fungi, as well as the use of the chitosan complex. The total number of contaminated microorganisms for chicken meat samples was not significantly different.

Keywords: chitosan complex, food poisoning, edible film

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INTRODUCTION

The degradable mechanism of food and its state of spoilage is contrary to the effectiveness of the preservation mechanisms that is used to prolong the food safety as long as possible without change in its qualities. So, the food preservation mechanisms have variety of difficulties as their routes include mechanisms against the principal of nature (Benbettaïeb *et al*. 2016). The most important techniques used to prolong the duration of food preservation are the use of packaging along with other techniques of cooling, freezing and others. The recent studies have shown that supporting of packaging with other conservation processes has advantage in prolonging the preservation period of many foods, thus providing food in non-productive seasons with high efficiency (Bedel *et al.* 2018, Plackett 2011). Chitosan is a cationic complex, and one of the most effective antioxidants, characterized by its high correlation with some metal ions, especially that stimulate oxidation processes (Tharanathan and Kittur 2003). The ability of chitosan to react with the mineral elements increased with the process of the acetylation. These properties are similar to the chitosan that is extracted from the fungi (Leceta *et al*. 2013). The effectiveness of chitosan is associated with the presence of hydroxyl and amino groups. Its antimicrobial activity is linked to its positively charged amino group which interacts with negatively charged microbial cell membrane promoting an increase in their permeability and causing disruptions that lead to cell death (Ziani *et al*. 2009). The watery membrane of the chitosan is characterized by its transparency and flexibility, as well as its good oxygen storage capacity. It can also improve its CO_2 permeability by conducting a methylation process on the resulted complex (Golmohammadi *et al*. 2014, Zhong and Xia 2008). It has been observed that

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the formed film was very stable. And also, the processes of permeability and transition did not deteriorate through it during storage period (Cardoso *et al*. 2016, Hosseini *et al*. 2015b). Several studies have indicated the antimicrobial activity of chitosan as an edible packaging material against Gram positive and negative bacteria as well as yeast and mold species that easily cause food spoilage (Liu *et al*. 2012), owing to its good film-forming properties (Zhao 2016). Chitosan offers real potential in the food industry applications. It has been classified as generally recognized as safe (GRAS) as recommended by the Food and Drug Administration (FDA, 2012). The aim of this study is to evaluate the effect of chitosan edible film or coating on the microorganisms that caused food poisoning in soft cheese and chicken breast samples at 4 ºC for 15 days.

MATERIALS AND METHODS

Samples Collection

The samples of chicken breast and soft cheese were collected from local markets in Erbil. Each sample was selected from fresh and single meal. They were cut and prepared in equal sizes with 80 g. Each one were keeped in a clean, sterile container and storage in the refrigerator until it is packaged. Sterilization conditions were used in preparation, packaging and storage (Trainer *et al*. 2010).

Preparation of Chitosan Coating Solution

Chitosan solution was prepared by dispersing 1.5 gm/gm of chitosan in 1% Sorbic acid to obtain a concentration of 1.5%. The solution was kept under the magnetic stirrer at 50 °C ± 2 ° C for 2 hours until complete dissolution of the chitosan (Leceta *et al*. 2013).

Preparation of Chitosan-based Edible Films

All the samples of chicken breast and soft cheese were packaged as indicated by (Poverenov *et al*. 2014, Seddigh *et al*. 2018) with some modification made by immersing each of the sample in the packaging materials solutions at 50 °C to ensure homogeneous distribution on the surface of the samples, as well as not to be destroyed by the effect of heat. For comparison, control samples were untreated by any type of packaging material. Film thickness of the packaging material was measured in 10 different points of the samples (Song *et al*. 2014). After drying and fixing the films, the samples were kept in sterile cork containers at 4 °C for testing and examination.

Experimental Assay

Tested were performed on the coated and uncoated chicken meat and cheese samples at 0, 3, 6, 9, 12 and 15 days. Each sample was subjected to testing at the rate of three replicates.

Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was applied to the Chitosan solutions as mentioned in CLSI, (2011) with some modulations as follows: serial dilution of chitosan solutions were prepared ranging from 1-10 ml. malt extract agar and muller hinton agar were prepared in the Erlenmeyer flask and sterilized by autoclaving at 121˚C for 15 min under 15 lbs pressure and cooled to 45 ˚C. The different concentrations of chitosan were added to the prepared media and poured into sterile Petri dishes and kept at a 4 °C until use. Overnight of bacterial suspension was prepared and compared with the McFarland standard solution. The suspension of *A. parasiticus* was also prepared and the numbers of the conidia were calculated using the Haemacytometer. The prepared plates were inoculated with (5) microliters of microbial suspension, and the dishes were left for a certain period until they dried before turning them over. The dishes were incubated at 37 ˚C for 24 hours for bacterial species and at 37 ° C for 72 hours for the isolation of fungi. The value of MIC was determined as a minimum concentration of antimicrobial agent, which prevents a visible growth in the culture media of bacterial or fungal isolates.

Determination the Antibacterial Activity of Chitosan Complex

The antimicrobial activity of chitosan was evaluated against the bacterial isolates obtained from (Department of Food Engineering –College of Chemistry and Minerals Engineering- Technical Istanbul University-Turkey). These isolates includes four isolates causing food poisoning: *Escherichia coli, Psedomonas flourescenes* as a model of Gram negative and *Staphylococcus aureus*, *Bacillus cereus* as a model of Gram positive (NCCLS, 2000). Muller hinton agar plates were inoculated with bacterial suspension by spreading 100 µl of revived culture on mueller hinton agar media with the help of spreader. Three wells were made using gel puncture (4mm) then 0.1ml of different concentration of packaging material (0.5, 1.0, 2.0 mg/ml). The plates were incubated at 37 ºC for 48 hours for bacterial species except in the case of *Pseudo*. *flourescens* which incubated at 30 °C for the same duration. The zone of inhibition was measured (mm) to determine the antibacterial activity of chitosan.

Determination the Antifungal Activity of Chitosan Complex

The experiment was performed after the preparation of chitosan complex and dissolve in distilled water to obtain a stock solution at a concentration of 1000 mg/ ml (Ziani *et al*. 2009). Then a specific volumes of chitosan complex was mixed with the sabouraud dextrose agar (SDA) after cooling to 50 °C to obtain the concentrations (50-100-250) mg/ ml at a rate of two replicates of each concentration. The treated SDA plates were inoculated

Table 1. Minimum inhibitory concentration of 1.5% chitosan against tested microbial types

Tested microbial types	Concentration of 1.5% chitosan at mg/5ml of dietary medium							
	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
E.coli	-	-	-	-	+	+	++	++
Ps.flourescens	-	-	-	+	+	++	++	+++
Staph.aureus	-	-	-	+	+	++	++	+++
B.cereus	-	-	-	+	++	+++	+++	++++
A.parasiticus	-	-	-	-	-	+	+	++

(-) no sensitivity, (+) weak sensitivity, (++) moderate sensitivity, (+++) high sensitivity, (++++) perfect sensitivity

Table 2. Minimum inhibitory concentration of 1.5% chitosan and 1% sorbic acid against tested microbial types

Tested microbial types	Concentration of 1.5% chitosan with 1% sorbic acid at mg/5ml of dietary medium							
	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
E.coli	-	-	+	++	+++	+++	++++	++++
Ps.flourescens	-	-	-	+	++	+++	++++	++++
Staph.aureus	-	+	+	++	+++	+++	++++	++++
B.cereus	-	-	+	++	+++	+++	++++	++++
A.parasiticus	-	-	-	-	+	++	+++	+++

(-) no sensitivity, (+) weak sensitivity, (++) moderate sensitivity, (+++) high sensitivity, (++++) perfect sensitivity

with the single-spore taken from a growing colony at the age of 7 days of fungi. Untreated plate of SDA was used as a negative control to compare the rate of growth with the treated plates. All plates were cultured with the same fungal. The dishes were incubated at a temperature of 28-30°C for 3-5 days. The diameter of the growing colony (the rate of perpendicular diameters) was measured and the rate of inhibition was calculated using the following equation:

Inhibition ratio = Rate of fungus diameter in control dishes- Rate of fungus diameter in treated dishes/ Rate of fungus diameter in control dishes

Estimation of the Total Numbers of Microbes

25 gm of each sample was mixed with 225 ml of 1% peptone in sterile conditions and homogenized for a minute. The required dilution was completed to the fifth dilution. To calculate the microbial numbers, 0.1 ml of the last dilution was spread on the surface of the solid medium. The total numbers of microbes were calculated using Plate Count Agar (PCA) at 35 °C for 24 - 48 hours (Zhang, and Peng, 2016). The numbers of *Pseudomonas spp*. in the meat samples was estimated after aerobically growing on cetrimide fusidin cephaloridine agar medium (CFC) at 30 °C for 48 hours (Siriken 2004). While calculation the total number of coliform bacteria were performed on MacConkey agar and Eosin methyl blue agar media at 35 °C for 24 -48 hours. Also, the total numbers of Lactic acid bacteria in the cheese samples were calculated on the MRS Agar medium at 35 °C for 48 hours (Begovic *et al*. 2011). Yeasts and molds were calculated using pour plate method after growing on the Potato Dextrose Agar medium at 30 °C for 24-48 hours (Çetin *et al*. 2010). The glass slides of growing fungal species were prepared using lactophenol blue and examined under light microscopy to confirm them, (Samson *et al*. 2000). Microbial species were calculated on the basis of logarithm of colony formation units (cfu/ g). The growing bacterial colonies were morphologically diagnosed using Gram stain and determined the shape and arrangement of the cells. (Winn *et al*. 2006).

STATISTICAL ANALYSIS

The experiment was performed under Complete Randomized Design (CRD), and the variance analysis was performed using the General Linear Model using SAS (2001). In the case of significant differences, the Duncan test (1955) was used to determine the significance of the differences between the different averages at a probability level of 0.05.

RESULTS AND DISCUSSION

Determination of the Minimum Inhibitory Concentration of Chitosan Complexes

The minimum inhibitory concentration (MIC) of the chitosan complex was determined against the microbes caused food poisoning (**Table 1**). The MIC against *E.coli* and *Pseudo*. *flourescens* were 1.25, 1.0 ml / 5 ml respectively. *S*. *aureus* and *B*. *cereus* were 1.0 ml / 5 ml of the nutriant medium. While the MIC of chitosan against *A. parasiticus* was 1.5 ml/ 5 ml of Nutriant medium. The enhancement of the chitosan package via the supplementation 1% of sorbic acid resulted in increased the sensitivity of *E.coli* at 0.75 ml/ 5 ml of the nutrient medium whereas the inhibitory effect against *Pseudo. flourescens* did not change. As showed in (**Table 2**) the MIC against Gram positive bacteria 0.5, 0.75 ml/ 5ml of the medium respectively. In case of the fungal strain the MIC was 1.25 ml/ 5 ml of the medium.

The effect of the chitosan complex with sorbic acid has increased the sensitivity of bacterial species, as shown in **Table 2**. There are no similar studies on the inhibitory action of both chitosan and sorbic acid. The recent studies are limited to the effect of chitosan or sorbic acid alone against some types of bacteria. The inhibitory effect of chitosan against microorganisms is due to the positive charge in the amine group (NH3+), which is effective in correlating with the cell wall components of the microorganisms that carry the negative charge, causing the cell wall and cellular membranes to be rupture (Helander *et al*. 2001).

The increasing effect of sorbic acid within the chitosan complex is due to the effect of acidity, which

Table 3. Antimicrobial activity of packages materials against some microbial types

Type of packages materials	Con. of packages materials (mg/ ml)	Zone of inhibition of packages materials activity against Tested microbial types				
		E.coli	*P.flourescens*	*S.aureus*	*B.cereus*	*A.parasiticus*
1	0.5	0.02±14d	0.03±12c	0.03±13d	0.02±14c	0.02±14c
	1.0	0.05±23c	0.06±21b	0.02±22c	0.09±23b	0.09±22b
	2.0	0.01±25b	0.01±23b	0.07±26b	0.06±24b	0.06±23b
2	1.0	0.09±28a	0.05±26a	0.08±31a	0.04±31a	-
3	1.0	-	-	-	-	0.04±29a

1- 1.5% packaging chitosan with 1% sorbic acid 2- Ciprofloxacin 3- Grisofolvin

a-d: Different letters in one column indicate significant differences between the averages at a probability level of 0.05. Rates are calculated for three replicates

Table 4. Rate of Microbial numbers in cheese pieces after packing and storage at 4 °C for 15 days

Types of packages complexes	Total numbers of microorganisms	The rate of spoilage microorganisms (cfu/ g) for cheese samples after storage at 4 ° C for 15 days					
		Storage days					
		0	3	6	9	12	15
1.5% chitosan- 1% sorbic acid	Total microbes	0.1d	0.1d	1c	2b	2b	3a
	LAB	0.1b	0.1b	0.1b	0.5b	0.8a	1a
	Coliform bacteria	0.1d	0.1d	1c	1c	2b	3a
	Fungi	0.1c	0.1c	1b	1b	2a	2a
Control groups	Total microbes	0.1f	4e	7d	9c	10b	12a
	LAB	0.1f	1e	2d	3c	4b	6a
	Coliform bacteria	0.1f	2e	4d	6c	8b	10a
	Fungi	0.1f	2e	3d	5c	6b	7a

a-f: Different letters in one rows indicate significant differences between the averages at a probability level of 0.05. Rates are calculated for three replicates

causes the change of environmental conditions, that leads to the inhibition of bacterial species because of their inappropriate growth of these species. Organic acids can be used as acidulates in edible films made from carbohydrate, proteins, and chitosan. Also they are used to enhance the antimicrobial activity of lipophilic acids. This acidity may denaturate the proteins that makeing up the cell wall. As well as its inhibitory effect on cellular membrane activity by altering the membrane charges and loss the functional effectiveness (Cagri *et al*. 2001).

The Inhibitory Effect of the Chitosan Film

Table 3 shows the effect of 1.5% chitosan and 1% sorbic acid film. The concentration of 2.0 mg/ ml has significantly increased the inhibitory effect to be 25, 23, 26, 24 and 23 mm respectively. The antimicrobial activity of the complex against the microbes under study is highly efficient compared to the antibacterial and antifungal antibiotic. The effectiveness of the chitosan complex is due to the combined efficiency of the chitosan and sorbic acids. The efficiency of the chitosan comes from the possession of positively charged ammonium (NH4 +) in the amino glucose units that interfering with the negatively charged compounds in the cell wall causes the breakdown the outer membrane of micro-organism and lead to an excessive leakage of essential elements, and cause bacterial death. The acidic effect of sorbic acid synergistic with chitosan, which lead to alter the pH level of the cytoplasm to the acidity and then inhibit the activity of the organelles, as well as altering the pathway of energy transfer in the cell, also causing the denaturation of proteins in cell wall and cellular membrane (Hazan *et al*. 2004, Hosseini *et al*. 2016).

Determination the Numbers and Types of Contaminated Microbes to Cheese and Chicken Meat Samples

Table 4 shows the total number of contaminated microbes of cheese samples in the case of chitosan/ sorbic acid film. The total number of microbes were 0.1, 0.1, 1, 2, 2 and 3 (log cfu/ g) and the total number of Lactic acid bacteria (LAB) were 0.1, 0.1 , 0.1, 0.5, 0.8 and 1,the coliform bacteria at 0.1, 0.1, 1, 1, 2 and 3 (log cfu/ g) and the total number of fungus were 0.1, 0.1, 1, 1, 2 and 2 (log cfu/ g) Respectively compared to the control group in the non-coated cheese samples mentioned above.

The use of packaging film of chitosan-sorbic acid significantly reduced the total numbers of microbes in chicken meat as shown in **Table 5** during the storage period 0.1, 1, 1, 1, 2 and 2 (log cfu/ g), *Pseudomonas spp* at 0.1, 0.1, 0.3, 1, 1 and 1 (log cfu / g), coliform bacteria were 0.1, 0.2, 0.3, 1, 2 and 2 (log cfu/ g). The total number of fungi were 0.1, 0.3, 0.4, 1, 1 and 1 (log cfu/ g) compared to the control samples of chicken meat mentioned above. These results agreed with (Butler *et al*. 1996, Leceta *et al*. 2013) who confirmed the inhibitory activity of the coated films of chitosan. The results showed that the coated films that included chitosan and the supported active substances added to it were significantly effective in controlling the growth of the microbes under study in the cheese samples during the storage period at 4ºC.

All samples were suitable for consumption at the end of the preservation period compared to the samples that reached to spoilage status at six days of cooling preservation. The efficiency of the coated films are due to multiple mechanisms, such as the ability of the coated film to prevent the arrival of microbes to samples of cheese or meat because the holes and pores between their molecules are not suitable for the arrival of microbial species to the samples. As well as discouraging the growth of microbes through consumption or use of some components. In addition to

Table 5. Rate of Microbial numbers in chicken meat after packing and storage at 4 °C for 15 days

Types of packages complexes	Total numbers of microorganisms	The rate of spoilage microorganisms (cfu/ g) for chicken breast samples after storage at 4 ° C for 15 days					
		Storage days					
		0	3	6	9	12	15
1.5% chitosan- 1% sorbic acid	Total microbes	0.1c	1b	1b	1b	2a	2a
	Pseudomonas spp.	0.1b	0.1b	0.3b	1a	1a	1a
	Coliform bacteria	0.1c	0.2c	0.3c	1b	2a	2a
	Fungi	0.1b	0.3b	0.4b	1a	1a	1a
Control groups	Total microbes	0.1f	4e	7d	10c	12b	15a
	Pseudomonas spp.	0.1f	2e	3d	5c	6b	8a
	Coliform bacteria	0.1f	1e	3d	5c	7b	9a
	Fungi	0.1f	1e	2d	4c	5b	6a

a-f: Different letters in one rows indicate significant differences between the averages at a probability level of 0.05. Rates are calculated for three replicates

its ability to maintain the internal conditions of preserved samples, especially the factors that encourage the growth of microbes found in food samples, such as oxygen or moisture, resulting in inhibiting microbial growth or reducing the corruption of coated food samples (Clarke *et al.* 2016, Ye *et al*. 2008).

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