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EVALUATION OF THE EFFECT OF CHITOSAN COATING ON MICROBIOLOGICAL AND OXIDATIVE PROPERTIES OF REFRIGERATED BEEF

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ABSTRACT
Beef is an animal food sensitive to deterioration with short shelf-life due to its rich nutrient content. Many studies concerned with the use of edible coating are carried out to increase the shelf life of beef. In this study, it was aimed to extend the shelf life of beef using chitosan. For this purpose, the bovine meat was coated with chitosan and stored at +4 ºC for 7 days. Total mesophilic aerobic bacteria, *Stapylococcus aureus*, *Pseudomonas* spp. counts and thiobarbituric acid values were analyzed. As a result, it was found that the chitosan coating reduced the TMAB, *Pseudomonas* spp. counts and TBARS values ($P < 0.05$) and inhibited all *S. aureus* up to day 5 of storage. According to the data obtained from this study, it has been concluded that chitosan can be used as a bio-preservative in the meat industry due to the antimicrobial and antioxidantive properties.
Keywords: Beef, chitosan, coating, *Staphylococcus aureus, Pseudomonas* spp., Thiobarbituric acid (TBARS).

KİTOSAN KAPLAMANIN SOĞUTULARAK SAKLANAN ETLERİN MİKROBİYOLOJİK VE OKSİDATİF ÖZELLİKLERİNE ETKİSİNİN BELİRLENMESİ

ÖZ
Sığır eti, zengin besin içeriğinden dolayı bozulmaya duyarlı ve kısa raf ömrüne sahip bir gıdadır. Bu nedenle, sığır etinin raf ömrünün artırılması için birçok çalışma yapılmaktadır. Bu çalışmaların ise önemli bir kısmı yenilebilir film kaplamalar üzerine yoğunlaşmıştır. Bu çalışmada, kitosan film kullanılarak sığır etinin raf ömrünün uzatılması amaçlanmıştır. Bu amaçla, çalışmada kullanılan sığır eti kitosanla kaplanmış ve + 4 ºC'de buzdolabında saklanmıştır. Toplam mezofilik aerobik bakteri sayımı (TMAB), *Stapylococcus aureus*, *Pseudomonas* spp. ve tiyobarbitürik asit (TBARS) sayısı analizleri yapılmıştır. Sonuç olarak, kitosan kaplamanın TMAB, *Pseudomonas* spp. ve TBARS sayısını düşürdüğü tespit edilmiştir ($p < 0.05$). Ayrıca, kitosan depolamanın 5. gününde tüm *S. aureus* 'u inhibe etmiştir. Nitekim bu çalışmadan elde edilen verilere göre kitosanın antimikrobiyal ve antioksidan özelliklerinden dolayı et endüstrisinde biyo-koruyucu olarak kullanılabileceği sonucuna varılmıştır.
Anahtar kelimeler: Kırmızı et, kitosan, kaplama, *Staphylococcus aureus, Pseudomonas spp,* Thiobarbituric acid (TBARS).

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INTRODUCTION

The dietary problems stemming from animal protein deficiency is of great importance to humans considering the rapidly growing human population. Therefore, as a food of animal origin, red meat species have an important role in human nutrition (Mehta *et al.,* 2015). Because of their properties (a suitable medium for microorganisms, suitable pH, high water activity, unsaturated fatty acid content, etc.), red meat types should be rapidly consumed and processed, which, if not, result in being confronted with the chemical, microbiological, and sensory spoilage of the products. Although spoilage affects the sensory properties such as color changes and putrefaction, chemical and microbiological spoilages are more of an issue than the deterioration of the sensory properties (Devatkal *et al.,* 2014). When meat samples are stored at temperatures above 20ºC, growth of mesophilic bacteria gives rise to spoilage, and, at lower temperatures, the dominant microflora comprise of mostly Gram-negative psychotropic bacteria species of different genus, such as *Pseudomonas*, *Moraxella*, and *Acinetobacter*. While rapidly growing on meat pieces as a result of their short generation periods, the *Pseudomonas* species firstly utilize glucose and, then amino acids, which results in the formation of malodorous compounds such as methyl sulfide, esters, and acids. Among Gram-positive bacteria, *Staphylococcus aureus* usually contaminates red meat through secondary contamination. Although growth of bacteria and bacterial toxin production usually do not cause deterioration in food quality, staphylococci can cause food poisoning when the toxins produced manage to reach human intestines (Addis and Sisay, 2015).

The lipid oxidation in meat is among the causes of quality degradation (Sharma *et al.,* 2017). Hence, the formation of peroxide, aldehydes, hydrogen sulfide, and ammonia is among the most important chemical changes. Undesirable toxic compounds are formed due to the lipid oxidation in red meats, which could also affect proteins through the complex chain reaction mechanism. Thereby, lipid oxidation leads to decrement in protein solubility and discoloration as well as losses in the nutritional value of meat (Estevez *et al.,* 2006; Ünal *et al.,* 2014). Along with their lipid content, muscle foods also contain approximately 1% phospholipids. Phospholipids are the first substrates of lipid oxidation and have a 100-fold greater surface area compared to triglycerides. Moreover, the unsaturation degree of phospholipids is much higher than that of fatty acids. The most susceptible compounds to the lipid oxidation in meat are the triglycerides that contain high amounts of phospholipids, free fatty acids, and unsaturated fatty acids, respectively (Decker *et al.,* 2005).

As it is the case in all food products, shelf-life is one of the most leading factors that demarcates the limits of quality and acceptability in meat and meat products. Red meats have a limited shelf life due to a multitude of reasons, which raises challenges in the transportation and storage (Olivera *et al.,* 2013). Today, certain systems including irradiation, microwave, ohmic and inductive heating, ultrasound, high-pressure, magnetic field, pulsed electric field, and ultraviolet light are used in meat preservation (Wang *et al.,* 2018). In recent years, edible coating materials are also used in food preservation (Aydin *et al*., 2017). Thin-layered non-synthetic packaging materials that are formed on the surface of food products and consumed together with the food. Thin-layered non-synthetic packaging materials that are formed on the surface of food products and consumed together with the food, obtained from natural resources, and controls moisture, gas, and solid movements. Edible films or coatings, apply to the outer surface of foods or between the food layers to protect foods against external factors and increase their shelf-lives. (Işık *et al.,* 2013). Thanks to their low costs, requirement simple technology and efficient preservation, edible coatings are gaining popularity in the food industry (Beikzadeh *et al.,* 2020; Duran and Kahve, 2016).

Chitosan is one of the most important edible films used worldwide, which is produced by deacetylation of chitin (Abdou *et al.,* 2008; Kuzgun and İnanlı, 2013). As a compound obtained using various methods, the most prominent features of chitosan are attributable to

its antimicrobial and antioxidant properties. Considering these properties, the studies on the use of chitosan as an edible film to increase the shelf-life of foods were studied by several researchers (Singh *et al.,* 2015; Kaya *et al.*, 2016; Hasheminejad and Khodaiyan, 2020). Chitosan is a polysaccharide-based film, applied to the outer surface of foods and is effective in the control of the physiological, morphological, and physiochemical changes in foods (Duran and Kahve, 2016). Chitosan films can control oxygen and moisture permeability and have antioxidant and antimicrobial effects on the food they are applied to (Roller and Covill, 1999; Coma *et al.,* 2002). The most highly regarded hypothesis on the antimicrobial effect of chitosan associates its antimicrobial activity with its polycationic properties. Indeed, through its interaction with the negatively-charged substances, it is effective against molds, yeasts, and bacteria. The dissociation constant (pKa) value of glucosamine residues is 6.3. Thus, below pH 6.3, the positive charge of the NH_3^+ group on the C-2 position of the glucose monomer of chitosan electrostatically interacts with the negatively-charged microbial cell membranes. This disrupts the integrity of the microbial membranes and therefore results in the separation of the cellular components from the cytoplasm and the ensuing leakage of these components from the cell wall (Liu *et al.,* 2004).

In this study, chitosan coating was applied to beef and stored in the refrigerator at 4 ± 1 °C. The aim of this study was to investigate the microbiological and oxidative changes as well as increasing shelf life of the beefs.

MATERIALS AND METHODS

Preparation of the meat samples and chitosan solution

In this study, the meat prepared from the loin (longissimus lumborum) of the bovine was used and collected from local market. Composition of meats were determined as follows; 4.01 g fat and 18.97 g protein per 100 g. The pH value measured on the first day was 6.3. Beefs were divided into two groups as coated with chitosan and uncoated. Six samples were prepared for each group, which included 100 g of cubes of meat (2x2 cm). The storage period was selected to be 7 days at 4 ºC and microbiological and chemical analyses were carried out on days 0, 3, 5, and 7.

Chitosan (moderate molecular weight and can dissolve in organic acids) was purchased from Sigma Aldrich (448877, USA). Chitosan solution was prepared with 2% acetic acid and 10 gr chitosan dissolved slowly in 500 mL acetic acid then stirred for two days in a magnetic stirrer (Velp Scientifica, F20520165, EU); hence, the concentration of the chitosan was adjusted to 2% (Cruz-Romero *et al.,* 2013).

The beef samples were immersed in the chitosan solution for 1 minute so that all surfaces are contacted with the solution. To allow the solution to dry on the meat surface, the samples were kept 1 h in a cabinet with cold air flow (Alveo KT, ASE.01, Turkey).

Microbiological analyses

For the microbiological analyses, 10 g of meat sample was homogenized with 90 ml of Maximum Recovery Diluent (Merck, 112535) in a stomacher (MAYO, HG-400, Australia). Appropriate dilutions were plated to get total mesophilic aerobic bacteria (TMAB) counts as previously described by Duran and Kahve (2020) in detail. Baird Parker Agar (BPA, Sigma Aldrich) was used for *Staphylococcus aureus*. The selected colonies were subjected to a coagulase assay and verified. In the *Staphylococcus aureus* analysis, the incubation temperature was 37 ± 2 °C for 48 h. After incubation, the black colonies with a clear zone were identified to be *Staphylococcus aureus* (ISO 6888–1, 1999). Additionally, *Pseudomonas* spp. count (ISO 13720, 2010) were determined using Pseudomonas Selective Agar Base (CFC agar, Merck) which was incubated at 30ºC ± 2 °C for 72 h. The blue-violet colonies surrounded by red-violet-colored zones were counted. The bacterial counts were given in cfu/g.

Thiobarbituric acid reactive substances (TBARS) analyses

Ten grams of beef samples were transferred to a stomacher (MAYO, HG-400, Australia) bag to be homogenized for 10 minutes. Then, a 4 N HCl

solution was added for the distillation process and 5 mL of TBA was added after the distillation. The distillates were placed in a boiling water bath and kept for 35 minutes and after than transferred to spectrophotometer tubes to measure the optical density. The density value obtained was multiplied by 7.80 and the results were given as mg of malonaldehyde in a 1000 gram sample (Tarladgis *et al.,* 1960).

Statistical analyses

All the experiments were triplicated, and SPSS Statistics Software (version 22, SPSS Inc., Chicago, USA) was performed for statistical analysis of variance (ANOVA). To compare two treatments, *t*-test was used, and ANOVA was used to evaluate the effect of storage. Differences among mean values were considered significant when $P < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

The results obtained from the study obtained is given in Table 1. The changes both within the groups and against time were investigated.

Table 1. The microbial and chemical changes in the beef during storage (4 ±1 ºC)

	0. Day	3. Day	5. Day	7. Day
	Total mesophilic aerobic bacteria (log cfu/g)			
Normal meat	4.51 ± 0.08^{d1}	5.44 ± 0.05^{c1}	5.79 ± 0.17^{b1}	6.02 ± 0.08^{a1}
Chitosan coated Meat	4.51 ± 0.08^{b1}	4.71 ± 0.06^{a2}	4.52 ± 0.16^{b2}	4.78 ± 0.18^{a2}
	Stapylococcus aureus (log cfu/g)			
Normal meat	1.00 ± 1.09^{b1}	1.46 ± 1.14^{b1}	2.05 ± 1.030^{a1}	2.62 ± 0.21^{a1}
Chitosan coated meat	1.00 ± 1.09^{a1}	0.46 ± 0.81^{b2}	ND	ND
	Pseudomanas spp. (log cfu/g)			
Normal meat	2.01 ± 0.01^{d1}	3.52 ± 0.22^{c1}	4.76 ± 0.15^{b1}	6.11 ± 0.09^{a1}
Chitosan coated meat	2.01 ± 0.01^{d1}	2.56 ± 0.07^{c2}	3.11 ± 0.26^{b2}	3.67 ± 0.14^{a2}
	TBARS value (mg/1000g)			
Normal meat	0.22 ± 0.01^{d1}	0.71 ± 0.01^{c1}	0.87 ± 0.06^{b1}	1.12 ± 0.12^{a1}
Chitosan coated meat	0.22 ± 0.01^{c1}	0.44 ± 0.02^{b2}	0.40 ± 0.05^{b2}	0.63 ± 0.06^{a2}

ND not detected.

Each value represents the mean of 6 samples ± standard deviation.

Bearing different superscripts row wise (alphabet) and column wise (numeral) differ significantly ($P < 0.05$).

The results revealed that the TMAB count in normal meat continuously increased during seven days of storage while, in the chitosan-coated group, the value at the 5th day of storage was close to the value at the first day. The increase in the TMAB count of the chitosan-coated meat was not high despite the considerable increase in the TMAB count of the non-chitosan-coated beefs ($P < 0.05$). The difference between the TMAB counts at the day zero and at the fifth day of storage was not statistically significant. The difference between the chitosan-coated samples and non-chitosan-coated samples reached a statistically significant level on the third day of the storage.

The *Staphylococcus aureus* counts in non-coated meat samples continuously increased during seven days of storage. The increases in the microorganism count both at the day zero and at

the seventh day of storage were statistically significant, while the microorganism count in the chitosan-coated group decreased in the third day and eventually reached zero in the fifth day.
The results indicated that the *Pseudomonas* spp. counts both in the normal meats and in the chitosan-coated meats significantly increased during storage. However, at the end of the storage, the *Pseudomonas* spp. count in the chitosan-coated group remained at a level that is half of non-coated group.

Table 1 shows that, at the end of the 7-day storage, the TBARS value in the normal meat was about 5-fold the initial level, while in the chitosan-coated group, the TBARS value increased on the 3rd day, remained constant on the 5th day, and re-increased on the 7th day. The increase in the TBARS value of the chitosan-coated group was 3-fold the initial level. The comparison between the coated and the non-coated group revealed that, at the end of the storage period, the difference between the groups was statistically significant.
The TMAB count in the chitosan-coated meats was lower than that of non-coated group. In a study in which chitosan was applied to ready-to-cook muttons using the coating material, the meats were stored for 14 days. In the meat samples, the initial TMAB value of 6.1 log cfu/g was reduced to 3.4 - 6.1 log cfu/g at the end of the storage (Kanatt *et al.,* 2013). In another study, meatballs were coated with chitosan and stored for 10 days and the number of TMAB was found close on the first day (İncili *et al.,* 2020). As seen in studies based on chitosan, it was successful at halting the increase of the TMAB count, which agrees with the results obtained in this study. The reason for this can be attributed to the potent antimicrobial and antifungal properties of chitosan. Although the antimicrobial property of chitosan was attributed to its polycationic properties, it may also be due to its chelator-like behavior, water-binding properties, activating the defense processes in the host tissue, and inhibiting mRNA synthesis through entering the nucleus (Liu *et al.,* 2004).

The differences between the groups that emerged at the end of the storage period revealed that chitosan had an inhibitory effect on *Staphylococcus aureus*. In another study on chitosan-coated meats, the meats were stored 48 h at 30º C and 10 days at 4º C; the *Bacillus subtilis IFO 3025, Escherichia coli RB, Pseudomonas fragi IFO 3458,* and *Staphylococcus aureus IAM 1011* bacteria were analyzed and the results showed that chitosan inhibited the growth of these bacteria species (Darmadji and Izumimoto, 1994). In a study in which chitosan was mixed with garlic oil, potassium sorbate, and nisin at certain concentrations, the activities of *E. coli, S. aureus, Salmonella trphimurium, L. monocytogenes*, and *B. cereus* were measured and the results showed that chitosan had an antimicrobial effect on all microorganisms (Pranoto *et al.,* 2005). In another study in which chitosan and peppermint were used as a complex, the *E.coli, P. fluorences, S. trphimurium, S. aureus,* and *B. cereus* bacteria were analyzed. The researchers determined whether the mixture they used influenced microbial activity. The initial *S. aureus* concentration of 6 log cfu/g was reduced to zero in a 24-hour period (Kanatt *et al.,* 2008). In a study focusing on increasing the shelf-life of minced meat, the meats stored at +3ºC was treated with chitosan and the researchers determined that chitosan had an inhibitory effect on *E.coli*, *S. aureus*, and *S. enteritidis* (Dehnad *et al.,* 2014). The results obtained in these studies are in agreement with the results obtained in this study. The bacteriostatic effect of chitosan on *S. aureus* was attributed to its antimicrobial and antifungal properties.

The results showed that the *Pseudomonas* spp. count in the chitosan-coated group was reduced. In a study in which modified packaging and antimicrobial packaging were combined to increase the shelf-life of beefsteaks, at the end of the 12-day storage period, the *Pseudomonas* spp. count in the control group increased from 2.16 log cfu/g to 7.21 log cfu/g. In the modified atmosphere-applied samples, the *Pseudomonas* spp. count was reduced from 1.79 log cfu/g to 1.58 log cfu/g. In the antimicrobial packaging group prepared by using nisin, the *Pseudomonas* spp. count increased from 1.72 log cfu/g to 7.77 log cfu/g (Storia *et al.,* 2012). The comparison of the study carried out by Storia (2012) to this study

reveals that, in reducing the *Pseudomonas* spp. count, chitosan coating could be more effective than nisin. In another study in which the combinations of the *Rosmarinus officinalis* essential oil with different packaging methods were used to increase the shelf-life of cold-stored bovine meats, at the end of the 10-day storage period, the *Pseudomonas* spp. count in the beefs to which active packaging and the *Rosmarinus officinalis* essential oil were applied increased from 2.5 log cfu/g to 7.4 log cfu/g (Sirocchi *et al.,* 2017). The comparison of the study carried out by Sirocchi (2017) to this study reveals that chitosan was more effective than active packaging with *Rosmarinus officinalis* essential oil in reducing the *Pseudomonas* spp. count.

Chitosan is known to affect both Gram-positive and Gram-negative bacteria. Among the bacteria affected by chitosan, Gram-positive bacteria are *Staphylococcus saprophyticus, Bacillus cereus,* and *Listeria monocytogenes*; Gram-negative bacteria are *Escherichia coli, Salmonella typhymurium, Pseudomonas aeruginosa, Aeromonas hydrophila, Shigella dysenteriae, Vibrio cholerae,* and *Vibrio parahaemolyticus* (Rejane *et al.,* 2009). Here, the action mechanism of chitosan is thought to be either through selective binding to metals or through the inhibition of mRNA synthesis by binding to DNA through entering the nuclei of microorganisms (Liu *et al.,* 2004). Moreover, the relatively higher antimicrobial activity of chitosan against Gram-positive bacteria compared to Gram-negative bacteria was also observed in the relevant studies (Kenawy *et al.,* 2020; No *et al.,* 2002; Goy *et al.,* 2009)

As seen in the Table 1, chitosan reduced the TBARS values. In a study in which chitosan was used to coat meats, the researchers reported that chitosan reduced lipid oxidation and putrefaction (Darmadji and Izumimoto, 1994). In another study, by examining the TBARS value in chitosan-coated and non-chitosan-coated ready-to-cook meat samples, the researchers determined that the TBARS value in all chitosan-coated samples was lower than that in non-coated samples (Kanatt *et al.,* 2008). In another study carried out with silver carp, the fish were coated with chitosan and stored 30 days at -3º C; the TBARS analysis of the samples showed that TBARS value in all non-coated samples was higher than those in the chitosan-coated samples (Fan *et al.,* 2009). These results agree with the results obtained in this study. TBARS value is a parameter that can measure lipid oxidation in meats. Chitosan formed a barrier by covering the surface of the red meats and thereby disconnected the food from the oxygen in the air and reduced the oxidation of the lipids in meat (Liu *et al.,* 2004). Through this barrier, the TBARS value was reduced in all chitosan groups when compared to normal meat.

CONCLUSION

There are many studies regarding chitosan's application on different food matrixes. The present study contributed these efforts by investigating the antimicrobial and antioxidative effects of chitosan on bovine meat samples. The results revealed that chitosan reduced the TMAB and *Pseudomonas* spp. counts while the number of *Staphylococcus aureus* eventually reached zero thanks to the bacteriostatic effect of chitosan. It also maintained the TBARS value, an indicator of the oxidative degradation, at a relatively low level compared to the control group. In conclusion, the study provided proofs of both the antimicrobial and barrier properties of chitosan. Future studies should focus on its use and applications at an industrial scale.

CONFLICT OF INTEREST

There are no possible conflicts of interest between the authors.

AUTHOR CONTRIBUTION

Halil İbrahim KAHVE performed the analyses and wrote the article. Ayhan DURAN directed the study.

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