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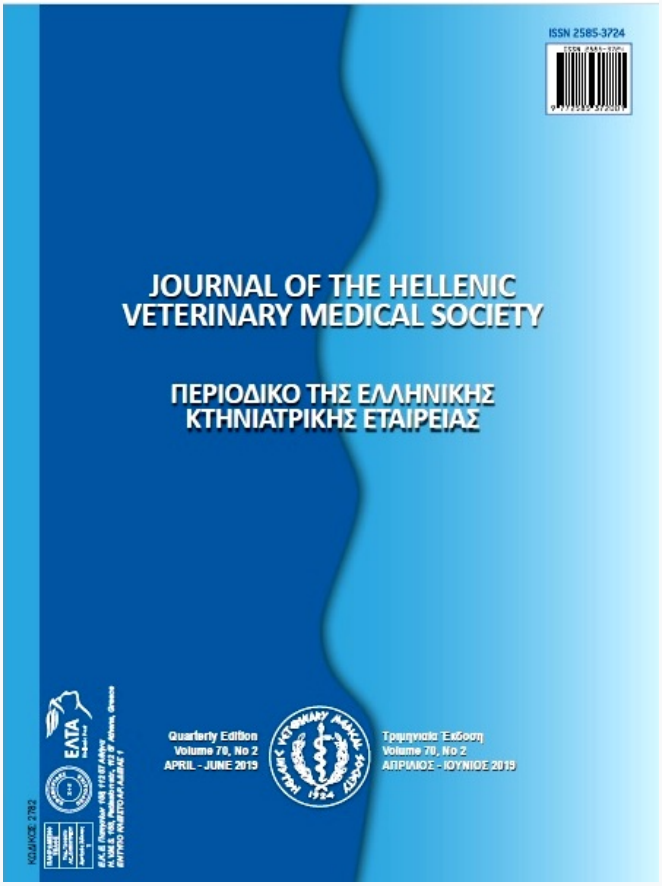

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Research article
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Effect of chitosan coating on the shelf life of ready-to-eat bovine meatballs and the control of *Listeria monocytogenes* growth on their surface during refrigeration storage

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Επίδραση επικάλυψης χιτοζάνης στο χρόνο ζωής προψημένων βόειων κεφτέδων και τον έλεγχο της ανάπτυξης της *Listeria monocytogenes* στην επιφάνεια τους κατά τη διάρκεια συντήρησής τους υπό ψύξη

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ABSTRACT. Edible chitosan coating on the surface of ready-to-eat (RTE) bovine meatballs was evaluated for its effect on their shelf life and the control of *Listeria monocytogenes* at 5 °C. *L. monocytogenes* was inoculated onto the surface of RTE bovine meatballs with and without edible chitosan coating. The samples were stored at 5 °C. Total aerobic viable count (TVC) and the bacterial counts of *L. monocytogenes*, lactic acid bacteria and Enterobacteriaceae were determined on days 0,1,7,14,21 and 28. The sensory characteristics were also evaluated at the same time spots by semi trained panelists. The results of the microbiological analysis depicted that the use of edible chitosan membranes reduced all of the microbial populations that were enumerated, and retarded their growth leading to the conclusion that they can prolong the shelf life of these products by 14 days. Moreover, the population of the inoculated *L. monocytogenes* was about 2 log CFU/g lower in the meatballs coated with chitosan, indicating an inhibitory effect of chitosan in the growth of *L. monocytogenes*. The sensory analysis showed that the samples coated with chitosan were satisfactorily accepted by the panelists even at day 28, in contrast to the samples without chitosan (control samples) which were unacceptable at day 14. These results indicate that edible chitosan coatings represent a potential agent in controlling *L. monocytogenes* on the surface of RTE meatballs as well as other RTE meat products, prolonging their shelf life without affecting their sensory characteristics.

Keywords: Chitosan, *L. monocytogenes*, Ready-To-Eat meatballs, shelf life

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ΠΕΡΙΛΗΨΗ. Η επίδραση εδώδιμων μεμβρανών χιτοζάνης στην επιφάνεια προψημένων (RTE) βόειων κεφτέδων αξιολογήθηκε ως προς το χρόνο ζωής τους και τον έλεγχο της ανάπτυξης της *Listeria monocytogenes* στους 5 °C. *L. monocytogenes* ενοφθαλμίστηκε στην επιφάνεια προψημένων βόειων κεφτέδων με ή χωρίς επικάλυψη χιτοζάνης. Τα δείγματα αποθηκεύτηκαν στους 5 °C και τις ημέρες 0,1,7,14,21 και 28 καταμετρήθηκαν οι μεταβολές των πληθυσμών των ολικών αερόβιων βακτηρίων, της *L. monocytogenes*, των οξυγαλακτικών βακτηρίων και των εντεροβακτηρίων. Τα οργανοληπτικά χαρακτηριστικά των δειγμάτων αξιολογήθηκαν τις ίδιες ημέρες από ομάδα ημιεκπαιδευμένων κριτών. Η ανάλυση των αποτελεσμάτων έδειξε ότι η χρήση εδώδιμων μεμβρανών χιτοζάνης μείωσε όλους τους μικροβιακούς πληθυσμούς που καταμετρήθηκαν και καθυστέρησε την ανάπτυξή τους οδηγώντας στο συμπέρασμα ότι μπορεί να επιμηκύνει το χρόνο ζωής των προϊόντων αυτών κατά 14 ημέρες. Επιπλέον, οι πληθυσμοί της *L. monocytogenes* ήταν περίπου κατά 2 log CFU/g μικρότεροι στους καλυμμένους με χιτοζάνη κεφτέδες. Συνεπώς, η χρήση χιτοζάνης είχε ανασταλτική επίδραση στην ανάπτυξη της *L. monocytogenes*. Η οργανοληπτική αξιολόγηση έδειξε ότι τα εμβαπτισμένα σε χιτοζάνη δείγματα έγιναν αποδεκτά από τους κριτές ακόμα και την ημέρα 28, σε αντίθεση με τα δείγματα χωρίς χιτοζάνη (δείγματα μάρτυρες) τα οποία ήδη από την ημέρα 14 δεν ήταν αποδεκτά. Από τα αποτελέσματα αυτά προκύπτει ότι οι εδώδιμες μεμβράνες χιτοζάνης είναι πιθανό να μπορούν να χρησιμοποιηθούν για τον έλεγχο της ανάπτυξης της *L. monocytogenes* στην επιφάνεια προψημένων κεφτέδων και άλλων προψημένων κρεάτων, επιμηκύνοντας το χρόνο ζωής τους χωρίς να υποβαθμίσουν τα οργανοληπτικά χαρακτηριστικά τους.

Λέξεις ευρετηρίασης*:* Χιτοζάνη, *L. monocytogenes*, προψημένοι κεφτέδες, χρόνος ζωής

INTRODUCTION

RTE products and especially the deli meats, are vulnerable to cross contamination of pathogens due to the multiple steps in their preparation, which can occur from contact with contaminated equipment and the environment (hoppers, slicers, peelers, conveyors, packaging machines etc) (Tompkin, 2002). Moreover, if these products are not processed after their final packaging, and because they are consumed without further cooking, if pathogenic contamination occurs, the chance of illnesses is increased (FSIS-USDA, 2005). Therefore, control of possible pathogens on RTE foods immediately prior to or after packaging is an important control point. Antimicrobial packaging provides a final defense hurdle for inactivation or inhibition of pathogens.

L. monocytogenes is a Gram- positive foodborne pathogen and represents a major food safety concern, especially in RTE foods (FAO, 2004) because of its ability to survive and grow at refrigeration temperatures. *L. monocytogenes* is associated with listeriosis, a disease with high fatality, affecting mainly immunocompromised persons, causing meningitis, and abortions in pregnant women (Beverly, 2004). Deterioration of foods, especially RTE, as well as contamination by pathogens, usually starts on the food surface. Consequently, food surface treatments and packaging after the treatments are of critical importance for food safety and quality (Malhotra et al., 2015). *L. monocytogenes* is a frequent surface contaminant of RTE meat products often occurring during the post-processing phase (Tompkin, 2002).

The increasing demands by the consumers concerning RTE foods with improved freshness and without chemical additives, led to the development of edible films (Kerry et al., 2006) which improve the

quality, the appearance and the safety, and increase the shelf life of RTE foods (Gennadios et al., 1997). Chitosan is one of the substances capable of creating such edible films (Coma, 2008).

Chitosan is a natural, nontoxic, biodegradable biopolymer which derives by the deacetylation of chitin, a main component of the shells of crustaceans such as, shrimp, crab and crawfish (No and Meyers, 2004). Chitosan as well as its oligomers, receive considerable attention due to their antimicrobial, antitumor and hypocholesterolemic abilities (No et al., 2002). Chitosan based edible coating and films, incorporated or not with other antimicrobial agents (essential oils, lauric alginate ester, allyl isothiocynate, nisin, etc) seem to be promising for their application in food preservation (Elsabee and Abdou, 2013; Kerch, 2015; Guo et al., 2017). The inhibitory effect and delay of *L. monocytogenes* growth by chitosan has been reported in several RTE meat products such as servelat sausage (Blom et al., 1997), ham steaks (Ye et al., 2008a) and deli turkey meat (Guo et al., 2014). The mechanism of the antibacterial activity of chitosan is not yet fully understood. However, a widely accepted assumption states that the positively charged chitosan molecules interact with the negatively charged microbial cell membranes, changing the cell permeability, causing a leakage of intracellular constituents and therefore the destruction of the microbial cell (Fang et al., 1994; Sudarshan et al., 1992; Papineau et al., 1991; Young et al., 1982).

The aim of the present study was the evaluation of the effect of chitosan edible coating on the shelf life and the control of *L. monocytogenes* in RTE bovine meatballs stored at 5 °C.

MATERIAL AND METHODS

1. Preparation of *L. monocytogenes* inoculum

L. monocytogenes serotypes Scott A and California (CA) obtained from the collection of the Laboratory of Milk Hygiene and Technology, of the School of Veterinary Medicine of Aristotle University of Thessaloniki, were used during this challenge study. The pure cultures were stored at -80 °C and sub-cultured twice in Tryptone Soy Broth (TSB, BioLab, Budapest, Hungary) at 35 °C for 24h before being used.

2. Preparation of chitosan solution

Chitosan with high molecular weight (>800.000Da, Aldrich Co, Germany) produced by crab shells was used to prepare 1g/100 ml chitosan solution in 1% v/v acetic acid under stirring for 24 hours. The solution was adjusted to a pH of 4 at 25 °C.

3. Preparation and thermal treatment of meatballs

Meatballs were prepared by mixing 800g of minced beef, 200g wheat breadcrumbs, 150g fresh onion, two egg yolks, 10g salt, 5g black pepper and 40ml olive oil. The meatballs were fried in olive oil bath at 180 °C until core temperature reached to 75 °C. They were allowed to dry and cool down to room temperature under a laminar flow before further treatments.

4. Preparation and treatment with chitosan solution of RTE meatballs

One group of the samples were dipped into the chitosan solution for 30 sec and placed on sterile trays under a laminar flow hood for 1h at room temperature 25 °C until the chitosan coating dried (group C). The rest of the samples were not dipped in the chitosan solution (group U-control samples). Overnight *L. monocytogenes* cultures of the two selected serotypes were centrifuged at 1500 rpm for 10 min and the bacterial pellet was resuspended in sterile peptone water and centrifuged again. This process was repeated twice and finally equal volume aliquots of each strain were combined and resuspended in peptone water and was then decimally diluted to 6.60 log CFU/g. An aliquot of 0.1 ml of the mixed culture was inoculated onto each meatball (18±1 g) to reach an initial inoculum of ca 5.30 log CFU/g. The samples remained for 10 minutes under a laminar flow hood in order to air-dry. They were then packaged, per tenth, in disks of expanded polystyrene on special absorbent meat papers, and the dishes were placed in plastic food bags.

Both trays and papers were previously sterilized by UV rays overnight under a laminar flow cabinet. The trays with the samples (inoculated with *L. monocytogenes* and uninoculated for sensory evaluation) were stored in an electronically controlled refrigerator (LBI-150M, Daichan Labtech Co. LTD Korea) at a temperature of 5±0.5 °C. Bacterial counts were determined at days 0, 1, 7, 14, 21 and 28.

5. Microbiological examination

The bacterial counts of the examined bacteria were determined at two samples of each group and the experiment repeated twice. The samples were stored at 5 °C. Total aerobic viable count (TVC) and the bacterial counts of *L. monocytogenes*, lactic acid

bacteria and Enterobacteriaceae were determined on days 0,1,7,14,21 and 28. More specifically, each meatball was mixed in a stomacher bag (Lab Blender 400, A. J. Seward and Co. Ltd., London, UK) for 60 sec with peptone water 0.1% w/v. Serial decimal dilutions were prepared and for the enumeration of *L. monocytogenes*, 0.1 mL of each dilution was streaked onto petri dishes with Agar Listeria Ottavani & Agosti (ALOA agar, LabM, Hal 10, Lancashire, United Kingdom) and incubated at 37 °C for 24-48h.

Total viable counts (TVC), Enterobacteriaceae and lactobacilli were counted on Tryptone Soy agar (Biolab, Budapest, Hungary), Violet Red Bile Glucose agar (VRBG PH EUR agar, BioLab, Budapest, Hungary) and De Man-Rogosa-Sharpe agar (MRS agar, BioLab, Budapest, Hungary) respectively. The counts were expressed as log CFU/g.

6. Sensory evaluation

Two groups of uninoculated meatballs (one group of meatballs with chitosan coating and another without) were stored for sensory evaluation, which was performed by a panel of eight semi-trained panelists at the same selected time spots of microbiological analysis. The sensory characteristics evaluated were color, flavor, taste and texture. Meatballs before consumption were heated in a microwave oven. Overall acceptability of the product (uniniculated meatballs with and without chitosan coating) was based on a 10-point numerical scale and a score of five was the lower limit of acceptability.

7. Statistical analysis

Analyses were based on two separate experiments with each mean Standard Deviation (± SD) being the average of the two determinations. The inhibitory effect of chitosan coating was analyzed using the General Linear Model (GLM) of Analysis of Variance (ANOVA) (STATA version 13, STATA Corp., Texas, USA). Differences at $p \leq 0.05$ were consider to be significant.

RESULTS

The population of TVC between the control samples and the samples coated with chitosan showed no significant difference at day 0 (5.31 log CFU/g) (Table 1). On day 1 the population of TVC in the samples coated with chitosan decreased from 5.31 to 3.97 log CFU/g, while the population of the control samples remained the same. From day 1 until the end of this study, the population of TVC in both sample groups increased. However, the difference between the two groups remained significant and at day 14 the population of TVC of the control samples reached 7.80 log CFU/g and meatballs were microbiologically unacceptable since the predominant flora was not the lactic acid bacteria (5.02 CFU/g) and spoilage is likely to be unacceptable at 10^7-10^8 CFU/g (Health Protection Agency, 2009). Their sensory characteristics were also unacceptable with a score of 3.25 (Table 2) which is very low. At the same time spot (day 14) the TVC population in coated with chitosan samples reached 5.77 log CFU/g. At day 28, the TVC population of the control samples was 8.90 log CFU/g, while in group C it was 6.73 log CFU/g and the sensory score still high (7.5). Therefore, the fact that there was a significant difference between the two groups leads to the conclusion that the use of chitosan has a beneficial effect in the shelf life of this product.

The *L. monocytogenes* counts on the RTE meatballs at day 0 were the same in both groups (5.19 log CFU/g), control and coated with chitosan. On day 1, the counts in the samples treated with chitosan were significantly lower from these in control samples. More specifically, on that day the population of *L. monocytogenes* in the control samples remained 5.19 log CFU/g, while in the treated with chitosan samples it decreased and reached 3.94 log CFU/g. From day 1 until day 28, the population of *L. monocytogenes* in both groups increased, along with their difference reaching its maximum at day 21 (3.16 log CFU/g).

The effect of chitosan in the population of Enterobacteriaceae was different compared to the rest of the bacterial counts (Table 1). On day 0 the Enterobacteriaceae counts of both the control samples and the samples coated with chitosan were similar (1.70 log CFU/g). At day 1 their counts were under the sensitivity of the method. Enterobacteriaceae could not be detected in the meatballs dipped in the chitosan until the end of the experiment, while the population in the control samples increased throughout the experimentation reaching 3.90 and 5.90 log CFU/g at day 14 and 21 respectively.

The lactic acid bacteria counts of the control samples and the samples coated with chitosan solution showed no difference at day 0 (Table 1). However, at day 1 the population in group C was 2.88 log CFU/g, while in group U 3.13 log CFU/g. This difference started decreasing until day 28 when both the control samples and the chitosan treated samples had similar population (6.39 and 6.40 log CFU/g respectively).

Table 1. Changes of the microbial counts (mean±SD) from the surface of RTE meatballs during their preservation at 5 °C for 28 days.

Day	Group of samples	Log CFU/g			
		TVC	*L. monocytogenes*	Enterobacteriaceae	Lactic Acid Bacteria
0	U*	5.31±0.03[a]	5.19±0.17[a]	1.70±0.03[a]	3.13±0.07[a]
	C**	5.31±0.16[a]	5.19±0.12[a]	1.70±0.04[a]	3.13±0.12[a]
1	U	5.31±0.23[a]	5.19±0.15[a]	<1	3.13±0.09[a]
	C	3.97±0.27[a]	3.94±0.21[a]	<1	2.88±0.33[a]
7	U	6.31±0.25[a]	6.07±0.05[a]	<1	5.48±0,28[a]
	C	4.52±0.37[a]	4.67±0.27[a]	<1	4.09±0.07[a]
14	U	7.80±0.42[a]	7.75±0.45[a]	3.90±0.13[a]	5.02±0.01[a]
	C	5.17±0.12[a]	5.02±0.0[a]	<1	4.54±0.21[a]
21	U	8.73±0.31[a]	8.89±0.52[a]	5.90±0.33[a]	5.61±0.14[a]
	C	5.77±0.30[a]	5.73±0.19[a]	<1	5.75±0.11[a]
28	U	8.90±0.43[a]	8.88±0.48[a]	6.13±0.11[a]	6.39±0.22[a]
	C	6.73±0.36[a]	6.72±0.22[a]	<1	6.40±0.28[a]

*Meatballs without chitosan coating
** Meatballs with chitosan coating
[a] Mean values for each microbial index at each day in the same column of different groups are not significantly different ($p>0.05$).

Table 2. Sensory evaluation scores of meatballs with and without chitosan coating during refrigeration storage.

Evaluation day	Evaluation parameter	Score		Overall acceptability	
		Group C*	Group U**	Group C	Group U
0	Taste	9***	10	9.25	9.25
	Color	10	9		
	Flavor	9	9		
	Texture	9	9		
1	Taste	9	9	9.25	9.25
	Color	10	9		
	Flavor	9	10		
	Texture	9	9		
7	Taste	9	8.5	9.25	8.5
	Color	10	7		
	Flavor	9	8		
	Texture	9	8.5		
14	Taste	8	4	8.25	3.75
	Color	9	4		
	Flavor	7	3		
	Texture	9	4		
21	Taste	8			8.25
	Color	9			
	Flavor	7			
	Texture	9			
28	Taste	8			7.5
	Color	7			
	Flavor	7			
	Texture	8			

* Group C=meatballs with chitosan coating
**Group U=meatballs without chitosan coating
***Mean values of the panelists' scores

DISCUSSION

In our study the use of chitosan films led to a reduction of 1.25 log CFU/g of the population of *L. monocytogenes* on the surface of RTE meatballs without preventing totally its growth, but could delay it significantly. The sensory evaluation demonstrated that the shelf life of RTE meatballs during their preservation at 5 °C does not exceed 28 days, while the use of chitosan improved their sensory characteristics.

Beverly et al. (2008) reported that the edible film of chitosan dissolved with acetic or lactic acid at 0.5% (w/v) or 1% (w/v) and stored at 4° C, on RTE roast beef could not prevent the growth of *L. monocytogenes*, but on day 14, *L. monocytogenes* counts were significantly different for all the chitosan-coated samples from the control by 2–3 log CFU/g and remained significantly different on day 28. This could be explained by the decreasing antimicrobial activity of chitosan films due to the decreased availability of amino groups on chitosan (Cargi et al., 2004; Comma et al., 2002). Ye et al. (2008a) reported that chitosan-coated plastic films were not able to control the growth of *L. monocytogenes* on ham steaks stored at 4 °C. A long term antilisterial effect was observed by chitosan film containing 0.001 g/cm^2 sodium lactate (SL) with the counts of *L. monocytogenes* being slightly lower than the initial inoculum. The SL treatment reduced the counts of *L. monocytogenes* from 2.7 to 1.5 log10 CFU/cm^2 during 10 weeks of storage. On week 10 *L. monocytogenes* started to grow, but its count at the end of the 12-week storage was still slightly lower than the initial one. Blom et al. (1997) reported that a mixture of 2.5% SL and 0.25% acetate prevented the growth of *L. monocytogenes* in servelat sausage without affecting the sensory acceptability of the sausage.

Chitosan-coated plastic films with 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL–0.6 mg/cm^2 potassium sorbate (PS) and 2.3 mg/cm^2 SL–500 IU/cm^2 nisin completely inhibited the growth of *L. monocytogenes* on smoked salmon for at least 6 weeks at refrigerated temperature (Ye et al., 2008b).

The antimicrobial activity of chitosan probably originates from its polycationic nature (Kim et al., 2003). The positively charged chitosan molecules interact with the negatively charged microbial cell membranes (Young et al., 1982; Papineau et al., 1991; Sudarshan et al, 1992; Fang et al, 1994). This antimicrobial activity was observed in our study, as the use of chitosan coating in RTE meatballs lead to a delay in the growth of TVC, *L. monocytogenes*, Enterobacteriaceae and lactic acid bacteria, resulting to a prolongation of their shelf life for about 14 days.

Latou et al. (2014) investigated the combined effect of dipping in a chitosan solution (1 g/100 ml) and packaging under modified atmosphere (MAP, 70% CO2, 30% N2) on shelf life extension of refrigerated chicken fillets. TVC were 3.9-4.9 log CFU/g lower on day 6 of storage in treated vs. untreated air-packaged samples with the strongest effect being shown by the combination of chitosan plus MAP. A similar reduction in LAB, Pseudomonads and Enterobacteriaceae was also observed during storage. Based on the microbiological and sensory data, shelf life of air-packaged, chitosan-treated, MAP-treated and chitosan/MAP treated samples was 5, 11, 12 and 14 days, respectively. Guo et al., (2014) investigated the antimicrobial efficacy of the coatings and films against *Listeria innocua* inoculated onto the surface of RTE deli turkey meat. Antimicrobial coatings with 1.94 mg/cm^2 of chitosan and 0.388 mg/cm^2 of LAE (Lauric Arginate Ester) reduced *L. innocua* by ca. 4.5 log CFU/cm^2. Nisin (486 IU/cm^2) showed less effectiveness than LAE (0.388 mg/cm^2) and addition of nisin to the antimicrobial coatings or films containing LAE (0.388 mg/cm^2) did not enhance the total antimicrobial effectiveness.

Kanatt et al. (2013) reported a microbiological shelf-life extension of 10 days using chitosan coating in in RTE cooked meat products (chicken balls, chicken seekh kababs and mutton seekh kababs). Chitosan coating eliminated fecal coliforms, lowered counts of *Staphylococcus* spp. and also retarded lipid oxidation in all the meat products during storage at 0-3 °C. The ability of chitosan coating to reduce microbial load has been reported by other researchers on various products. Darmadji and Izumimoto (1994) reported that chitosan at a concentration of 1 g/100 ml reduced microbial counts by an average of 1-2 log CFU/g in minced beef patties stored at 4 °C for 10 days. Also, addition of chitosan at 1% in fresh pork sausages reduced counts by 0.5-1.5 log CFU/g (Soultos et al., 2008). In other studies, the combined use of rosemary extract or thyme along with chitosan extended the shelf-life of fresh pork sausages and chicken product stored under refrigeration (Georgantelis et al., 2007; Giatrakou et al., 2010). Petrou et al. (2012) reported the extension of the shelf life at 4 °C of a chicken breast meat product treated with chitosan (1.5% w/v) or its combination with oregano oil (0.25 % v/w) by

10 days. Sagoo et al. (2002) demonstrated that total viable counts, yeasts, and molds were reduced by approximately 1-3 log CFU/g on skinless and standard sausages dipped in a 1% chitosan solution before storage at 7 °C for 18 d.

Mohan et al. (2012) reported that the chitosan coating improved the water holding capacity, drip loss and textural properties significantly of double filleted Indian oil sardines compared to untreated samples. The sensory characteristics during chilled storage were acceptable up to 8 and 10 days for 1 and 2% chitosan treated samples respectively, compared to only 5 days for untreated samples.

CONCLUSIONS

The results of microbiological analyses, sensory evaluation and the significant reduction of *L. monocytogenes* on RTE meatballs during chilled storage, indicate that chitosan coatings represent a promising means for the shelf life extension and improvement of microbiological safety of RTE meat products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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