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In vitro Effects of Chitosan on the Survival of *Listeria monocytogenes*

Ali GUCUKOGLU[1], Yeliz YILDIRIM[2], Göknur TERZİ GÜLEL[1], Murat ERDEM[3], Ufuk Tansel SIRELI[4]

[1]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun-TURKEY
[2]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Erciyes University, Kayseri-TURKEY
[3]Department of Chemistry, Faculty of Science, Anadolu University, Eskişehir-TURKEY
[4]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ankara University Ankara-TURKEY

Summary: The aim of this study is to evaluate the in vitro effects of different molecular weights of chitosan on the growth of three *Listeria monocytogenes* strains isolated from mayonnaise-based salad and of a *L. monocytogenes* reference strain (ATCC 7644). All *L. monocytogenes* isolates were numerically adjusted to $2.0x10^5$ cfu/mL and were treated with 0.1% chitosan solutions that had been prepared by dissolving low, medium and high molecular weight chitosan in 1% acetic acid at different pH values (4.0, 4.5 and 5.0) in vitro. All *L. monocytogenes* isolates were inhibited 3 log levels following 24 h incubation in a low molecular weight chitosan solution at pH 4.0, whereas 2 log levels of inhibition were observed for medium and high molecular weight chitosan solutions. The effect of different molecular weighted chitosan solutions and pH on *L. monocytogenes* strains in vitro was found to be statistically significant.

Key Words: Chitosan, *Listeria monocytogenes*, mayonnaise based salad

Kitosan'ın İn vitro Koşullarda *Listeria monocytogenes* Üzerine Etkisi

Özet: Bu çalışmanın amacı, farklı moleküler ağırlıktaki kitosan solusyonlarının mayonez bazlı salatalardan elde edilen üç *Listeria monocytogenes* saha izolatı ile *L. monocytogenes* (ATCC 7644) referans suşu üzerindeki etkilerini in vitro koşullarda değerlendirmekti. İn vitro koşullarda bütün *L. monocytogenes* izolatları sayısal olarak $2.0x10^5$ cfu/mL'ye ayarlandıktan sonra farklı moleküler ağırlıktaki (düşük, orta ve yüksek) kitosanların %1'lik asetik asit içersinde çözündürülmek suretiyle farklı pH değerlerine (4.0, 4.5 ve 5.0) sahip % 0.1'lik solusyonları hazırlandı. Her bir izolat hazırlanan kitosan solusyonlarıyla muamele edildi. Düşük moleküler ağırlıklı ve pH değeri 4 olan kitosan solusyonuna maruz bırakılan bütün *L. monocytogenes* suşlarının 24 saat sonunda 3 log düzeyinde inhibe olduğu, orta ve yüksek moleküler ağırlıklı kitosan solusyonuna maruz bırakılanlarda ise 2 log düzeyinde bir inhibisyon şekillendiği gözlemlendi. Çalışmada in vitro koşullarda farklı moleküler ağırlıklı ve farklı pH değerine sahip kitosan solusyonlarının *L. monocytogenes* üzerine istatistiksel olarak farklı inhibitorik etkiler ortaya koyduğu belirlendi.

Anahtar Kelimeler: Kitosan, *Listeria monocytogenes*, mayonez bazlı salata

Introduction

Listeria monocytogenes is a gram-positive, intracellular bacterium. Epidemiological studies conducted on *L. monocytogenes* indicate that it causes serious public health problems as a result of the consumption of contaminated food (17, 26, 33). As for all pathogens of food origin, the use of food additives has become more prominent in recent years in order to protect the public health and reduce the risk of listeriosis. From this aspect, inclusion of chitosan into the Generally Recognized as Safe (GRAS) category by the Food and Drug Administration (FDA) and its use as a food preservative has been the topic of research in many studies (10).

Chitosan was first discovered in 1859 by Rouget through boiling chitin and concentrated potassium

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hydroxide together. Chitin is present in shellfish, in the external skeleton of crustaceans, in the cell walls of some fungi and in planctons (19). Chitosan is obtained by the deacetylation of chitin. Through the removal of acetyl groups, reactive amino (NH_2) groups appear in the structure of chitosan. These free amino groups constitute the basis for the major physical and chemical properties associated with chitosan (1, 6, 25, 27, 31). Chitosan may be utilized in many areas owing to its antibacterial, antifungal and antitumor effects in addition to its properties in terms of heavy metal, protein and oil/fat absorption and biodegradation. The antimicrobial effect of chitosan stems from its polycationic property. Chitosan interferes with the cell wall components of gram-positive and gram-negative bacteria through electrostatic interactions. Another mechanism mentioned in some studies in the literature is its binding to the bacterial nucleolus which leads to the inhibition of mRNA and protein synthesis (6, 8, 21, 25, 27 - 29, 31).

In this study, the in vitro effect of low (75-85% deacetylated, viscosity 20-200 cps), medium (75-85% deacetylated, viscosity 200-800 cps) and high (≥ 75% deacetylated, viscosity 800-2000 cps) molecular weight chitosan solutions at different pH, were screened against three *L. monocytogenes* isolates from mayonnaise based salad and a *L. monocytogenes* strain (ATCC 7644) and compared to those known in the literature.

Materials and methods

A total of 50 (250 g each) mayonnaise based salad samples were collected from various grocer shops in Ankara (Turkey) and were taken to the laboratory in cool box (2-4 ^{0}C) to be used as material.

Preparation of chitosan solutions

One g of the chitosan formulations in powder form of low (Aldrich-44886-9, 75-85% deacetylated- viscosity 20-200 cps), medium (Aldrich-44887-7, 75-85% deacetylated-viscosity 200-800 cps) and high (Aldrich-41941-9, ≥75% deacetylated-viscosity 800-2000 cps) molecular weight were dissolved in 1% acetic acid (Sigma, 242853), these were then, readjusted to 100 ml to obtain 1% chitosan solutions with varying molecular weights. The low, medium and high molecular weight chitosan solutions were prepared in duplicate and the pH of the solutions belonging to each group were adjusted to 4.0, 4.5 and 5.0 with 0.1 M NaOH (Sigma, 8045) and 0.1 M HCl (Sigma, 1758). Following the preparation of the study groups, three 1% acetic acid solutions without chitosan were prepared to constitute the control groups.

Detection of L. monocytogenes

The mayonnaise based salad samples were brought to the laboratory under cold chain conditions and were analyzed for the presence of *Listeria* spp. as suggested by USDA-FSIS (14, 20). *Listeria* test kits (API®-Biomerieux) were used to identify *L. monocytogenes* isolates. The primer pair corresponding to *hly*A gene sequence (5'-GAA TGT AAA CTT CGG CGC AAT CAG-3'; 5'-GCC GTC GAT GAT TTG AAC TTC ATC-3') was used in the PCR confirmation of *L. monocytogenes* strains obtained from the samples (7). The specific bands obtained after the PCR protocol reported by Aznar and Alcaron (5) were evaluated through the use of a DNA marker and positive control (*L. monocytogenes* ATCC 7644). DNA bands of 388 bp weight which are specific to the *hlyA* gene were regarded as positive. Verified isolates were stored at +4^{o}C on Tryptone Soya Agar (TSA, Oxoid CM0131) to determine the antibacterial activity of chitosan solutions.

Assays for antibacterial activity

All isolates and the reference strain (*L. monocytogenes* ATCC 7644) stored at +4 ^{o}C on Tryptone Soya Agar were recovered in Brain Heart Infusion broth (BHI, Oxoid CM 0225) at 37 ^{o}C for 24 h. Decimal dilutions were prepared and viable cell counts were obtained by plating on Oxford-Listeria Selective Agar (MOX, Difco 0225-0218) after 24-48 h incubation at 35^{o}C. Each of the isolates and the reference strain were adjusted to give final bacterial concentrations of $2.0x10^5$ cfu/ml in 9 mL of BHI broth. One ml of each chitosan solutions were added to BHI broth to give final chitosan concentrations of 0.1% and these were then incubated at 37 ^{o}C for 24 h. Enumeration of the viable cells was carried out on Oxford-Listeria Selective Agar after 24 h storage.

Statistical analysis

The experiment was repeated two times on different days taking the average of the results. The package programme (SPSS,14,01 no: 9869264) was used to make two way variance analyze and were compared with Tukey multicomparing test.

Results

Chitosan is included in the GRAS category and this structure can be used as an antimicrobial packaging agent and as a preservative food additive (10). Nonetheless, different molecular weights and vis-

cosities of chitosan are reported to have an important effect on the antimicrobial mechanism of action (1, 8). In order to better evaluate the inhibition potential of chitosan, different molecular weights and pH values have been tried. In many countries, the regulatory agencies establish “a zero tolerance” policy for *L. monocytogenes* in ready to eat (RTE) food products including mayonnaise based salads (2, 3, 4, 30). Therefore the isolates have been selected from mayonnaise based salads in this study. The inoculation concentration of the agent was deliberately kept much higher than the probable contamination levels in food in order to observe the inhibition potential of different chitosan solutions. Since the shelf life of RTE food products is quite short, analyses were carried out at 0 hours and at the end of 24 hours.

Three *L. monocytogenes* strains isolated from mayonnaise based salads and a reference strain (*L. monocytogenes* ATCC 7644) were tested to determine the antibacterial activity of chitosan solutions after 24 h storage.

The mean log reduction observed for all *L. monocytogenes* strains affected by chitosan solutions of different concentrations and pH values at the end of the 24th hour of analysis are reported in Table 1.

In the study, in vitro effects of different molecular weight of chitosan solutions (Low moleculer weight-LMW, Middle moleculer weight-MMW, High moleculer weight-HMW and control) and pH values (4, 4.5, 5) on *L. monocytogenes* isolates at 0 and 24th hour were evaluated by two way variance analyse. Obtained results indicated that the effects of different molecular weight of chitosan solutions (Low moleculer weight-LMW, Middle moleculer weight-MMW, High moleculer weight-HMW and control) and pH values (4, 4.5 and 5) on *L. monocytogenes* isolates were statistically significant (Table 2)

Table 1. In vitro effect of the different moleculer weight and pH of chitosan on *L. monocytogenes* strains

pH	Chitosan	Time (hour)	*L. monocytogenes* Strain 1 (Log cfu/ml)	*L. monocytogenes* Strain 2 (Log cfu/ml)	*L. monocytogenes* Strain 3 (Log cfu/ml)	*L. monocytogenes* ATCC 7644 (Log cfu/ml)
4.0	LMW	0	3.30	3.41	3.04	3.0
		24	2.53	2.66	2.60	2.60
	MMW	0	3.30	3.48	3.30	3.30
		24	3.0	3.0	2.93	3.30
	HMW	0	3.92	3.72	3.56	3.60
		24	3.48	3.30	3.30	3.30
	Control	0	3.48	3.53	3.20	3.43
		24	7.85	7.82	7.60	7.48
4.5	LMW	0	3.52	3.56	3.56	3.08
		24	2.70	2.78	2.66	2.75
	MMW	0	3.48	3.70	3.85	3.53
		24	3.41	3.48	3.08	3.48
	HMW	0	3.98	3.78	3.90	3.90
		24	3.60	3.48	3.75	3.60
	Control	0	3.90	3.88	3.78	3.60
		24	8.0	8.0	7.85	7.82
5.0	LMW	0	3.60	3.95	3.64	3.30
		24	3.20	3.60	3.48	2.98
	MMW	0	3.70	3.90	3.78	3.85
		24	3.56	3.60	3.56	3.48
	HMW	0	4.18	4.08	4.11	4.30
		24	3.78	3.78	3.78	3.66
	Control	0	4.08	4.30	4.48	4.48
		24	8.78	8.60	8.48	8.60

LMW : Low moleculer weight (75-85% deacetylated,Viscosity 20-200 cps)
MMW : Middle moleculer weight (75-85% deacetylated, Viscosity 200-800 cps)
HMW : High moleculer weight (≥ 75% deacetylated, Viscosity 800-2000 cps)

In the study, the inhibition effects of LMW and MMW chitosan solutions found to be higher than HMW and control group on 0. hour whereas all chitosan solutions were found effective than control group on 24th hour and the difference were found statistically significant (p<0.001) (Table 3). Besides, the difference on inhibition effects of all chitosan solutions at different pH values were found statistically significant on 0 and 24th hour (p<0.001) (Table 4).

The PCR confirmation of three *L. monocytogenes* isolates identified by the classical culture technique and using the Listeria test (API®-Biomerieux) from the mayonnaise based salads and of the *L. monocytogenes* strain ATCC 7644 are shown in Figure 1.

Discussion

pH was found to be effective for all isolates when

Table 2. Two way variance analyze table of the effects of different molecular weight of chitosan solutions and pH values (4, 4.5 and 5) on *L. monocytogenes* isolates at 0 and 24th hour

Hour	Varation source	Sum of square	Df: degree of freedom	Mean square	F statistics	Significance
0. hour	pH	2.623	2	1.311	47.986	P<0.001
	Chitosan	1.939	3	0.646	23.649	P<0.001
	Chitosan X pH	0.367	6	0.061	2.240	P<0.05
24.hour	pH	3.313	2	1.657	86.454	P<0.001
	Chitosan	211.757	3	70.586	3.683.664	P<0.001
	Chitosan X pH	0.560	6	0.093	4.873	P<0.001

Table 3. Descriptive statistic values of different chitosan consantrations on 0 and 24. hour

Hour	Chitosan groups	Mean ± Sem	Significance
0. hour	LMW	3.41 ± 0.05a	p<0.001
	MMW	3.60 ± 0.05b	
	HMW	3.92 ± 0.05c	
	CONTROL	3.85 ± 0.05c	
24.hour	LMW	2.88 ± 0.04a	p<0.001
	MMW	3.32 ± 0.04b	
	HMW	3.57 ± 0.04c	
	CONTROL	8.07 ± 0.04d	

a,b,c,d: Difference on the group means with different letters are statistically significant

Table 4. Descriptive statistic values of different pH values on 0 and 24. hour

Hour	pH groups	Mean± Sem	Significance
0. Hour	4	3.41 ± 0.04a	p<0.001
	4.5	3.69 ± 0.04b	
	5	3.98 ± 0.04c	
24.Hour	4	4.17 ± 0.03a	p<0.001
	4.5	4.40 ± 0.03b	
	5	4.81 ± 0.03c	

a,b,c,d: : Difference on the group means with different letters are statistically significant.

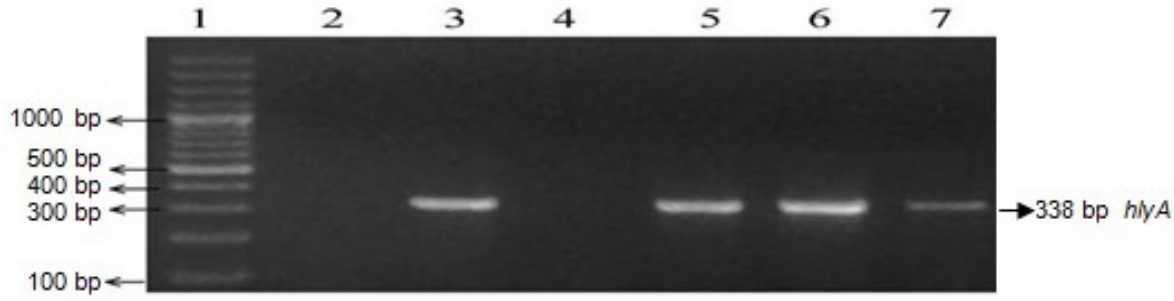

Figure 1. Representative gel electrophoresis picture of PCR detection of *hlyA* gene of *L. monocytogenes* with product size of 338 bp.

1. 100-bp DNA ladder **2.** Negative control (*Eschrerichia coli* ATCC 25922) **3.** Positive control (*L. monocytogenes* ATCC 7644) **4.** *hlyA* gene negative *Listeria* spp. **5.** *hlyA* gene positive *L. monocytogenes* strain 1 **6.** *hlyA* gene positive *L. monocytogenes* strain 2 **7.** *hlyA* gene positive *L. monocytogenes* strain 3

considering the values at the end of the 24th hour. The bacterial counts for all isolates with no chitosan addition was around 7 logs for pH = 4.0, 8 log levels at pH = 4.5 and was higher than 8 logs at pH = 5.0. Although *L. monocytogenes* is able to grow in quite wide range of pH (4.3-9.6), the optimum pH range is 6.0-8.0. The ability of the agent to grow at low pH values is thought to depend on many factors including the incubation temperature, type of acid and nutrient content (15).

In the present study, the inhibition effect of chitosan was determined to be high at low molecular weight and pH values (Table 1). Low pH value and low molecular weight rank among the most significant factors affecting solubility (1, 8). Chitosan has three reactive functional groups. These are an amino group at the C-2 position and additional primary and secondary hydroxyl groups at the C-3 and C-6 positions, respectively. In order for chitosan to become soluble, the NH_2 functional group at the C-2 position of the D-glucosamine section needs to be saturated with protons. Since the glucosamine parts of this chemical structure carry protonated free amino groups in acidic medium, the amount and position of the glucosamine determines the charge distribution of the chitosan molecule. Change in the charge density affects the solubility and binding properties of chitosan. It is reported that chitosan that has dissolved at low pH values provides inhibition at a higher activity. The antimicrobial properties of chitosan are based upon its polycationic structure. Related to that, chitosan

is charged positive at low pH values and reacts with negatively charged structures. As a result of the electrostatic reaction between the NH^{3+} groups of chitosan and the negatively charged phosphoryl groups in the cell wall phospholipid component, the permeability of the cell wall changes and an antimicrobial effect is observed through the oozing of the intracellular material to the outside of the cell (1, 6, 8, 18, 21, 25, 27, 31).

The inhibitory power of the chitosan solutions varied among the isolates. *L. monocytogenes* strains isolated from the mayonnaise based salads were more resistant than the reference strain *L. monocytogenes* ATCC 7644. This situation indicates that the multi-resistance mechanism is much more developed in the wild-type isolates than in the reference strain. Several researchers have highlighted a synergetic relation between the acid and the osmotic shock responses of *L. monocytogenes* (9, 32). *L. monocytogenes* has many stress response proteins that enable its growth in a host and outside of a host in harsh environmental conditions. For instance, the LisRK transduction system is related to the virulence of the pathogen together with its acid and ethanol tolerance as well as its oxidative stress adaptation (16). The heat shock protein DnaK enables the sustenance of the viability of *L. monocytogenes* at high temperatures, in acidic media and during phagocytosis by the macrophages (13). GroES and GroEL are the most significant heat shock proteins of the bacterium playing a role at high temperatures, low pH values and in cellular infections (12). The Sigma B factor enables *L. monocytogenes* to maintain its viability and reproduction outside the host in unsuitable conditions (acid, osmotic and oxidative stress, low temperature and carbon deficiency) (11).

No et al. (23) determined a value of 2.38 log cfu/mL in a low molecular weight solution of chitosan following an inoculation of 6.36 log cfu/ml at 37 ^{o}C for 24 hours. They determined 3.10 log cfu/mL reduction in high molecular weight chitosan solution in terms of in vitro inhibition concerning *L. monocytogenes* using chitosan of varying molecular weights dissolved in acetic acid and they determined this value as 8.31 log cfu/mL for the control group. The values obtained in this study are in accordance with the results obtained by these researchers.

Chitosan has been reported to be effective against gram positive, gram negative and anaerobic bacteria and many fungus species in studies conducted to determine the inactivation effects of different chitosan concentrations on various microorganisms. Liu et al. (18) reported that *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 strains treated with 0.5% chitosan solution at a pH of 5.4 caused 1 log reduction in both species at the end of 5 minutes whereas at the end of 120 min chitosan was reported to provide 4 log inhibition on the *E. coli* ATCC 25922 strain and 1 log inhibition on the *S. aureus* ATCC 25923 isolate. In a study by Sagoo et al. (24), the cell count of *Saccharomycodes ludwigii* treated in vitro with 0.05% chitosan concentrations with a pH adjusted to 6.2 was reduced by 1 log at the end of 60 minutes. A high concentration of chitosan (0.5%) has been found to result in an inactivation by 2 logs on the number of *Lactobacillus viridescens* and by 1 log on the number of *Listeria innocua*. However, *Torulaspora delbrueckii* and *Salmonella* Enteritidis PT4 were reported as resistant to chitosan of the same concentration (0.5%). Both study results are in accordance with the results of our study as regards inhibition, although the microorganisms and the chitosan concentrations that were used in the treatments were different.

No et al. (22) applied chitosan (Mw 2025 kDa) dissolved in 1% acetic acid in vitro on gram positive *L. monocytogenes* and *S. aureus* and on gram negative *Salmonella Enteritidis* and *E. coli* at a concentration of 0.05%. At the end of 48 hours at 37^{0} C, researchers reported an inhibition by 1 log on average in *S. aureus* and by 2 logs in other bacteria. The results display similarities although the chitosan concentration used in their study was lower than that was used in the present study.

In conclusion, the inhibition effect of low molecular weight chitosan solution dissolved at low pH values was higher on different *L. monocytogenes* isolates in vitro. It will be an effective control measure to use chitosan as a preservative to prevent health hazards associated with consumption of foods contaminated with *L. monocytogenes* or to extend the shelf-life of food. Higher antibacterial activity of chitosan at lower pH suggests that the addition of chitosan to acidic foods will support its effectiveness as a natural preservative. Besides further studies are needed to evaluate the effects of chitosan in different matrix and conditions (pH, water activity, storage temperature) as well as different contamination levels which can influence the effect of chitosan.

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Correspondence
Assist. Prof. Dr. Ali GÜCÜKOĞLU
Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology
Atakum/Samsun
Tel: +90 362 3121919/2812
Fax: +90 362 4576922
E-mail: aligucuk77@hotmail.com