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Antibacterial activity of chitosan biofilm for the conservation of fertile and table eggs

[*Atividade antibacteriana de biofilme de quitosana para conservação de ovos férteis e de mesa*]

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ABSTRACT

The aim of this study was to develop a chitosan biofilm against *Salmonella enteritidis*, for the conservation of fertile and table eggs. Two experiments were performed. Experiment 1: 400 specific pathogen-free table eggs were divided in a completely randomized design into four treatments, five replicates and each replicate with 20 table eggs. Experimental groups were assigned to control and 1, 5 and 10% chitosan treatment. The eggs were immersed in the chitosan solution. They were then exposed to *Salmonella enteritidis* and stored for 1, 24, 96 and 168h at 4ºC. The eggs were then washed with 10mL of physiological saline solution. Experiment 2: 80 specific pathogen-free fertile eggs were tested, the assays were assigned to control and 1, 5 and 10% chitosan treatment. Each treatment had 20 fertile eggs. The eggs were immersed in the chitosan solution. They were individually weighed and incubated. Egg weight, humidity loss, and hatchability (weight and length of newly hatched chicks) characteristics were assessed. In Experiment 1, comparison between treatments showed differences (P< 0.05) in the total recovered of *Salmonella enteritidis* on eggshell, with the lower values in 5 y 10% chitosan treatment at 96 y 168h respectively. In Experiment 2, chitosan did not show any effect on the egg weight and chick weight, where the average was 57.44 and 38.23g respectively. The humidity loss and chick length showed differences (P< 0.05), with the lower values in 5 y 10% chitosan treatment. The antibacterial activity of chitosan biofilm provide a practical tool against *Salmonella enteritidis* in fertile and table eggs because the chitosan did not affect egg weight and chick weight, relevant parameters in the poultry industry.

Keywords: chitosan, fertile eggs, table eggs, *Salmonella enteritidis*

RESUMO

O presente estudo teve como objetivo desenvolver um biofilme de quitosana contra Salmonella enteritidis, *para conservação de ovos férteis e de mesa. Dois experimentos foram realizados. Experimento 1: 400 ovos de mesa livres de patógenos especificados foram divididos em delineamento inteiramente casualizado em quatro tratamentos, cinco repetições e cada réplica contendo 20 ovos de mesa. Grupos experimentais foram designados para controle e 1, 5 e 10% de tratamento com quitosana. Os ovos foram imersos em solução de quitosana. Em seguida foram expostos a* Salmonella enteritidis, *e armazenados por 1, 24, 96 e 168h a 4ºC. Após, os ovos foram lavados com 10mL de solução salina fisiológica. Experimento 2: 80 ovos férteis livres de patógenos especificados foram testados. Os ensaios foram atribuídos a controle e 1, 5 e 10% de tratamento com quitosana. Cada tratamento teve 20 ovos férteis. Os ovos foram imersos em solução de quitosana. Em seguida foram individualmente pesados e incubados. Peso dos ovos, perda de umidade e características de eclodibilidade (peso e comprimento dos pintinhos recém-nascidos) foram avaliados. No Experimento 1, a comparação entre tratamentos*

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mostrou diferenças (P< 0,05) na quantidade total recuperada de Salmonella enteritidis *na casca, com os menores valores em 5 e 10% de tratamento com quitosana a 96 e 168h respetivamente. No experimento 2, a quitosana não mostrou nenhum efeito no peso do ovo e no peso do pintinho, onde a média foi de 57,44 e 38,23g respetivamente. A perda de umidade e comprimento do pintinho apresentaram diferenças (P< 0,05), com os menores valores em 5 e 10% de tratamento com quitosana. A atividade antibacteriana do biofilme de quitosana, fornece uma ferramenta prática contra* Salmonella enteritidis *em ovos férteis e de mesa, pois a quitosana não afetou o peso do ovo e peso do pintinho, parâmetros relevantes na indústria avícola.*

Palavras-chave: quitosana, ovos férteis, ovos de mesa, Salmonella enteritidis

INTRODUCTION

According to the World Health Organization (WHO) report published in 2015, the foodborne disease is a leading cause of mortality and morbidity, causing an estimated 600 million human infections/year and 42,000 deaths/year (Havelaar *et al.*, 2015). It is estimated that *Salmonella enteritidis* (*SE*) causes 93.8 million human infections and 155,000 deaths annually worldwide (Aspinall *et al.*, 2016). In the United States, *SE* affects up to 2 million human infections/year and 452 deaths/year, with an estimated cost of $15 billion (Scharff, 2015). Therefore, the Commission Regulation (CR) No. 2073/2005, concerning microbiological criteria for foodstuffs established the absence of *Salmonella* spp. in 25g of food as a requirement to gaining market access, particularly for developing economies (Lake *et al.*, 2015).

To achieve this objective, efficient cleaningand disinfection methods are necessary to totally eradicate problem strains in farms and food environments. In this way, the development of antibacterial biofilms and coatings from food-grade biopolymers has advanced significantly during recent years (Costa *et al.*, 2017; Dhumal and Sarkar, 2018). Among the alternatives tested, polycationic biopolymers are among the most notable due to their high biocompatibility and antimicrobial activity (Venkataraman *et al.*, 2019). In this regard, the seafood processing industry generates approximately 100 billion t of chitin/year from the marine invertebrates, e.g. exoskeletons of crustaceans, mollusks and shrimp (Hamed *et al.*, 2016). However, there are some limitations to using chitin. Two of its major drawbacks, high viscosity, and insolubility in water, are due to the chitin's own structure and molecular weight (Younes and Rinaudo, 2015). Therefore, its derivative chitosan has been receiving increasing attention as invaluable biopolymer (Bano *et al.*, 2017).

The chitosan is a linear polysaccharide that consists of glucosamine and *N*-acetyl-β-D-glucosamine units, obtained after deacetylation of chitin, which consist of β-(1-4)-2-amino D-glucose and β-(1-4)-2-acetamido-D-glucose linked through 1,4-β-glucosidic bonds (Younes and Rinaudo, 2015). It is a polycationic biopolymer with a unique structure, including amino groups (-NH_2) in its backbone, that grants it the high reactivity (Thakur and Voicu, 2016). Therefore, they have received great attention due to their physical, chemical and antibacterial activity (Verlee *et al.*, 2017). The use of antibacterial biofilms and coatings from food-grade biopolymers during processing can extend the shelf life of food and reduce health risks and economic losses of foodborne disease (Randazzo *et al.*, 2018).

The table eggs are among the most consumed food worldwide. However, one of the constraints limiting the acceptance of table eggs is the suspicion about their bacteriological quality (Antunes *et al.*, 2016). Contamination of table eggs with *SE* is a recurring fact, due to the ability to be transmitted both vertically (from layer to egg) and horizontally (from the environment) (Baron *et al.*, 2016). The eggs carrying *SE* on the eggshell or in shell membranes could lead to contamination of the chick (Ketta and Tůmová, 2016). Also, eggs are highly susceptible to internal quality deterioration and bacterial growth during storage (Park *et al.*, 2016b). Therefore, the aim of this study was to develop a chitosan biofilm against *SE*, for the conservation of fertile and table eggs.

MATERIALS AND METHODS

Deacetylated 95% food-grade chitosan was obtained commercially (Paragon Specialty Products., Rainsville, Alabama, United States) and was used in all experiments. The chitosan molecular weight was 350kDa with a viscosity of 800mPa, and particle size of 100 United States mesh (sieve size 0.152mm). Chitosan was prepared by dissolving it in a solution containing 0.5% (w/v glacial acetic acid (J41A08; Mallinckrodt Baker Inc., Phillipsburg, New Jersey, United States).

A poultry isolate of *SE*, selected for resistance to nalidixic acid (NA) (N-4382; Sigma-Aldrich., St. Louis, Missouri, United States), was used for all experiments. *SE* was grown in tryptic soy broth (N-22092; Sigma-Aldrich., St. Louis, Missouri, United States) for approximately 8h. The cells were washed three times with 0.9% physiological saline solution (PSS), by centrifuging at 1864 x *g* for 10min using a portable centrifuge (Porta-Spin C828; UNICO., Dayton, United States). The concentration of the stock solution was determined with a UV/Vis spectrophotometer (ES-218; KONTROLab., Guidonia, Italy) at 525nm. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1mL/replicate) spread on brilliant green agar (278820; Becton-Dickinson Co., Franklin Lakes, United States) plates containing 25µg/mL novobiocin (N-1628; Sigma-Aldrich., St. Louis, Missouri, United States) and 20µg/mL of NA.

Experiment 1. 400 specific pathogen-free (SPF) table eggs were divided in a completely randomized design into four treatments, five replicates and each replicate with 20 table eggs. Experimental groups were assigned to control and 1, 5 and 10% chitosan treatment. The eggs were immersed in chitosan solution for 30s and allowed to dry. They were then exposed to *SE* (10^5CFU/mL) for 30s and were transferred to sterile bags and stored for 1, 24, 96 and 168h at 4ºC. The eggs were then washed with 10mL of PSS. Enumeration of *SE* (CFU/mL) was performed by spread-plating.

Experiment 2. 80 SPF fertile eggs were tested, the assays were assigned to control and 1, 5 and 10% chitosan treatment. Each treatment had 20 fertile eggs. The eggs were immersed in chitosan solution for 30s and allowed to dry. Hatching egg was incubated at constant temperature (37.7ºC) in a single-stage incubation (Chick Master) and relative humidity was maintained at a constant 50% throughout incubation. At 18d of incubation, eggs were weighed and transferred to hatching baskets. Egg weight and humidity loss were quantified. On the day of hatch, chick weight and chick length were quantified according to quality criteria in the poultry industry.

One-way analysis of variance (ANOVA) was performed between and within groups of all data using (SPSS…, 2013), and differences were considered to be significant at a level of (P< 0.05), means were separated using the Tukey multiple range tests.

RESULTS

The total recovered (CFU/mL) of *SE* on eggshell, determined from 400 table eggs are shown in Table 1. The comparison between treatments showed differences (P< 0.05), with the lower values in 5 y 10% chitosan treatment at 96 y 168h respectively.

The effect of treatments (control, 1, 5 and 10% chitosan) on egg weight, humidity loss, chick weight, and chick length, determined from 80 fertile eggs incubated are shown in Table 2. The chitosan did not show any effect on the egg weight and chick weight, where the average was 57.44 and 38.23g respectively. The humidity loss and chick length showed differences (P< 0.05), with the lower values in 5 y 10% chitosan treatment.

Table 1. Total recovered (CFU/mL) *Salmonella enteritidis* on eggshell at different times of storage, *n*= 400 table eggs

	Time of storage at 4 ºC (h)			
Chitosan (%)	1	24	96	168
0	2.53±0.74[a]	2.23±0.78[a]	2.58±0.90[a]	2.98±1.24[a]
1*	3.70±0.71[a]	3.82±0.69[a]	1.39±0.33[b]	0.92±0.30[b]
5*	2.79±0.60[a]	1.06±0.37[b,c]	0.83±0.27[c]	1.34±0.35[b]
10*	2.73±0.31[a]	1.47±0.34[b]	1.29±0.50[b,c]	1.01±0.41[c]

Data expressed as log_{10} mean±standard deviation; *significant differences were obtained between groups indicated with different letters; *P< 0 .05.

Table 2. Effect of treatments (control, 1, 5 and 10% chitosan) on egg weight, humidity loss, chick weight and chick length, *n*= 80 fertile eggs incubated/chicks during the first day post-hatch

	Chitosan (%)			
Quality criteria	0	1	5	10
Egg weight	57.69±1.20[a]	57.23±1.51[a]	57.52±1.26[a]	57.35±1.52[a]
Humidity loss*	10.86±1.09[a]	10.34±1.37[a,b]	9.41±0.93[c]	9.56±1.01[b,c]
Chick weight	38.40±1.61[a]	38.17±1.83[a]	38.45±1.81[a]	37.92±1.58[a]
Chick length*	18.01±0.52[a]	17.72±0.30[a]	16.62±0.67[b]	16.66±0.45[b]

Data expressed as mean±standard deviation; *significant differences were obtained between groups indicated with different letters; *P< 0 .05.

DISCUSSION

The *SE* biofilm formation under different environmental conditions is a public health problem because it can survive in the long term around poultry farms and contaminate meat and eggs or by-products, through the food chain to reach the consumer (Antunes *et al.*, 2016; Shah *et al.*, 2017). Biofilms are microbial communities of bacterial cells enclosed in a self-produced polymeric matrix of 97% water and 3% exopolysaccharides (EPS), outer membrane proteins, lipopolysaccharides (LPS), nucleic acids and fatty acids, that attaches to different types of surfaces, inert and alive (Barreto *et al.*, 2016).

The *SE* biofilms are difficult to eliminate because they are well protected against antibiotics and disinfectants. Therefore, the factors that induce or prevent the formation of bacterial biofilms are currently topics of interest in the poultry industry (Lamas *et al.*, 2018). In this way, the following *log* reductions in total recovered (CFU/mL) of *SE* on eggshell were observed on chitosan as a "secondary cuticle", in comparison to control (Figure 1).

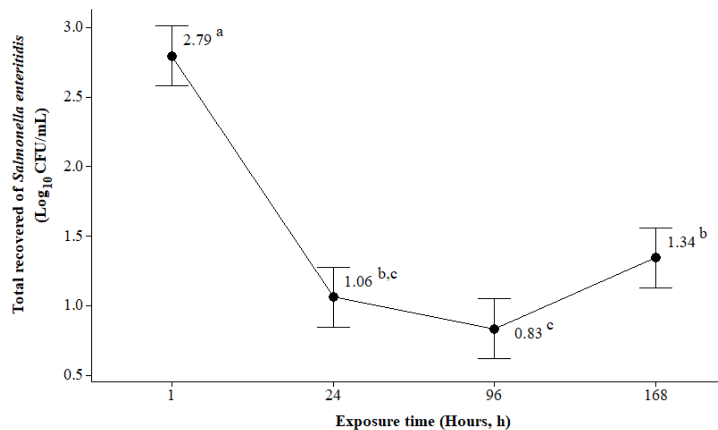

Figure 1. Effect of 5% chitosan on total recovered CFU/mL of *Salmonella enteritidis* on eggshell at different times of storage, *n*= 400 table eggs.

The differences in chitosan efficacy (Figure 2), is thought to be a result of varying bacterium cell surface charge and differing cell wall/membrane structure (Verlee *et al.*, 2017). These results suggest that chitosan coatings abort *SE* biofilms formation by permeabilizing bacterium cells as they contact the surface of chitosan biofilm (Younes and Rinaudo, 2015). The positively charged (cationic) chitosan polymer compromises the integrity of the *SE* membrane due to their reactive amino groups ($-NH_2$) (Thakur and Voicu, 2016). This activity of chitosan biofilm seems to echo reports of engineered antibacterial surfaces in which cationic biocides have been covalently immobilized (Verlee *et al.*, 2017). In addition, the water solubility of the chitosan oligosaccharides enabled their penetration across the extracellular EPS y LPS matrix of *SE* biofilms, leading to enhanced biofilm killing (Thakur and Voicu, 2016).

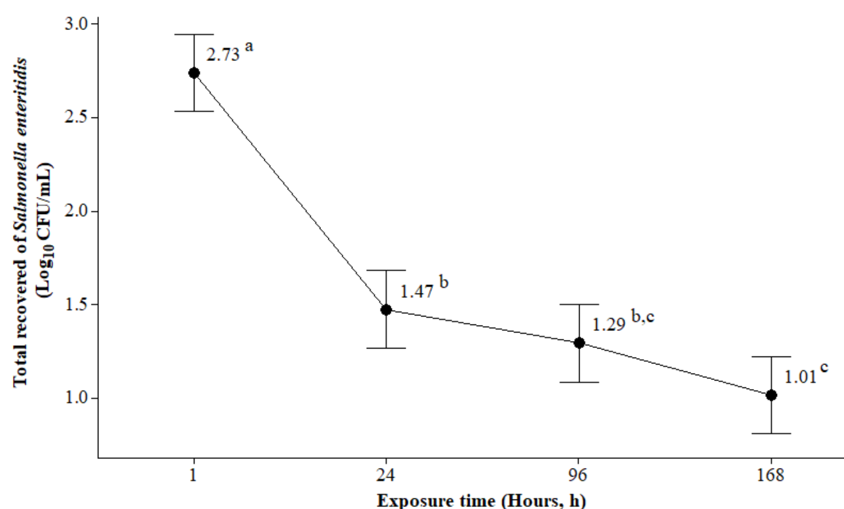

Figure 2. Effect of 10% chitosan on total recovered CFU/mL of *Salmonella enteritidis* on eggshell at different times of storage, *n*= 400 table eggs.

During incubation, egg water content is absorbed by the embryo and embryonic annexes e.g. amniotic cavity and allantois (Ketta and Tůmová, 2016), and a small portion is lost to the evaporation of water molecules through the eggshell pores (Park *et al.*, 2016a). This loss of water is associated with egg heat loss by evaporation and conduction and is important not only for egg heat loss but also for the formation of the air chamber (Menconi *et al.*, 2014). The chitosan has a mucoadhesive property due to the union of its hydroxyl group (-OH) and the amino groups ($-NH_2$) of mucin, and the electrostatic interactions between the positively charged amines of chitosan and the negatively charged sialic acid residue of the mucin (Shi *et al.*, 2005; Pradhan and Sahoo, 2017).

However, we have not observed any negative effect on the loss of water through the eggshell pores (Figure 3). The eggshell allows physical exchanges between the egg and the external environment and includes heat transfer and the exchange of carbon dioxide (CO_2) and dioxygen (O_2). In this sense, it's known that chitosan has a selective permeability to gasses, CO_2 and O_2 (Costa *et al.*, 2017; Dhumal and Sarkar, 2018), and high resistance to heat due to hydrogen-bonded bridges (Randazzo *et al.*, 2018).

Although more studies of the fertile egg of old hens where the shell is more porous are still needed, in the present study the antibacterial activity of chitosan biofilm provided a practical tool against *SE* in fertile and table eggs, because the chitosan did not affect egg weight and chick weight (Table 2), relevant parameters in the poultry industry.

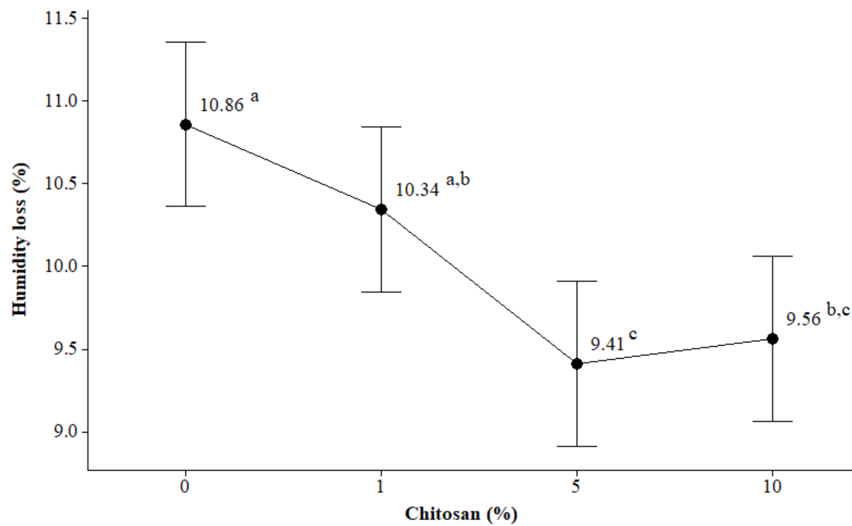

Figure 3. Effect of 0, 1, 5 and 10% chitosan on humidity loss, *n*= 80 incubated fertile eggs.

CONCLUSIONS

The surfaces coated with chitosan biofilm resisted the formation of *Salmonella enteritidis* biofilm. The comparison between treatments showed differences in the total recovered *Salmonella enteritidis* on the eggshell, the lowest values being obtained with the chitosan treatments. These results support the hypothesis that chitosan biofilm interacts with *Salmonella enteritidis* through a combination of electrostatic, hydrophobic and chelating properties. Because the reactive amino groups ($-NH_2$) present in the chitosan biofilm and positively charged, bind to negatively charged cell surface molecules such as lipopolysaccharide and outer membrane proteins. At the same time, chitosan can chelate metal ions which are crucial for the stability of the bacterial cell membranes, and thus cause the disruption of cell surface structure. Overall, these data support further investigations for larger-scale application of a chitosan biofilm that offers good antibacterial activity against *Salmonella enteritidis* in fertile and table eggs, because the chitosan did not affect egg weight and chick weight, relevant parameters in the poultry industry.

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