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Efficiency of chitosan synergism with clove essential oil in the coating of intentionally contaminated Tambaqui fillets

Eficiência do sinergismo de quitosana com óleo essencial de cravo no revestimento de filés de tambaqui contaminados intencionalmente

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Highlights:
Gram-positive bacteria are more sensitive to chitosan and clove essential oil than Gram-negative bacteria.
The edible coating of chitosan with clove essential oil significantly reduced the number of *L. monocytogenes* and *S. aureus* in the Tambaqui fillets.
Clove essential oil potentiated the antimicrobial effect of chitosan on Tambaqui fillets.

Abstract

The edible coating of chitosan with clove essential oil (CEO) was studied for its ability to reduce the microbial growth of pathogens (*Escherichia coli* O157:H7 CDCEDL933, *Listeria monocytogenes* CERELA, *Salmonella* Enteritidis ATCC13076, *Staphylococcus aureus* ATCC43300, and *Pseudomonas aeruginosa* ATCC27853) in Tambaqui fillets kept under refrigeration. In *in vitro* tests, chitosan showed higher antimicrobial activity against *S. aureus* and *L. monocytogenes* (MIC 0.5%), and CEO for *L. monocytogenes* (MIC 0.08%). Based on the antimicrobial activity of chitosan and CEO, Tambaqui fillets were subjected to different treatments, T1: chitosan 2%; T2: chitosan 2% + CEO 0.16%, and T3: chitosan 0.5% + CEO 0.08%, kept at 4 ºC for 72 h. The chitosan coating, incorporated with CEO, inhibited microorganisms in Tambaqui fillets and enhanced coating efficiency ($p < 0.05$). It was most effective against *L. monocytogenes* and *S. aureus* at the lowest CEO concentration (0.08%). Chitosan coating in combination with CEO enhanced the antimicrobial effect of pathogens on Tambaqui fillets, increased their shelf life under refrigeration, and was more effective against Gram-positive pathogens than Gram-negative pathogens.
Key words: Edible coating. *Listeria monocytogenes*. Natural antimicrobials. Pathogens.

Resumo

O revestimento comestível de quitosana com óleo essencial de cravo (OEC) foi estudado por sua capacidade em reduzir o crescimento microbiano de patógenos (*Escherichia coli* O157:H7 CDCEDL933, *Listeria monocytogenes* CERELA *Salmonella* Enteritidis ATCC 13076, *Staphylococcus*

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aureus ATCC43300 e *Pseudomonas aeruginosa* ATCC27853) em filés de tambaqui mantidos sob refrigeração. Nos testes *in vitro*, a quitosana apresentou maior atividade antimicrobiana para *S. aureus* e *L. monocytogenes* (CIM 0,5%) e o OEC para *L. monocytogenes* (CIM 0,08%). Com base na atividade antimicrobiana da quitosana e OEC, os filés de Tambaqui foram submetidos a T1: quitosana a 2%; T2: quitosana 2% + OEC 0,16% e T3: quitosana 0,5% + OEC 0,08%, mantidos a 4 ºC por 72 h. O revestimento de quitosana, incorporado ao OEC, inibiu os micro-organismos nos filés de Tambaqui aumentando a eficiência do revestimento ($p< 0,05$); e foi mais eficaz para *L. monocytogenes* e *S. aureus* na menor concentração do OEC (0,08%). O revestimento de quitosana quando combinado ao OEC aumentou o efeito antimicrobiano de patógenos nos filés de Tambaqui, aumentando sua vida útil sob refrigeração, sendo mais eficaz contra patógenos Gram positivos do que os patógenos Gram negativos.
Palavras-chave: Antimicrobianos naturais. *Listeria monocytogenes*. Patógenos. Revestimento comestível.

Introduction

Worldwide aquaculture production continues to grow faster than any other food sector (Food and Agriculture Organization [FAO], 2018). In Brazil, Tambaqui (*Colossoma macropomum*) is the most predominant native fish species produced via aquaculture and is second overall, with the non-native Nile tilapia being the most produced species. Tambaqui, which originates from the Amazon River basin, makes up 27.7% of the total fish production in Brazil (Seafood Brazil, 2018).

The search for healthier and less processed foods has contributed to the increased commercialization of fish fillets, due to their nutritional value and ease of which it can be preparation (Yu, Li, Xu, Jiang, & Xia, 2017). When compared to other types of animal meat, fish have a shorter shelf life due to postmortem changes caused by endogenous biochemical reactions (Yu et al., 2018) and, their chemical composition, which favors the rapid growth of pathogenic microorganisms, leading to spoilage (Chaparro-Hernández et al., 2015).

One way to protect fish and fish fillets while minimizing microbial spoilage is the application of edible coatings (EC), that act as a barrier against moisture, oxygen, and light, thereby preventing dehydration, lipid oxidation, and color degradation (Martínez, Salmerón, Epelde, Vicente, & Vega, 2018). Polysaccharide coatings are the most commonly used type of EC. Chitosan, a polymer derived from chiton, is an ideal biopolymer for food application. It is a natural, biodegradable, biocompatible, and displays antimicrobial and antioxidant properties. Chitosan also has the ability to form edible films and coatings, and has been classified as a Generally Recognized as Safe (GRAS) product since 2001 (United State Food and Drug Administration [USFDA], 2013). When combined with lipids such as essential oils, chitosan EC can display optimized characteristics (Dehghani, Hosseini, & Regenstein, 2018).

Essential oils are rich in bioactive compounds, making these lipids suitable for incorporation into EC, as they enhance the preservative effect of the coating by attributing sensory characteristics to the food (Sivakumar & Bautista-Nanos, 2014). The multifunctional bioactivity of clove essential oil can be attributed to its main compound, eugenol (Heredia-Guerrero et al., 2018).

Although several studies have reported the efficacy of chitosan in preserving the quality of freshwater fish such as grass carp (Yu et al., 2018), Nile tilapia (Alsaggaf, Moussa, & Tayel, 2017) and rainbow trout (Ozogul et al., 2017), there are no reports of chitosan associated with clove essential oil – CEO used in the microbiological preservation of Tambaqui fillets kept under refrigeration. The objective of this study was to evaluate the efficacy of chitosan association with clove essential oil in the edible coating of intentionally contaminated Tambaqui fillets.

Material and Methods

Microorganisms

The microorganisms used were *Escherichia coli* O157:H7 CDCEDL933, *Listeria monocytogenes* CERELA, *Salmonella* Enteretidis ATCC13076, *Staphylococcus aureus* ATCC43300 and *Pseudomonas aeruginosa* ATCC27853. The strains were activated on tryptone soy agar (Himedia®, Mumbai, India) and incubated at 37 ºC for 24 h.

Chitosan antimicrobial activity

High molecular weight chitosan (310 kDa) with a 91.5% deacetylation degree, obtained from Polymar Indústria e Comércio LTDA (Fortaleza, Ceará, Brazil), was used. The stock solution was prepared by dissolving chitosan 2% (w/v) in 1% (v/v) lactic acid solution with agitation using a magnetic stirrer for 24 h. Aliquots of 50 µL of the bacterial suspension (10^7 CFU mL^{-1}) were inoculated into nutrient agar plates (Himedia®) along with 100 µL of chitosan solution at different concentrations (0.0625%, 0.125%, 0.25%, 0.5%, 1%, and 2%) and incubated at 37 °C for 24 h, in triplicate. As control, nutrient agar plates inoculated with sterile water and 1% lactic acid solution were used.

Chitosan inhibition rate (IR) was calculated using the equation:

$$\text{IR}\ (\%) = \frac{\text{N1 - N2}}{\text{N1}} \times 100$$

Where IR is the percentage inhibition rate; N1 is the number of colonies on sterile water plates and N2 is the number of colonies after treatment at different concentrations of chitosan (Wang & Wang, 2011).

The minimum inhibitory concentration (MIC) was defined as the lowest concentration capable of reducing 99% of viable bacteria and the minimum bactericidal concentration (MBC) was defined as the lowest concentration capable of eliminating 99.9% of viable bacteria (Kuete et al., 2008).

Antimicrobial activity of clove essential oil

Clove essential oil (*Syzygium aromaticum,* CEO) was obtained commercially in 10 mL volumes from Terra Flor Aromatherapy© (Alto Paraíso, Goiás, Brazil). The antimicrobial activity of the CEO was tested via plate diffusion disc technique according to the M2-A8 protocol of the National Committee for Clinical Laboratory Standards [NCCLS] (2003), adapted for natural products. The bacterial suspension (10^7 CFU mL^{-1}) was seeded on Mueller-Hinton agar plates (Himedia®) and filter paper discs of 6 mm in diameter were imbibed with 10 µL CEO. The reading was obtained after 18 - 24 h of incubation at 37 ºC by growth inhibition halos using a digital caliper.

To determine the MIC, 1 g of CEO was diluted using methanol to a concentration of 640 mg mL^{-1} (Solution I). A second 1:100 dilution was performed using Mueller-Hinton broth (Himedia®) at a concentration of 6.4 mg mL^{-1}. The MIC was determined based on Clinical and Laboratoy Standards Institute document M07-A10 (CLSI, 2018) with final concentrations at 0.32%, 0.16%, 0.08%, 0.04%, 0.02%, 0.01%, 0.005%, and 0.0025%, in triplicate. Readings were obtained with 0.01% (w/v) sodium resazurin dye (Sigma-Aldrich, Sant Louis, EUA). The MIC and MBC are previously defined in section 2.2.

Edible coating of Tambaqui fillets

The 500 mL coating solution was composed of chitosan (10 g; final concentration of 2%) and 85% lactic acid (5.88 mL; final concentration of 1% w/v). Chitosan powder was dissolved by stirring continuously for 2 h (Cahú et al., 2012) then the pH was adjusted to 6.0. CEO (0.4 g and 0.8 g) was then subsequently added to achieve a final concentration of 0.08% and 0.16%, respectively.

Fresh Tambaquis weighing approximately 6 kg were commercially purchased, filleted into 100 g portions, and separated into groups according to

the test microorganism (*E. coli, L. monocytogenes, S.* Enteritidis*, S. aureus* and *P. aeruginosa*). The antimicrobial activity of the chitosan + EOC coating was evaluated according to Barbosa et al. (2009), with adaptations. Initially, microbiological analysis was performed on the fillets to verify the presence of the test-pathogens. For intentional contamination, the fillets were inoculated with a bacterial suspension of 10^6 CFU mL^{-1} for each indicator pathogen. After 30 minutes of contact, the fillets (9 per treatment) were immersed in 500 mL in the following coating treatments for 10 seconds: T1 (chitosan 2%), T2 (chitosan 2% + CEO 0.16%), T3 (chitosan 0.5% + CEO 0.08%), and control (sterile water). The samples were then dried for 1 minute at 25 °C. Each fillet was subsequently packaged using a plastic polyethylene film and were stored at 4 ºC ± 1 °C for 72 h. Analyses for each pathogen were performed at 24, 48 and 72 h intervals (Silva et al., 2010). All assays were performed in triplicate.

Statistical analysis

The dates were subjected to analysis of variance (ANOVA) and means evaluated by Tukey test at 5% significance ($p<0.05$). The analyses were performed using the statistical program R Core Team (R Core Team [R], 2017).

Results and Discussion

Chitosan antimicrobial activity

Chitosan was more effective against Gram-positive bacteria than Gram-negative bacteria, especially *S. aureus,* whose growth was inhibited with 0.5% chitosan (Table 1). The MIC and MBC of chitosan were only found for *L. monocytogenes* and *S. aureus* (Table 1). It was not possible to determine the antimicrobial activity of chitosan at concentrations greater than 2% as it became more viscous at high concentrations, making it difficult to carry out the test. The antimicrobial activity of chitosan against *L. monocytogenes* and *S. aureus* was attributed to a greater amount of positive charges present in the chitosan molecule as its concentration increases. The protonated amino groups ($NH3^+$) within chitosan selectively bind to the cell membrane of negatively charged microorganisms and alter their cell activity and membrane permeability (Cai et al., 2015). This behavior was also observed by Wang and Wang (2011): by analyzing the antimicrobial activity of chitosan and its derivatives, they observed the inhibition of *S. aureus* due to an increased concentration of chitosan.

For Gram-negative bacteria, the chitosan inhibition rate varied, revealing that it was not influenced by an increase in concentration. The highest inhibition rate (93.87%) was obtained for *P. aeruginosa*, while *E. coli* showed greater resistance to chitosan with an inhibition rate below 58.98% and high lactic acid interference (Table 1). The antimicrobial action of chitosan is influenced by several factors, such as environmental conditions, chemical substrates present in the environment, structural conditions such as the degree of deacetylation, molecular weight, and forms derived from chitosan and its concentration, among others (Hosseinnejad & Jafari, 2016).

Table 1
Chitosan inhibition rate (%) against food-borne pathogenic microorganisms

Microorganisms	Chitosan Concentration (w / v) / Inhibition Rate (%)								
	LA 1%	2	1	0.5	0.25	0.125	0.0625	MIC	CBM
E. coli O157:H7	52.40	16.09	08.32	19.36	54.70	33.19	58.98	-	-
L. monocytogenes	05.20	100.0	97.20	96.80	81.30	72.00	58.20	2.00	2.00
P. aeruginosa	60.40	93.87	88.68	86.65	78.09	71.08	81.85	-	-
S. Enteretidis	40.64	76.42	62.65	61.05	54.98	66.80	69.00	-	-
S. aureus	12.56	100.0	100.0	100.0	99.62	98.84	93.27	0.25	0.50

LA: lactic acid; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration
- not inhibition.

Among Gram-negative microorganisms, *E. coli* O157:H7 has been considered one of the most acid-resistant serovars (Mittelstaedt & Carvalho, 2006). A 1% solution of lactic acid showed significant inhibition of the pathogen *E. coli* O157:H7 (Table 1). The undissociated form of lactic acid (weak organic acid), when accumulated in the cell membrane, is thought to promoted membrane disruption by inhibiting essential metabolic reactions (Jay, 2005). The resistance presented by *E. coli* O157:H7 to chitosan has been related to several adaptive genetic mechanisms of virulence, and is necessary for its survival (Khachatryan, Hancock, Besser, & Call, 2005).

Antimicrobial activity of clove essential oil

The antimicrobial activity of CEO in the disc diffusion test showed weak inhibition of *P. aeruginosa* [<13.9 mm (Silveira et al., 2012)], moderate inhibition of *S.* Enteretidis, *E. coli,* and *S. aureus* (14 - 18 mm), and strong inhibition ($p<0.05$) of *L. monocytogenes* (> 18 mm) (Table 2).

Table 2
Antimicrobial activity of clove essential oil by disc diffusion and broth dilution against pathogenic microorganisms of food interest

Microorganisms	Antimicrobial activity					
	Disc diffusion (mm)		Broth dilution (**µg** mL^{-1})			
	Inhibition halos	CLO	MIC	MBC	CLO	Methanol
E. coli O157:H7	15.08 ± 0.41c	30.38	1600	-	60	NI
L. monocytogenes	19.36 ± 0.63a	26.03	800	-	30	NI
P. aeruginosa	09.75 ± 0.52d	21.09	3200	-	120	NI
S. Enteretidis	15.51 ± 1.57c	28.53	1600	-	30	NI
S. aureus	17.19 ± 0.42b	31.51	1600	-	60	NI

Area of inhibition expressed as the average of three repetitions ± standard deviation. Degree of inhibition: < 13.9 mm: weak; 14 - 18 mm: moderate; >18 mm: strong (Silveira et al., 2012). NI: no inhibition, CLO: chloramphenicol. MIC: Minimum Inhibitory Concentration. MBC: Minimum Bactericidal Concentration. High activity: 50 - 500 µg mL^{-1}; moderate activity: 500 - 1500 µg mL^{-1}; weak activity: > 1500 µg mL^{-1} (Sartoratto et al., 2004). Averages followed by the same letter in the column are not significantly different ($p<0.05$).

The results of the diffusion disc test differed from the data obtained from the broth dilution test (MIC), with CEO showing weak antimicrobial activity against *P. aeruginosa, S.* Enteretidis*, E. coli,* and *S. aureus* [>1500 **µg** mL^{-1} (Sartoratto et al., 2004)], and moderate antimicrobial activity for *L. monocytogenes* (500 - 1500 **µg** mL^{-1}) (Table 2). The concentrations obtained in the MIC experiments were found be bacteriostatic.

The greater sensitivity of Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) to CEO is related to the simple structure of their cell wall, compared to the complexity of that of Gram-negative bacteria that are polysaccharide-rich, inhibiting the penetration of antimicrobial substances (Burt, 2004). The bactericidal activity of CEO has been attributed to its majority constituent, eugenol (low molecular weight), and the lipophilic components of essential oils that promote high cell membranes penetration with extravasation of other cellular components, including intracellular proteins, and cell death (Devi, Nisha, Sakthivel, & Pandian, 2010).

When comparing the two methods used (disk diffusion and MIC), results indicate that the diffusion method can be used as a preliminary method because it determines the sensitivity of many microorganisms to certain substances, is easy to perform, and requires small amounts of sample, presenting semiquantitative results (Kalemba & Kunicka, 2003).

Antimicrobial potential of chitosan coating associated with clove essential oil in intentionally contaminated Tambaqui fillets

To ensure that initial number of bacteria in the fish fillets was the same as that in the inoculum used in the initial contamination, microbial analysis of the fillets in fresh fish was performed before treatments, which showed that the fillets had zero counts for the test bacteria (Table 3). All treatments were effective in reducing *S. aureus* in Tambaqui fillets in the first 24 h of storage, with a reduction of 2.9 log CFU g^{-1} cycles in T2 (Table 3) when compared to the control. Total inhibition of *S. aureus* was obtained at 24 h in T3, and at 48 h in T1 and T2. The control reduced ~ 2 log CFU g^{-1} cycles (due to immersion in water), maintaining the same growth (bacteriostatic effect) until the end of storage ($p<0.05$). The T3 treatment showed a higher efficacy in pathogen reduction after 24 h ($p<0.05$), demonstrating that the synergism of chitosan and CEO at lower concentrations is more effective.

Fillets contaminated with *L. monocytogenes* also showed growth reduction ($p<0.05$) in the T3 treatment after 48 h (Table 3). The chitosan + CEO effect (T2 and T3) had the best antilisterial effect, with a reduction of ~ 3 log CFU g^{-1} cycles after 24 h in T2 treatment, and 2.3 log CFU g^{-1} cycles in T3 (Table 3). The microbial reduction in the control sample was not significant ($p>0.05$) (Table 3).

Similar to the results observed in *in vitro* tests, the treatments were more effective in reducing Gram-positive bacteria compared to Gram-negative bacteria. The coating was more effective for *P. aeruginosa*, particularly at the lowest concentration (T3), which showed a reduction of 3.46 log CFU g^{-1} cycles after 72 h compared to the control (Table 3).

The edible coating was more efficient against *S.* Enteritidis (reduction of 2.27 log cycles of CFU g^{-1}) after 48 h in treatment T2. In *E. coli* O157:H7*,* the T3 treatment showed less reduction in the first 24 h compared to T2 treatment, and at 72 h, the microbial reduction values in both treatments (T2 and T3) did not differ significantly (Table 3).

Table 3
Effect of chitosan coating and its association with clove essential oil on Tambaqui fillets intentionally contaminated with pathogenic microorganisms, kept under refrigeration (4 ºC)

Microorganisms	Initial Cont.	Time (h)	Control	T1	T2	T3
			log CFU g^{-1}			
S. aureus	0.0	0	6.00Aa	6.00Aa	6.00Aa	6.00Aa
		24	4.16Ab	4.28Ab	3.10Bb	0.00Cb
		48	4.27Ab	0.00Bc	0.00Bc	0.00Bb
		72	4.13Ab	0.00Bc	0.00Bc	0.00Bb
L. monocytogenes	0.0	0	6.00Aa	6.00Aa	6.00Aa	6.00Aa
		24	5.58Aab	3.31BCb	3.00Cb	3.70Bb
		48	5.29Abc	3.53Bb	2.19Cc	0.00Dc
		72	4.85Ac	3.19Bb	0.00Cd	0.00Cc
S. Enteretidis	0.0	0	6.00Aa	6.00Aa	6.00Aa	6.00Aa
		24	5.10Ab	4.69Bb	4.22Cb	4.74Bb
		48	5.18Ab	4.55Bb	3.73Cb	4.52Bb
		72	4.79Ac	4.08Bc	3.66Cb	4.22Bc
E. coli O157:H7	0.0	0	6.00Aa	6.00Aa	6.00Aa	6.00Aa
		24	6.06Aa	5.25Bb	5.27Bb	4.93Bb
		48	6.11Aa	5.05Bb	5.68Aab	4.81Bb
		72	5.72Aa	4.20Bc	4.56Bc	4.65Bb
P. aeruginosa	0.0	0	6.00Aa	6.00Aa	6.00Aa	6.00Aa
		24	5.62Ab	3.98Cb	4.31Bb	3.00Db
		48	5.41Ab	3.88Bb	3.47Cc	2.98Db
		72	5.08Ac	3.24Bc	2.93Bd	2.54Cc

Initial Cont: Initial contamination of fish. Control: uncoated sample; T1: chitosan 2%; T2: chitosan 2% + CEO 0.16%; T3: chitosan 0.5% + CEO 0.08%. Means followed by equal letters do not differ from each other by the Tukey test ($p<0.05$), and uppercase letters between rows and lowercase letters between columns are compared.

The difference in inhibition of Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*E. coli, P. aeruginosa* and *S.* Enteretidis) bacteria by the chitosan + CEO association (Table 1) can be attributed to chitosan's molecular weight (>310 kDa). High molecular weight of chitosan forms a film around Gram-positive bacterial cells, preventing nutrient absorption (Devlieghere, Vermeiren, & Debevere, 2004).

Another factor that interferes with coating efficiency is the bacterial cell envelopes. The cellular envelope of *L. monocytogenes* and *S. aureus*, composed mainly of peptide glycans, allows easier entry of molecules into the cell. The cell membrane of *E. coli, P. aeruginosa,* and *S.* Enteretidis are composed of lipopolysaccharides, phospholipids, and lipoproteins that act as a barrier against the penetration of high molecular weight molecules such as chitosan (Mohamed & Al-Mehbad, 2013).

Upadhyay et al. (2015) analyzed the effect of chitosan associated with phytochemicals and their results indicated that the incorporation of 0.4% eugenol significantly increased the antimicrobial coating efficacy, reducing *L. monocytogenes* counts by at least 4 - 5 log CFU g^{-1} at the end of 42 days of storage at 4 °C when compared to that of the control.

The antimicrobial efficacy of chitosan coating on Tambaqui fillets can be further attributed to the oxygen barrier that reduces the number of bacteria, specifically aerobic bacteria such as *P. aeruginosa* (Jeon, Kamil, & Shahidi, 2002).

Although the antimicrobial and antioxidant activities of essential oils have been demonstrated previously (Yu et al., 2018), one of the major limitations of their use is the negative effect they have on the taste of food; this may affect consumer preference for the fish fillets. Vieira et al. (2019) performed sensory analysis of chitosan + CEO in Tambaqui fillets and reported that after treating the Tambaqui fillets with chitosan (2%) and chitosan + CEO (0.16% and 0.08%, respectively), several parameters such as color, texture, and aroma were well accepted by the tasters, and a score >5 was assigned for these parameters (I liked it, I liked it a lot); however, the tasters assigned a score of 4 (neither like or dislike) for flavor in samples containing CEO. According to the authors, this response may be due the fact that clove is not a common ingredient in Bahian cuisine.

Conclusion

The synergism of chitosan coating with CEO is most effective in inhibiting Gram-positive microorganisms. Results indicate that CEO-associated chitosan coating is a potential alternative to improve the safety of chilled fishery products as it reduces spoilage-related microorganisms such as *P. aeruginosa,* as well as other important pathogens associated with food such as *S. aureus* and *L. monocytogenes*.

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