Antimicrobial evaluation of gelatin–based films incorporated with chitosan in the conservation of fish fillets

Avaliação antimicrobiana de filmes à base de gelatina incorporados com quitosano na conservação de filtes de peixe

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**ABSTRACT**
Materials obtained from biodegradable polymers can be an alternative to reduce the environmental impact caused by petroleum–derived polymers. Materials of different origins
have been considered as a raw material with technical and economic feasibility for the development of packaging films. However, it is required that these alternative materials, in addition to being biodegradable, have beneficial properties in food preservation. In this context, biodegradable films based on fish gelatin, and fish gelatin with chitosan incorporation, were prepared and characterized in terms of their mechanical properties (tensile strength (TS) and elongation (E)), permeation (WVP), and antimicrobial activity in the conservation of tilapia fillets (Tilapia rendalli). Regarding the mechanical and permeation properties, the results showed that the incorporation of chitosan to the gelatin films promotes an increase in TS and E, and a reduction in WVP. As for the antimicrobial property in the conservation of tilapia fillets, both films showed satisfactory activity against the pathogenic microorganism Staphylococcus aureus. These results indicate that fish gelatin–based films with chitosan incorporation are promising as active packaging in the conservation of fish fillets.

Keywords: biodegradable films, active packaging, Staphylococcus aureus.

ABSTRACT
Os materiais obtidos a partir de polímeros biodegradáveis podem ser uma alternativa para reduzir o impacto ambiental causado pelos polímeros derivados do petróleo. Materiais de diversas origens têm sido considerados como matéria-prima com viabilidade técnica e econômica para o desenvolvimento de filmes para embalagens. Porém, é necessário que esses materiais alternativos, além de serem biodegradáveis, tenham propriedades benéficas na preservação de alimentos. Nesse contexto, filmes biodegradáveis à base de gelatina de peixe e gelatina de peixe com incorporação de quitosana foram preparados e caracterizados quanto às suas propriedades mecânicas (resistência à tração (TS) e alongamento (E)), permeação (WVP) e atividade antimicrobiana em a conservação de filés de tilápia (Tilapia rendalli). Em relação às propriedades mecânicas e de permeação, os resultados mostraram que a incorporação de quitosana aos filmes de gelatina promove aumento de TS e E, e redução de WVP. Quanto à propriedade antimicrobiana na conservação de filés de tilápia, ambos os filmes apresentaram atividade satisfatória contra o microrganismo patogênico Staphylococcus aureus. Esses resultados indicam que filmes à base de gelatina de peixe com incorporação de quitosana são promissores como embalagens ativas na conservação de filés de peixe.

Palavras-chave: filmes biodegradáveis, embalagem ativa

1 INTRODUCTION
Both quality and food safety are crucial concerns for producers and researchers, while preserving the natural state of the material is a challenge (Guillard, et. al., 2018). New technologies are continually being studied to overcome these challenges. An important technology to provide fresh, high quality and safe food products is the use of biodegradable and biocompatible polymers in the production of packaging (Çakmak, et al., 2020). Biodegradable films are used as packaging materials to improve the fresh quality of food products, form a gas barrier against the surrounding atmosphere, decrease moisture loss from food material and reduce the use of non–degradable packaging materials based on petroleum (Marsh and Bugusu, 2007; Rego, et al. 2020).
In this context, protein–based films stand out, as they have excellent nutritional value, relatively low cost, good film formation capacity, in addition to functional groups, such as amino groups (–NH₂) and carboxyl groups (–COOH) that enable the interaction with other materials of interest (Koshy, et al., 2015). However, despite all the advantages, in general, protein–based films have weak mechanical properties, high solubility and high water vapor permeation, factors that limit their use (Wittaya, 2012). However, the physical and microbiological performance of fish gelatin–based films can be improved by combining, in their matrix, other renewable materials, such as chitosans (Pérez–Córdoba, et al., 2018).

Chitosan is a cationic polysaccharide, obtained from the deacetylation of chitin (Moura, et al., 2015). It has unique biological characteristics, such as biodegradability, biocompatibility, non–toxicity, and antimicrobial and antibacterial properties (Priyadarshi and Rhim, 2020). In addition, chitosan has an excellent ability to form films, which makes it a material with great potential for the development of active food packaging, either alone or in blends with other natural products (Franco, et al., 2020). In this context, chitosan, when combined with gelatin, can provide antibacterial activity to films and an improvement in mechanical properties, in addition to contributing to the conservation of food products, especially highly perishable products, such as fish fillets (Baptista, Horita and Sant’ana, 2019).

Foods such as fish fillets are highly perishable because they have some characteristics, such as, high water content, lower proportion of connective tissue, high content of autolytic enzymes, lower pH drop after death and high microbiota (Oliveira, et al., 2017). These peculiarities lead to a rapid deterioration of the fish, which are responsible for large losses during postmortem storage. Therefore, one of the ways to extend the fish's useful life is to apply appropriate conservation methods. In this sense, biodegradable films combined with refrigeration have stood out for promoting improvements in food quality, since it protects them from biological, physical and chemical deterioration, resulting in a long and safe life (Tharanathan, 2003).

In this work, fish gelatin–based films and fish gelatin–based films incorporating chitosan were produced. Both were evaluated for their mechanical properties (tensile strength (TS) and elongation (E)), water vapor permeability (WVP), optical surface (SEM), and when the inhibition of Gram–positive bacteria (Staphylococcus aureus) in the conservation of tilapia fillets (Tilapia rendalli).
2 MATERIALS AND METHODS

2.1 MATERIALS

All reagents used were of analytical grade. Fish skin gelatin (bloom number 220), chitosan (85% deacetylation degree, 120 kDa molecular weight) and glycerol (99.5% purity) were purchased from Sigma–Aldrich, Brazil. To carry out the microbiological analysis, the fish fillets were placed in a suitable container and kept refrigerated for 24 hours at –18ºC, for subsequent microbiological analysis.

2.2 FILM PREPARATION

2.2.1 Fish Gelatin–Based Films

Gelatin–based films were made according to Bandeira, et al., (2015). The fish gelatin solution, containing 1.8 g (w/v) of gelatin, was stirred for 30 min on a magnetic stirrer (FISATOM 752A, Brazil) at 300 rpm and a temperature of 30 ± 2ºC. Afterwards 0.2 g of glycerol (as a plasticizer) was added, and the pH was adjusted to 4.0 using 0.1 mol L⁻¹ NaOH solution. The filmogenic solution was then stirred for another 2 hours and filtered through a funnel with Whatman filter paper nº 4. Then, 50 mL of the film–forming solution was poured into plexiglas plates (17 cm²). The schematic representation of the process of preparing fish gelatin film with glycerol is shown in Figure 1.

![Figure 1. Schematic representation of the fish gelatin film.](image)

The plates containing the film–forming solution were placed in an oven with forced air circulation for 24 hours at 40ºC. Then, the films were removed from the plates and placed in a desiccator at 25 ± 1ºC for 48 hours before characterization.
2.2.2 Films Based On Fish Gelatin With The Incorporation Of Chitosan

Initially, 0.2 g of each chitosan sample was dissolved in 20 mL of acetic acid solution (0.1 mol L\(^{-1}\)) under constant stirring at 300 rpm in a magnetic stirrer (Marte, MAG – 01H, Brazil) for 2 hours. Subsequently, 30 mL of fish gelatin solution (5.3% w/v) and 0.2 g of glycerol (as a plasticizer) were added to each solution, and the pH was adjusted to 4.0 using 0.1 mol L\(^{-1}\) NaOH solution (Bandeira, et al., 2015). The solution was stirred at 300 rpm for 2 hours to form the mixture. Then, the solutions (50 mL) were poured onto the plexiglas plates. The schematic representation of the production process of fish with chitosan film with glycerol uptake is shown in Figure 2.

**Figure 2.** Schematic representation of the preparation of the film based on fish gelatin with the addition of chitosan.

The plates containing the film–forming solution were placed in an oven with forced air circulation for 24 hours at 40\(^\circ\)C. Then, the films were removed from the plates and placed in a desiccator at 25 ± 1\(^\circ\)C for 48 hours before characterization.

2.3 FILM CHARACTERIZATION

2.3.1 Mechanical Properties (Ts And E)

The mechanical properties (TS and E) were measured by a texture analyzer (Stable Microsystems SMD TA.XP2i, UK) according to the standard ASTM D–882–02 (ASTM 2000a), aided by Texture Expert Exceed software 2.61 (Stable Micro Systems, Godalming, England). The film samples were cut to sizes 25 mm wide and 100 mm long. Then they were fixed to the equipment with a 50 N load cell, with an initial separation distance between the claws of 50 mm and a traction speed of 2 mm s\(^{-1}\).
2.3.2 Water Vapor Permeability (Wvp)

The water vapor permeability (WVP) of biopolymer films was determined by the gravimetric method at 25°C, using the standard method E0096–00 (ASTM, 2000b). The samples of each film, in the form of discs (50 mm in diameter), were fixed in permeation cells, containing granulated anhydrous calcium chloride (CaCl₂), and these were stored in desiccators at 25°C and 75% relative humidity (RH). From the weight gain of the granulate CaCl₂, measured at 24 hours intervals for 7 days, it was possible to determine the water vapor transfer through the film, according to Equation (1).

\[
WVP = \frac{m_{ab}}{t} \times \frac{e}{A \times \Delta P}
\]

where, WVP to water vapor permeability (g m Pa⁻¹ s⁻¹ m⁻²), \(m_{ab}\) is moisture absorbed weight (g), \(t\) is the duration of the tests (s), “\(e\)” is the average film thickness (m), \(A\) is the exposed film surface area (m²) \(\Delta P\) is the partial pressure difference across the film (Pa).

2.3.3 Scanning Electron Microscopy (SEM)

The evaluation of the surface of two films and of the blends was performed by scanning electron microscopy (SEM), using an electron microscope (Jeol, JSM 6060, Japan). The samples were placed on stainless steel supports (stubs) and metallized with gold. The analysis was performed on the acceleration voltage of 10 kV and magnification of 500 times (Li, et al., 2010).

2.4 MICROBIOLOGICAL EVALUATION OF GELATIN FILMS AND FILMS WITH INCORPORATION OF CHITOSAN IN THE CONSERVATION OF TILAPIA FILLETS

To perform the antimicrobial activity of films, the fillets were previously thawed under refrigeration at 6 ± 1°C for 24 hours. Afterwards, the fillets were packed with different films (gelatin and gelatin/chitosan), and stored in a refrigerator for 48 hours at 6 ± 1°C. After this time, the analyzes were carried out to verify the presence of *Staphylococcus aureus*, according to the standard method described by Tallent et al., (2016).

The samples were first homogenized in stomarc (MARCONI – MA440, Brazil) with lactated broth (25 g of the sample and 225 mL of broth) and incubated at 35°C for 48 hours. After preparing and diluting the samples, aliquots of 0.1 mL of the dilutions were seeded in the spread plate method on plates containing Baird–Parker agar (BP) and incubated at 35°C for 48
hours. The plates with typical and atypical colonies were counted and from each plate, on average, three to five colonies were selected, and they were transferred to tubes containing Brain Heart Infusion (BHI) broth. These tubes were incubated at 37ºC for 6 hours to perform the coagulase test. After the period of incubation and turbidity of the broths, 0.5 mL were transferred to tubes containing 0.5 mL of rabbit plasma with rehydrated EDTA, and the incubation was carried out at 37ºC. The coagulase reaction is considered positive when the tubes form a firm clot after 6 hours of incubation.

3 RESULTS AND DISCUSSION
3.1 FILM CHARACTERIZATION
3.1.1 Mechanical Properties (Ts And E) And Water Vapor Permeability (WVP)
Films and/or blends produced for application in food packaging, need to present good mechanical properties and extensibility mainly due to external stress (Cazón, et al., 2017). In addition to mechanical properties, films with low water vapor permeability are necessary, because one of the main functions of food packaging is to prevent/reduce the transfer of moisture between food and the environment (Siracusa, 2012). The values of mechanical properties (TS and E) and water vapor permeability (WVP) of fish gelatin films and fish gelatin films with chitosan addition are shown in Table 1.

Table 1. TS, E and WVP values of fish gelatin and fish gelatin/chitosan films.

| Film                  | TS* (MPa) | E* (%) | WVP* (g m Pa⁻¹ s⁻¹ m⁻² × 10¹¹) |
|-----------------------|-----------|--------|---------------------------------|
| Fish gelatin          | 12.8 ± 1.7 | 5.8 ± 1.3 | 1.62 ± 0.02                     |
| Fish gelatin/chitosan | 17.6 ± 1.5 | 8.2 ± 1.1 | 1.38 ± 0.01                     |

* Values for each sample are means ± standard deviation (n = 3).

It is observed in Table 1, the film prepared with fish gelatin, showed lower values TS and E, and the higher WVP value when compared with films in which chitosan was added. This behavior is mainly due to the types of bonds and interactions that occur between gelatin/glycerol, and gelatin/glycerol/chitosan.

For better understanding, a schematic of the interactions between the components of the films is shown in Figure 3 (a, b and c).
It can be seen in Figure 3 (b) that the interactions existing in the gelatin and glycerol film are basically hydrogen bonds, and that the addition of the polyalcohol promotes a separation of the protein chains in relation to films without glycerol as shown in the Figure 3 (a), as these form hydrogen bonds between the hydroxyl groups of the polyalcohol and the carboxylic acids present in the protein chain. When chitosan was added, TS and E increased and WVP decreased (Table 1), and this may have occurred because in addition to hydrogen bonds, electrostatic interactions were promoted between protonated amino groups and chitosan carboxylic acids, in addition to hydrogen bonds with glycerol. These new connections and interactions promoted
a closer approximation between the polymer chains, causing an increase in TS, a slight increase in E and a decrease in WVP, due to the difficulty of forming permeation ducts.

These results confirm that as for the mechanical properties and water vapor permeation, the blends of fish gelatin with the incorporation of chitosan are quite promising with regard to the replacement (although in part) of petroleum–derived materials, since the values of TS and WVP, were within the range of values found for synthetic films, as mentioned by Coutinho et al., (2003) who found TS values in the range of 5.2^{-11} MPa and Dias et al., (2013) with WVP values around 1.78 ± 0.29×10^{-11} (g m Pa^{-1} s^{-1} m^{-2}) for low density polyethylene films.

SCANNING ELECTRON MICROSCOPY (SEM)

Figure 4 (a) and (b) shows the microscopic images of the surface of gelatin films and gelatin films with the incorporation of chitosan, respectively.

Figure 4. SEM of the films (a) fish gelatin and (b) fish gelatin incorporating chitosan.

It can be seen in the SEM images that the gelatin film surface (Figure 4 (a)) has a smooth and homogeneous appearance. When chitosan was added to the gelatin films (Figure 4 (b)), they showed fibrous structures on the surface. These results corroborate the behaviour of the mechanical properties and water vapor permeability.

3.1.2 Microbiological Evaluation Of Films Against Staphylococcus Aureus In The Conservation Of Tilapia Filets

Both films were tested as active packaging against Staphylococcus aureus, in the preservation of tilapia fillets. Figure 5 presents the photographic images of the tilapia fillets packed with the films of pure gelatin and gelatin with the incorporation of chitosan, as well as the respective images of the plates containing typical and atypical colonies for Staphylococcus aureus and the test tubes containing the samples colonies.
Figure 5. Fillets packed with films (a) gelatin and (b) gelatin/chitosan, and the respective results for *Staphylococcus aureus*.

Tilapia fillet packed with active film

*Staphylococcus aureus*

It can be seen in the images in Figure 5 (a) and (b) that, both the gelatin film and the gelatin/chitosan film, presented negative results for the investigated microorganism. These results corroborate the results of Matiacevich et al., (2013), who studied the thermo–physical and antimicrobial properties of gelatin–based films of different origins (bovine and salmon skins) and verified the impact of the incorporation of chitosan in these films. The authors concluded that chitosan in the proportions of 0.5 and 1.0%, improved the physical performance of gelatin films, especially when using fish gelatin. The authors found that both films of pure gelatin, as well as gelatin with chitosan, had an antimicrobial effect against *E. coli*, *L. monocytogenes* and *Salmonella thyphimurium*, films produced from salmon skins were more effective than those of bovine origin in antimicrobial activity. The authors further suggest that this result (mainly the antimicrobial activity of pure gelatin) could be related to the presence of oligopeptides with antimicrobial properties (similar to the amino groups present in the polysaccharide polymer chain), as a result of the partial hydrolysis of gelatin. According to Pereda et al., (2011), films composed of gelatin/chitosan have improved the mechanical and transport properties compared to single component films. However, the antimicrobial activity of chitosan depends on different factors, such as pH, interaction with other components and...
molecular weight, and it is necessary to consider that the antibacterial properties are a combination of the effect of chitosan and acetic acid. According to Liu et al., (2006), the acetic acid used as a chitosan solvent with concentrations above 200 ppm (0.02% w/w) showed antibacterial activity against *E. coli* at pH 5.4. Therefore, the authors concluded that the antimicrobial effect observed in the films is attributed to the combination of chitosan and added acetic acid.

However, the mechanisms of antimicrobial action of these materials are not clearly detailed, since each component can exhibit a unique mechanism of action that is specific to a specific range of foods and microorganisms. Among these mechanisms, some have been identified, such as damage to the cell wall, interaction and rupture of the cytoplasmic membrane, damage to membrane proteins, leakage of cellular components, coagulation of the cytoplasm and depletion of the proton motor force. All of these effects produce the death of the microorganism by modifying the structure and composition of the bacteria cells (Biji, et al., 2015).

4 CONCLUSION

The results obtained for the films produced from these biomaterials, showed to be very promising as an active packaging, as they presented good physical–chemical and microbiological properties. The addition of chitosan in the gelatin–based films, promoted a significant increase in the TS of the films and decreased the E. In relation to WVP, it decreased with the addition of chitosan, because the increase in molecular interactions, caused an approximation of the polymer chains, reducing the molecular spaces for diffusion of water vapours through the films.

Regarding the surface analysis, it showed that the pure gelatin films presented a smooth and homogeneous structure, but when chitosan was incorporated, they presented fibrous structures on the surface, which suggests a change in molecular interactions and a possible molecular reorganization.

Regarding the microbiological analysis for the bacterium *Staphylococcus aureus*, the films totally inhibited their development in the packaged fillets, indicating that both films are quite promising in the application of antimicrobial packaging in the conservation of fish fillets.

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