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Efecto Inhibitorio de Películas de Quitosano y Papaína Activadas con Plasma en *Streptococcus mutans*

Inhibitory Effect of Plasma-Activated Chitosan-Papain Films on *Streptococcus mutans*

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Resumen
El quitosano es un polímero natural con gran potencial en diversas aplicaciones, ha sido ampliamente utilizado en diferentes áreas como la agricultura, para producir bactericidas y fungicidas, entre otros. La papaína es una enzima proteolítica, se utiliza comúnmente para el tratamiento de heridas, ablandamiento de carne, clarificación de cerveza, entre otras aplicaciones, posee propiedades medicinales y ha generado un interés significativo en acelerar el proceso de cicatrización y su actividad antimicrobiana. El objetivo de este trabajo es evaluar el efecto inhibidor de las películas de quitosano y papaína activadas con plasma sobre *Streptococcus mutans*. Las películas de quitosano-papaína se prepararon utilizando el método de fundición, incorporando quitosano y papaína. Las propiedades estructurales y térmicas de las películas se analizaron mediante espectroscopía infrarroja por transformada de Fourier (FTIR), revelando interacciones entre el quitosano y la papaína. Un ensayo de actividad enzimática confirmó la función proteolítica de la papaína. La eficacia antimicrobiana de las películas se evaluó mediante el método de difusión en disco de Kirby-Bauer, mostrando resistencia en todos los tratamientos con zonas de inhibición mínimas (< 10 mm). Aunque las zonas de inhibición eran pequeñas, las películas inhibieron el crecimiento bacteriano directamente donde se colocaron. El estudio concluye que las películas de quitosano-papaína exhiben cierta actividad antimicrobiana contra *S. mutans*. Sin embargo, se necesita una mayor optimización para mejorar su eficacia

Palabras clave: Papaína, películas, plasma, quitosano, *Streptococcus*.

Abstract
Chitosan is a natural polymer with great potential in various applications, has been widely used in different areas such as agriculture, to produce bactericides and fungicides, among others. Papain is a proteolytic enzyme, is commonly used for wound treatment, meat tenderizing, beer clarification, among other applications, it possesses medicinal properties and has generated significant interest in accelerating the healing process and its antimicrobial activity. The objective of this paper is to evaluate the inhibitory effect of chitosan and papain films activated with plasma on *Streptococcus mutans*. Chitosan-papain films were prepared using the casting method, incorporating chitosan and papain. The structural and thermal properties of the films were analyzed using Fourier Transform Infrared (FTIR) Spectroscopy, revealing interactions between chitosan and papain. An enzymatic activity assay confirmed the proteolytic function of papain. The antimicrobial efficacy of the films was assessed using the Kirby-Bauer disk diffusion method, showing resistance in all treatments with minimal inhibition zones (< 10 mm). Although the inhibition zones were small, the films inhibited bacterial growth directly where they were placed. The study concludes that chitosan-papain films exhibit some antimicrobial activity against *S. mutans*. However, further optimization is needed to enhance their efficacy.

Keywords: Chitosan, films, papain, plasma, *Streptococcus*.

INTRODUCTION

The consumption of crustaceans generates between 70–80% of waste, mainly from viscera and exoskeletons (García Zapata & Roca Ortega, 2014). In Mexico, the fishing industry produces approximately 60,000 tons of waste annually, with shrimp farming representing a major contributor to coastal pollution (Castellano, 2017; Gutierrez Avella, 2016; Martínez Sánchez et al., 2014). In response to environmental concerns, increasing attention has been directed toward biodegradable and low-impact materials.

Chitosan, obtained from the deacetylation of chitin, is a naturally abundant, renewable, biocompatible, and non-toxic polymer with strong biofilm-forming capacity (Moreno et al., 2012; Singla & Chawla, 2010). It is mainly extracted from the exoskeletons of shrimp, crabs, and other crustaceans (Ahmed & Ikram, 2016), and its large-scale production has been established in several regions, including Japan, North America, Poland, and Russia (Singla & Chawla, 2010). Chitosan can be processed into various forms such as powders, pastes, films, and fibers (Ahmed & Ikram, 2016).

Structurally, chitosan is an amino-polysaccharide composed of repetitive units of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose (Figure 1b). Its nitrogen content is approximately 6.89 %, which provides basic character due to the free amino groups (Giraldo, 2015). Chitosan is soluble in diluted organic acids such as acetic, lactic, and formic acids, and it is classified as chitosan when the degree of deacetylation of chitin (Figure 1a) is ≥50 % (Ahmad et al., 2015; Ahmed, 2015; Ahmed et al., 2014; Ahmed & Ikram, 2016; Giraldo, 2015; Skotak et al., 2011).

Chitin is the second most abundant natural biopolymer after cellulose and is the main structural component of the exoskeletons of crustaceans and insects (Giraldo, 2015). It is the primary source of chitosan, typically obtained by alkaline deacetylation. This process involves grinding the raw material, followed by deproteinization and demineralization, and finally treatment with concentrated sodium hydroxide at high temperatures (Singla & Chawla, 2010).

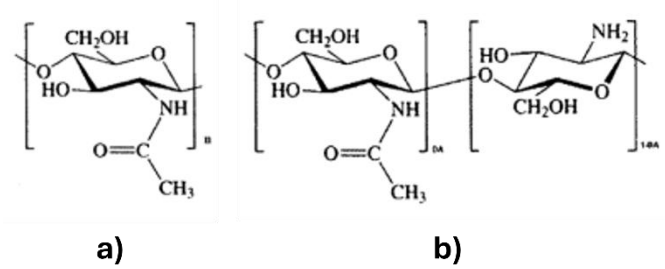

Figure 1. (a) Structure of chitin, (b) Structure of chitosan.Taken from Marguerite Rinaudo (2006).

Chitosan exhibits several unique characteristics, including biodegradability, biocompatibility, and non-toxicity (Ayala, 2015). These properties have promoted their use in agriculture (as bactericides, fungicides, and seed coatings), in healthcare (for wound dressings, bactericidal creams, and healing gels), and in wastewater treatment (Velásquez-Lárez, 2006). Furthermore, chitosan is an excellent biofilm-forming polymer, making it valuable for food packaging, surgical sutures, and drug delivery systems (Moreno et al., 2012; Singla & Chawla, 2010).

Biofilms are thin layers of biodegradable material with versatile applications across the food, medical, and agro-industrial sectors. They can incorporate active substances that provide additional properties, such as antimicrobial or enzymatic activity (Orozco Velandia, 2021). Biofilms are generally prepared by either the casting method, which involves dissolving or dispersing the biopolymer and evaporating the solvent, or by compression molding, which exploits the thermoplastic behavior of proteins and polysaccharides under pressure, temperature, and humidity (Orozco Velandia, 2021). Additives such as papain or advanced surface treatments like cold plasma can be integrated to further enhance their functional performance.

Papain is a cysteine protease from *Carica papaya* latex with well-documented proteolytic activity. It hydrolyzes peptide bonds at residues such as arginine, lysine, and glutamine, and is widely used in wound treatment, meat tenderization, and beer clarification (Areas et al., 2015). Structurally, papain consists of 212 amino acids, with cysteine at the catalytic site, a molecular weight of 33 kDa (precursor: 38.9 kDa), and three

disulfide bonds (Cys22–Cys63, Cys56–Cys95, Cys153–Cys200). It presents as a white amorphous powder, slightly hygroscopic, insoluble in water and most organic solvents, but soluble in ethanol and methanol. Its optimal activity occurs at 65 °C and pH 5–7, with an isoelectric point of 9.6. Due to its short shelf life, papain requires storage in a dry, cool environment (Diaz Aliaga, 2019; Areas et al., 2015).

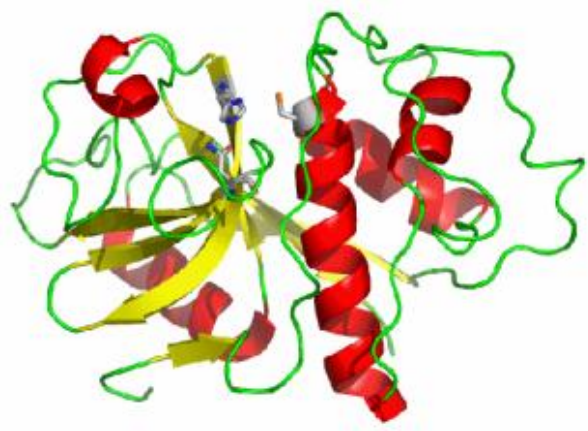

Figure 2. Structure of papain. Taken from Proteolytic Enzymes (2009)

Oral health involves not only caries and gingivitis but also soft tissue lesions affecting the palate, tongue, and floor of the mouth, which can cause pain and impair basic functions such as eating and speaking (Abid et al., 2015; Cárdenas, 2016; Singh et al., 2015; Misrachi & Espinoza, 2005). Infections often complicate wound healing, slowing tissue repair and favoring the proliferation of pathogens such as Streptococcus mutans, a major contributor to caries and periodontal disease (Katsura et al., 2001).

Papaya contains bioactive compounds, including proteolytic enzymes such as papain, with recognized antimicrobial and healing properties (Areas et al., 2015). Clinical and experimental studies have shown that papain-based formulations accelerate healing, reduce exudate, and lower infection rates by degrading devitalized tissue and modulating inflammation (Soares et al., 2015; Areas et al., 2015).

Wound healing is a complex but organized process involving inflammation, proliferation, angiogenesis, and granulation tissue formation, and remodeling through collagen deposition and contraction. Its progression is influenced by factors such as tissue environment, extent of injury, and systemic conditions (González Tuero et al., 2001).

In addition, plasma technology has emerged as a method to enhance the properties of polymeric materials. Plasma treatment modifies surfaces by introducing functional groups and improving hydrophilicity, without altering the bulk structure of the material (Reséndiz, 2005; Nageswaran & Neogi, 2015). Depending on the gas and parameters used, plasma can generate reactive species that interact with polymers, enhancing compatibility with biological systems. Both glow discharge (frequency plasma) and arc plasma are commonly applied, differing in their electrical potential characteristics (Eswaramoorthy & McKenzie, 2017).

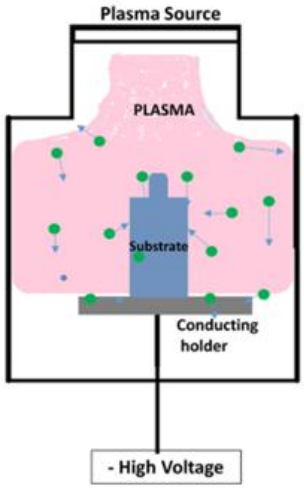

Figure 3. Schematic diagram of a glow discharge. Taken from Eswaramoorthy & McKenzie, 2017.

The application of plasma in wound dressings is still in its infancy, the purpose of these dressings is to prevent infection and promote the healing process (Eswaramoorthy & McKenzie, 2017), there are different types of dressings, these can be natural or synthetic polymers, fibers, gels, foam, among others, depending on the type of wound to be treated (Breitwieser et al., 2013). Considering this, the objective of this document is to evaluate the inhibitory effect of chitosan and papain films activated with plasma on *Streptococcus mutans*.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Streptococcus mutans*, provided by the Department of Nanobiosciences at the Chemical Sciences Faculty of the Universidad Autónoma de Coahuila (Saltillo, Mexico).

Preparation of chitosan-papain films

The extraction of chitin was from waste and shrimp residues provided by a local restaurant of Saltillo, Coahuila, México (Las Brazas). The shrimp were washed and ground, then demineralized in an acidic medium (generally hydrochloric acid, HCl), followed by alkaline hydrolysis to extract the chitin. Once the chitin is obtained, deacetylation is carried out in a basic medium, followed by washing and dehydration to obtain chitosan. Papain, casein, cysteine, trichloroacetic acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Co. Glycerol and phosphate salts (PBS) were purchased from Jalmek (Mexico). All reagents were of analytical grade and used as received.

The films were prepared following the methodology of Orozco et al. (2021). Using the "casting" method, the films were prepared by dissolving 1 % w/v chitosan and 0.1 % w/v papain in an aqueous solution containing 1 % v/v acetic acid, and then incorporating 1 % v/v glycerol as a plasticizer. 40 ml of the obtained solution was poured into disposable plates and then placed in stoves at 45 °C for 48 h. The films were manually recovered and placed between sheets of paper for storage.

Plasma treatment of films

For the plasma treatment, frequency plasma (PF) and arc plasma (PA) were utilized, provided by the materials department of the Faculty of Chemical Sciences. The films were placed inside a container (Figure 4a and 4b) and subsequently subjected to treatments of 30 and 90 s of PF and PA, while maintaining conditions of 40 W and 500 Hz.

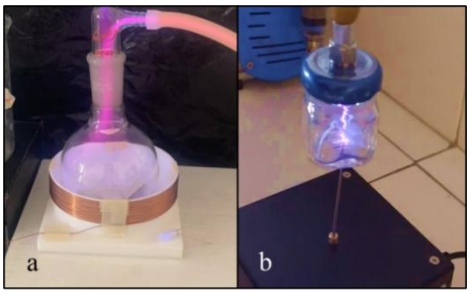

Figure 4a. Frequency Plasma (PF) **Figure 4b**. Arc Plasma (PA)

FTIR Spectroscopy

Infrared spectroscopy (FTIR) was used to characterize the presence of specific chemical groups in the film of chitosan and chitosan-papain. FTIR spectra were obtained in a Perkin-Elmer Spectrophotometer Model GX. Spectra were recorded at 500 to 4000 cm-1 wavelengths with 30 scans and a resolution of 4 cm-1. Spectra were run directly from the solid films without any method of preparation.

Thermal Analyses

For thermogravimetric analysis (TGA), the equipment used was a PerkinElmer Pyris TGA, which was used to determine the weight variation of the samples upon heating. Approximately 10 mg of each sample was placed in an alumina pan with an empty pan used as a reference. Samples were heated from 30 to 800 ºC at a heating rate of 20 ºC/min-1 under an airflow of 20 mL·min-1. The Universal Analysis 2000 software, version 4.7, from TA Instruments, was used to plot the thermogravimetric (TG) curves. OriginPro 8.5 was used to analyze the curves.

Enzymatic Activity Assay

The proteolytic activity of papain was determined as previously described by Câmara da Silva Melo et al. (2023), with modifications. The reaction mixture was 2 mL of 0.01 M phosphate buffer (PBS) at pH 8.0, which contains 0.5 mM EDTA, 5 mM cysteine, and 1.0 ml of 2.0 % w/v casein, as well as 1.0 ml of free enzyme solution,

or pectin-trapped enzyme preparation previously suspended in 1 ml of 0.05 M PBS. The reaction mixture was stirred and heated to 37° C for 20 min; the reaction was subsequently stopped with the addition of 3.0 % w/v trichloroacetic acid. Then, the supernatant was centrifuged for 30 min at 1800 rpm, and the supernatant was measured at 280 nm, showing the presence of amino acids as a product of the enzymatic reaction.

Inhibition assay

The preparation of antibiograms was carried out following the Kirby-Bauer disk diffusion method, as described by Hudzicki in the 2009 article "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol." A standardized solution of 0.5 MacFarland of *Streptococcus mutans* was prepared, and this was verified by measuring optical density at 625 nm. Subsequently, Mueller-Hinton agar plates were inoculated with 100 µl of the suspension in Petri dishes. The films were then cut into disks and added to the inoculated Petri dishes along with their corresponding controls. The Petri dishes were incubated for 24 hours. After the incubation period, the inhibition zones around each disk were measured in millimeters (mm).

Statistical Analysis

For the statistical analysis, a completely randomized design was employed, and a mean difference and Tukey test were performed. The statistical package SPSS was used, with the data representing the averages of each replicate for each treatment, and the response variable was the diameter of each inhibition zone in mm. After analyzing the significance level ($p < 0.05$), a Tukey test was conducted for multiple mean comparisons.

RESULTS AND DISCUSSIONS

Chitosan-papain films

Figure 5 shows four types of prepared films: chitosan sigma films (QS), chitosan extracted films (QE), chitosan sigma films with papain (QSP), and chitosan extracted films with papain (QEP). The main difference between the films is color: the films containing papain are darker.

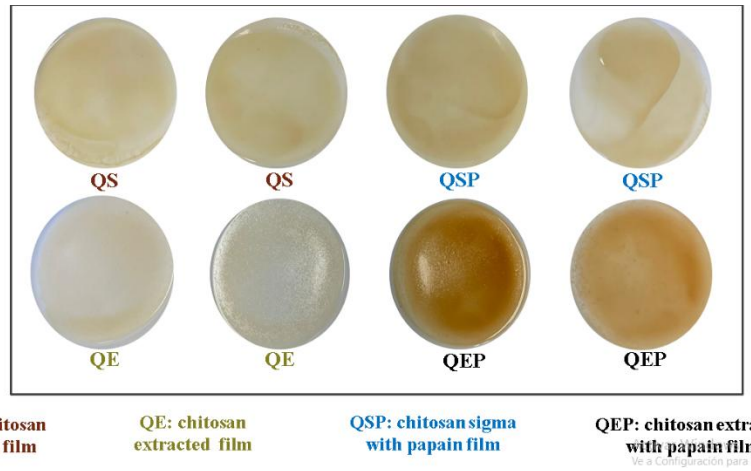

Figure 5. Chitosan film and chitosan with papain films

Papain interactions with chitosan by thermal analyses and FTIR.

Figure 6 shows the FTIR spectra of the extracted chitosan powder and the extracted chitosan film, showing the functional groups of chitosan in both. The OH/NH stretching band around 3437 cm^{-1} is prominent in both QE powder and QE film, indicating the presence of hydroxyl and amine groups, which are characteristic of chitosan, the C-O stretching at 2883 cm^{-1} and the C-N stretching at 1381 cm^{-1} are observed in the QE powder and film, confirming the presence of chitosan's polysaccharide backbone, the amide I band (C=O stretching) near 1644 cm^{-1} and the amide II band (N-H bending) around 1421 cm^{-1} suggest the presence of acetylated residues in the chitosan structure, the C-O-C stretching band at 1021 cm^{-1}, common in chitosan, is also present in both the powder and film, indicating the retention of the polysaccharide structure after film formation, Likewise, in figure 7 shows the papain characteristics peaks, the broad peak around 3400-3300 cm^{-1} in the papain powder (P powder) corresponds to OH and NH stretching, indicative of proteins. This peak shifts slightly in the PQEP film, likely due to interactions between papain and chitosan. The C-H stretching band at 2901 cm^{-1} and the C=O stretching band around 1638 cm^{-1} are characteristic of papain's protein structure, observed in both P powder and PQEP film. The C-S stretching band around 1074 cm^{-1} is

also observed, which is associated with papain's cysteine residues, a key functional group in papain's enzymatic activity. This band is present in the PQEP film, indicating successful incorporation of papain into the chitosan matrix.

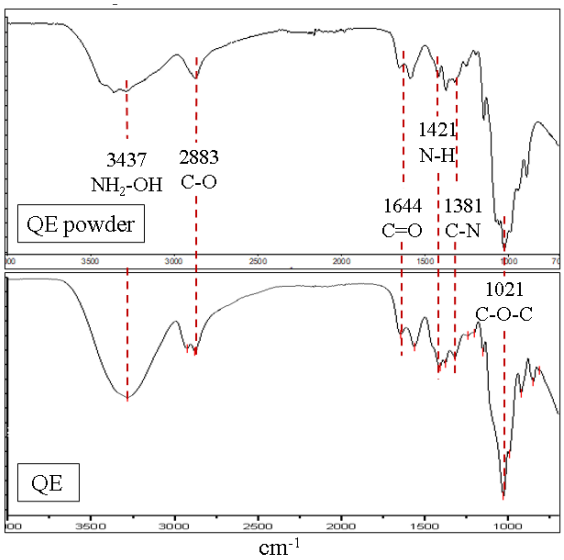

Figure 6. Chitosan extracted powder FTIR and chitosan extracted film

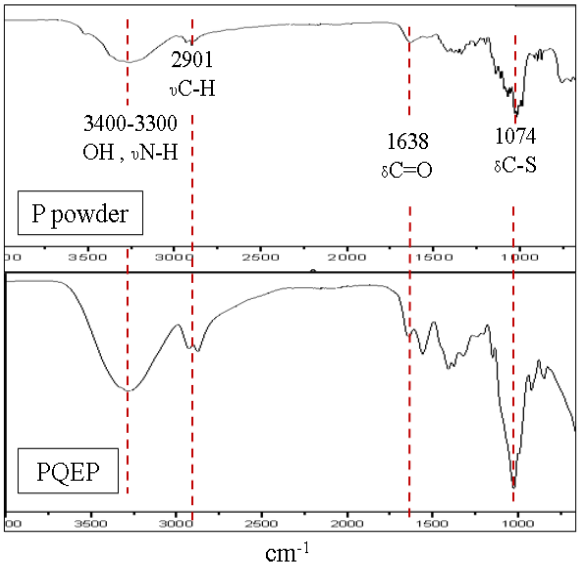

Figure 7. Papain powder FTIR and chitosan with papain film (QEP) FTIR.

The shifts and broadening in the OH/NH region (3400-3300 cm^{-1}) and the amide bands suggest strong interactions, possibly hydrogen bonding, between chitosan and papain within the PQEP film. The presence of characteristic peaks for both chitosan and papain in the PQEP spectrum indicates that the functional groups from both components are preserved, confirming the successful formation of the chitosan-papain composite film. The FTIR spectra of the films do not show any new peaks or significant shifts indicative of chemical modification or the formation of new covalent bonds between chitosan and papain. This suggests that the film-forming process primarily involves physical blending rather than chemical crosslinking.

Figure 8 shows the FTIR of all plasma-treated films, where it is observed that no grafted functional groups are present. This indicates that the films successfully underwent plasma erosion treatment at times 30 s and 90 s, as shown by the absence of additional functional groups to which each molecule corresponds.

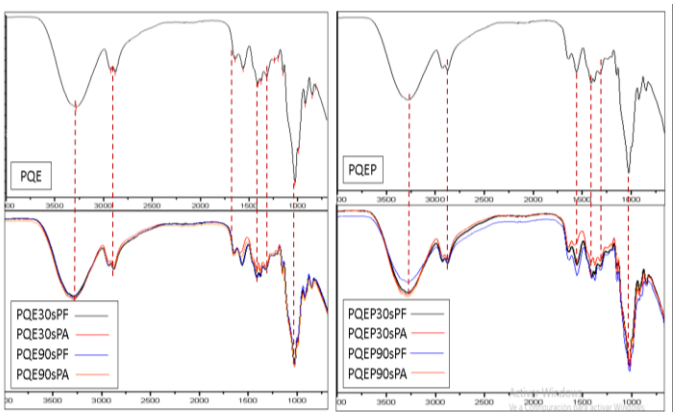

Figure 8. FTIR of PQE and PQEP films treated with frequency plasma and arc plasma at 30s and 90s.

The TGA curves show the mass loss of the films as the temperature increases from around 100 °C to 800 °C. Figure 9 shows that all the films follow a similar degradation pattern, with two main degradation stages. The first stage (around 100 °C - 200 °C) likely corresponds to the loss of water and low molecular weight compounds. The second stage (around 250 °C - 600 °C) corresponds to the thermal degradation of chitosan, papain, and any other organic components this result can be compared to that of Camara Da Silva (2023) in their article they mentioned that in membrane Ch (Chitosan) 52 °C, the first event had a loss of 3 % and the second event occurred in 278 °C with

a loss of 68 % of mass. In ChPap (chitosan and papain), 2.5 % at 52 °C resulted in a 3.2 % loss, and at 287.14 °C, a 67.12 % loss. In ChPap5 % at 56 °C, there was a loss of 2.4 %, and at 280 °C, there was a loss of 68.63 %. Papain powder presented only one event at 314 °C with a loss of 54.04 % of mass (Câmara da Silva Melo et al., 2023).

The films treated with plasma (both frequency plasma (PF) and arc plasma (PA)) for 30 s and 90 s generally show a slight shift in thermal stability compared to the untreated films (PQE, PQEP, PQS). The differences, though subtle, suggest that plasma treatment may influence the thermal degradation profile, potentially through changes in surface chemistry or crosslinking induced by plasma.

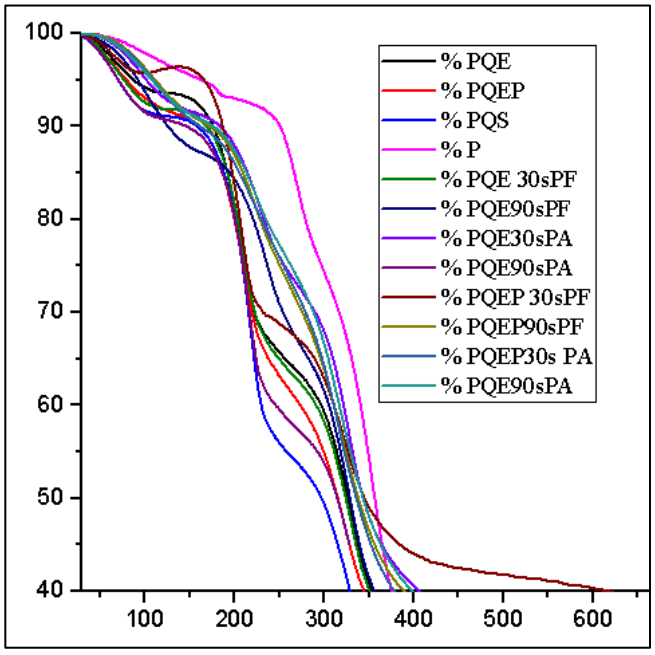

Figure 9. TGA of PQE and PQEP films treated with frequency plasma and arc plasma at 30s and 90s

Among the plasma-treated samples, the film treated with frequency plasma for 30 seconds (PQEP 30s PF) appears to have slightly higher thermal stability, with a more gradual mass loss than the others. This could imply that a short plasma treatment enhances the film's resistance to thermal degradation. On the other hand, prolonged plasma treatment (90 s) does not significantly improve thermal stability and might even slightly reduce it. The presence of papain in the films (PQEP, PQEP 30 s PF, PQEP 90 s PF, etc.) does not drastically alter the thermal degradation pattern compared to films without papain (PQS, PQE). However, films containing papain exhibit slightly different degradation profiles, which may be due to interactions between chitosan and papain, thereby affecting thermal stability.

The TGA curves suggest that while plasma treatment affects the overall thermal stability of the films, it does not cause significant chemical changes or grafting of new functional groups, as indicated by the consistent degradation patterns across the samples.

In conclusion, the TGA analysis indicates that plasma treatment can modify the thermal stability of chitosan-papain films, with frequency plasma at shorter durations showing the most promise for enhancing thermal properties. However, the overall impact of plasma treatment is subtle, and the inherent properties of chitosan and papain dominate the thermal degradation behavior.

Measurement of the diameter of the inhibition zone of the treatments.

In Table 1, the meaning comparison of the calculated diameter in mm is presented. It can be observed that these values were relatively low. Additionally, the Tukey test at 95 % confidence indicates that the inhibitory effects of the treatments did not differ significantly, even with the addition of plasma treatment to the films. According to Hudzicki (2009), in the article "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol," the inhibition zones generated against *Streptococcus mutans* indicate resistance in all treatments, as the zones are small (< 10 mm). This result does not align with findings in other research; for example, Chen et al. (2012) found that water-soluble chitosan has a significant effect on dental pathogens and reported that mouthwash containing water-soluble chitosan demonstrated antibacterial activity comparable to that of commercial mouthwashes, exceeding 99.91 % in both in vitro and in vivo experiments. Costa et al. (2013) worked with chitosan of different molecular weights and found that higher-molecular-weight chitosan has greater efficacy against oral pathogens, suggesting that chitosan has the potential to be an alternative to traditional antimicrobials in oral health.

Table 1. Diameters of the inhibition zone for each of the treatments.

Tratament	Inhibition (mm)
Film extracted chitosan (PQE)	0,0
Film extracted chitosan/papain (PQEP)	8,0[b]
Film extracted chitosan_ Frequency Plasma 30s (PQE 30sPF)	8,5[b]
Film extracted chitosan/papain_ Frequency Plasma 30s (PQEP 30sPF)	0,0
Film extracted chitosan_ Arc Plasma 30 s (PQE 30sPA)	0,0
Film extracted chitosan/papain_ Arc Plasma 30 s (PQEP 30sPA)	8,2[b]
Film extracted chitosan_ Frequency Plasma 90s (PQE 90sPF)	0,0
Film extracted chitosan/papain_ Frequency Plasma 90s (PQEP 90sPF)	7,7[b]
Film extracted chitosan_ Arc Plasma 90s (PQE 90s PA)	0,0
Film extracted chitosan/papain_ Frequency Plasma 90s (PQEP 90s PA)	0,0
Papain (P)	0,0
Film chitosan sigma/papain PQS	8,5[b]
Control (A)	26[a]

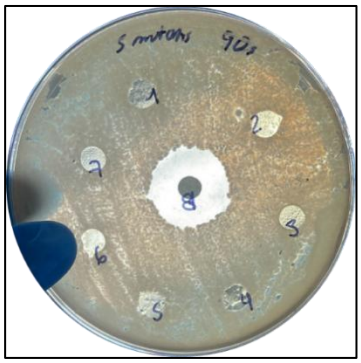

Figure 10. Inhibition test result.

On the other hand, although the obtained zone was minimal, there was no bacterial growth at the film site, as shown in Figure 10; this can be attributed, regardless of chitosan's antimicrobial activity, to its natural characteristics mean the antimicrobial effect occurs without migration of active agents through the agar; in other words, it does not diffuse into the agar. Therefore, it only inhibits microorganisms in direct contact with chitosan. This is explained by Hosseini et al. (2008). The same occurred in the study by Hernández-Ochoa et al. (2011), in which chitosan films with different molecular weights and mixed with different extracts were evaluated. As a result, the chitosan films at the three molecular weights studied, without the addition of antimicrobials (control), did not exhibit inhibition zones against the tested strains. Inhibition was only observed in the area where the films were placed.

CONCLUSIONS

Plasma-treated films showed an antimicrobial effect of 32 % against *Streptococcus mutans* compared to the control for 30 seconds and 90 seconds of resistance; however, the films showed inhibition at the site of application, so their antimicrobial effect is still not ruled out.

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