

## Antimicrobial potential of a bioactive coating based on chitosan incorporated with clove essential oil in hamburger-like meat product

Potencial antimicrobiano de revestimento bioativo a base de quitosana incorporado com óleo essencial de cravo-da-índia em produto cárneo análogo a hambúrguer

Potencial antimicrobiano del recubrimiento bioactivo a base de quitosano incorporado com aceite essencial de clavo em um produto cármico similar a uma hamburguesa

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### **Abstract**

The food industry is looking for strategies to prevent microbial growth in order to ensure food safety and shelf life. However, the use of synthetic preservatives, such as nitrate and nitrite in meat products, entails risks to human health. An alternative is the utilization of essential oils, widely known for their antimicrobial properties. This work aimed the antimicrobial potential of a bioactive coating based on chitosan incorporated with clove essential oil in hamburger-like meat product. Through the analysis of antimicrobial activity by diffusion in agar and broth, there was an action against Gram-positive and Gram-negative bacteria. Regarding *Staphylococcus aureus* and *Escherichia coli*, the minimum inhibitory concentration (MIC) was 3.74 mg/mL and the minimum bactericidal concentration (MBC) was 374.33 mg/mL for both. In the micro atmospheric diffusion test, CEO reduced by up to 70 and 76% of the *E. coli* and *S. aureus* bacteria development, respectively. CEO was applied as an active component in chitosan-based coatings in hamburger-like meat, in which it was able to promote the control of microbial proliferation of Total Coliforms, Coliforms at 45 °C and Coagulase-Positive *Staphylococcus* throughout 7 days of storage under refrigeration. It is concluded that the bioactive coating based on chitosan incorporated with clove essential oil promotes microbiological control in hamburger-like meat product.

**Keywords:** *Syzygium aromaticum*; Antimicrobial activity; Bioconservative; Hamburger; Eugenol.

### **Resumo**

A indústria alimentícia busca por estratégias que previnam o crescimento microbiano, a fim de garantir a segurança e a vida útil dos alimentos. No entanto, o uso de conservantes sintéticos, como nitrato e nitrito em produtos cárneos, acarreta riscos à saúde humana. Uma alternativa é a utilização de óleos essenciais, os quais são amplamente conhecidos por suas propriedades antimicrobianas. Este trabalho teve como objetivo avaliar o potencial antimicrobiano de um revestimento bioativo à base de quitosana incorporado ao óleo essencial de cravo-da-índia em um produto cárneo análogo a hambúrguer. Por meio da análise da atividade antimicrobiana por difusão em ágar e

caldo, houve ação contra bactérias Gram-positivas e Gram-negativas. Em relação a *Staphylococcus aureus* e *Escherichia coli*, a concentração inibitória mínima (CIM) foi de 3,74 mg/mL e a concentração bactericida mínima (CBM) foi de 374,33 mg/mL para ambos. No teste de difusão em micro-atmosfera, o óleo essencial de cravo-da-índia (OEC) reduziu em até 70 e 76% o desenvolvimento das bactérias *E. coli* e *S. aureus*, respectivamente. O OEC foi aplicado como um componente ativo em revestimentos à base de quitosana em produto carne análogo a hambúrguer, no qual foi capaz de promover o controle da proliferação microbiana de Coliformes Totais, Coliformes a 45 °C e *Staphylococcus* Coagulase-Positivo ao longo de 7 dias de armazenamento sob refrigeração. Conclui-se que o revestimento bioativo à base de quitosana incorporado ao óleo essencial de cravo-da-índia promove o controle microbiológico em produtos cárneos do tipo hambúrguer.

**Palavras-chave:** *Syzygium aromaticum*; Atividade antimicrobiana; Bioconservante; Hambúrguer; Eugenol.

### Resumen

La industria alimentaria busca estrategias que eviten el crecimiento microbiano para garantizar la seguridad y la vida útil de los alimentos. Sin embargo, el uso de conservantes sintéticos como el nitrato y el nitrito en productos cárnicos presenta riesgos para la salud humana. Una alternativa es utilizar aceites esenciales, que son ampliamente conocidos por sus propiedades antimicrobianas. Este trabajo tuvo como objetivo evaluar el potencial antimicrobiano de un recubrimiento bioactivo a base de quitosano incorporado al aceite esencial de clavo en un producto cárnico análogo a la hamburguesa. Al analizar la actividad antimicrobiana por difusión en agar y caldo, se encontró acción frente a bacterias Gram positivas y Gram negativas. En cuanto a *Staphylococcus aureus* y *Escherichia coli*, la concentración mínima inhibitoria (MIC) fue de 3,74 mg/mL y la concentración bactericida mínima (CBM) fue de 374,33 mg/mL para ambos. En la prueba de difusión en micro-atmósfera, el aceite esencial de clavo (AEC) redujo el desarrollo de las bacterias *E. coli* y *S. aureus* hasta en un 70 y 76%, respectivamente. AEC se aplicó como componente activo en recubrimientos a base de quitosano en un producto cárnico similar a una hamburguesa, que pudo controlar la proliferación microbiana de Coliformes Totales, Coliformes a 45 °C y Estafilococos Coagulasa Positivos durante 7 días de almacenamiento refrigerado. Se concluye que el recubrimiento bioactivo a base de quitosano incorporado al aceite esencial de clavo promueve el control microbiológico en productos cárnicos tipo hamburguesa.

**Palabras clave:** *Syzygium aromaticum*; Actividad antimicrobiana; Bioconservante; Hamburguesa; Eugenol.

## 1. Introduction

The industry implements different conservation methods during the production and marketing of products of animal origin, in order to delay or prevent microbiological, chemical or physical changes that make them unfit for human consumption (Macwan et al. 2016). However, food safety issues are one of the main concerns associated with public health, since outbreaks of foodborne diseases are annually recorded worldwide (Burt, 2004; Macwan et al., 2016).

Allied with this fact, the addition of synthetic preservatives to foods is confronted with the increase in consumer demand for products of natural origin (Calo et al., 2015). Such preservatives can cause complications to human health, such as the use of nitrate, nitrite and their respective salts of sodium (Na) and potassium (K), which have a carcinogenic potential when consumed excessively (Sindelar & Milkowski, 2011; World Health Organization, 2015).

Essential oils are among the alternatives with the potential to replace synthetic preservatives in meat products, either partially or totally. These compounds are derived from the specialized metabolism of aromatic plants. Plants trigger natural defense mechanisms against edaphoclimatic and pathogenic factors, synthesizing biologically active molecules, such as terpenes and phenolic compounds, through their specialized metabolism, providing essential oils with an antimicrobial capacity (Burt, 2004).

The clove essential oil (*Syzygium aromaticum* L.) has demonstrated prominent antimicrobial activity against important foodborne pathogenic bacteria (Radünz et al., 2019; Ghabraie et al., 2016; Hosseini et al., 2015; Scopel et al., 2014). Meanwhile, the direct application of essential oils to the food matrix can cause undesirable sensory interferences, due to the characteristic odors and flavors (Dannenberg et al., 2016; Otoni et al., 2016).

Nonetheless, this limitation can be reduced with the implementation of essential oil in edible coatings, avoiding its direct incorporation into the food and, thus, concentrating its action on the food surface, where microbial contamination is

more intense (Appendini & Hotchkiss, 2002; Coma, 2008). In addition, the volatility and instability of essential oil under environmental conditions, such as temperature, light, and oxygen, can be reduced (Radünz et al., 2019).

Among the coating options, there is chitosan, a polysaccharide derived from chitin, which is a component synthesized by a variety of living organisms (Kurita, 2006). Chitosan is used in the preparation of coatings as a natural and non-toxic product, capable of forming films with antimicrobial properties (Gómez-Estaca et al., 2010).

In view of the potentiality of clove essential oil as an antimicrobial in foods, the objective of the present study was to assess its antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* and evaluate the effect of edible coatings based on chitosan incorporated with clove essential oil on the microbiological characteristics in a hamburger-like meat product.

## 2. Methodology

### Extraction and characterization of essential oils

Samples of dried clove flower buds were purchased from local businesses in the city of Pelotas, RS. The clove essential oils were extracted according to methods described in the Brazilian Pharmacopeia (Brazil, 2010) by hydrodistillation for 3 h using a Clevenger apparatus (Vidrolabor, Brazil).

Clove essential oil compounds were evaluated by gas chromatography. A 1  $\mu$ L sample of essential oil was diluted with 1:20 (v:v) n-hexane and injected into a gas chromatograph coupled with a mass spectrophotometer GCMS-QP2010 Ultra (Shimadzu™, Kyoto, Japan) with an automatic injector AOC20i (Shimadzu™, Kyoto, Japan) and a capillary column OV-5MS (30 m  $\times$  0,25mm  $\times$  0,25 $\mu$ m).

The elution gradient started at 60 °C for 1 min in isothermal mode followed by heating to 180°C at a rate of 5°C/min, remaining at 180 °C for 1 min in isothermal mode, followed by heating to 280°C at a rate of 40°C/min, remaining at 280 °C for 1.5 min in isothermal mode, for a 30 min total run time. The injection temperature was 200°C with a flow rate of 1 mL/min using split mode helium gas (ratio 50:1). The sweep range was 40 to 450 m/z with the solvent cut in 3 min. The interphase temperature was 270 °C, and the ion source temperature was 260 °C. The mass spectrometer was operated with 70 eV electronic impact ionization.

Identification of compounds was based on comparison of mass spectra with the NIST11 library (Mass Spectra Library, USA). Concentrations were presented as percentages of area under each peak relative to total area.

### Microorganisms

For the determination of the antimicrobial potential *in vitro*, two microorganisms were used, one Gram-positive bacteria: *Staphylococcus aureus* (ATCC 10832) and one Gram-negative: *Escherichia coli* O157:H7 (ATCC 43895).

The analyses for enumeration of Total Coliforms, Coliforms at 45 °C (Thermotolerants) and Coagulase-Positive *Staphylococcus* in a hamburger-like meat product were performed according to the procedures proposed by the American Public Health Association (APHA) with modifications (Downes & Ito, 2001).

### Disk diffusion

Initially, the CEO antimicrobial activity was determined by the disk diffusion technique (CLSI, 2012). The bacterial cultures were suspended in saline NaCl (0.85%), obtaining a concentration of  $1.5 \times 10^8$  log CFU mL<sup>-1</sup> (0.5 McFarland). In the technique, three small equidistant disks (diameter 5 mm) were made with a sterile aluminum cylinder in the center of the Petri dishes containing Mueller-Hinton agar (Oxoid®). The cell suspension was inoculated on the agar surface with the aid of a sterile swab, and 10  $\mu$ L of essential oil was added to each disk. As a negative control, 10  $\mu$ L of sterile distilled water was used.

The plates were incubated at 37 °C and the readings were taken after 24 h of incubation, in which the existence of inhibition halos was analyzed, quantifying the existing ones with calipers, expressing them in millimeters.

### **Minimum Inhibitory Concentration and Minimum Bactericidal Concentration**

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were performed using the methods described by Cabral et al. (2009) with modifications. CEO was tested in four different concentrations: 374.33 mg/mL, 74.86 mg/mL, 37.43 mg/mL and 3.74 mg/mL. Microbial growth was assessed by turbidity readings at a wavelength of 620 nm employing a spectrophotometer (Biochrom EZ Read 400) at the time of preparation and after 24 h of incubation. MIC was considered the lowest concentration of essential oil that prevents bacterial growth in the culture medium.

To detect MBC, 15 µL aliquots from each well, with inhibition in the MIC test, were inoculated in Petri dishes containing Brain Heart Infusion agar (KASVI®). The lowest concentration without growth in this new medium was considered MBC.

### **Antimicrobial activity in micro-atmosphere**

The antimicrobial activity in the micro atmosphere was evaluated using the technique proposed by Ghabraie et al. (2016) with modifications. Aliquots of 0.1 mL of the bacteria cell suspensions ( $10^8$  log UFC mL<sup>-1</sup>) were inoculated by implementing the technique of spreading BHI agar (KASVI®) on the surface of Petri dishes. Sterile filter paper discs (15 mm) were placed on the lid of each plate, in which different volumes of CEO (100, 50, 25 and 12.5 µL) were added and immediately closed and sealed with parafilm film, with inverted (coating down) incubation at 37 °C for 24 h. The antimicrobial action was expressed by the cell count (CFU) percentage reduction of treatments with essential oil compared to a control containing sterile distilled water.

### **Preparation of chitosan coating**

The chitosan coating was prepared according to the methodology proposed by Moradi et al. (2011) with modifications. The chitosan solution was formulated by dissolving 2 g of chitosan in 100 mL of glacial acetic acid (1% - SYNTH®) under magnetic stirring overnight. Then, 1 mL of glycerin (1% - SYNTH®) and 0.1 mL of Tween 80 (0.1% - SYNTH®) were added to the solution. CEO was added to the solution at a concentration of 1% (v / v). The mixtures were homogenized in Ultra-Turrax (Metabo) at 27,000 rpm for 20 min.

### **Antimicrobial activity *in situ***

A hamburger-like meat product was created as a food matrix following the recommendations of Terra (2005). The ingredients used in the standard formulation of hamburger-like meat were 73% lean meat, 7% pork fat, 20% soy protein and 1.5% salt.

Initially, to prepare the standard formulation, the chilled beef and pork fat were cut into cubes. Posteriorly, the cubes were ground with the addition of salt in a meat grinder (Bermar Indústria e Comércio®) with a 6 mm disc. After the process, the hydrated soy protein was added and, after obtaining the homogeneous meat mass, 30 g of the meat mass were removed and individually wrapped with plastic PVC films (Polyvinyl chloride) and molded in Petri dishes (60 x 15 mm).

The synthetic preservative sodium nitrite (100g/100 kg - Duas Rodas Industrial®) was added to the meat mass at the end of the process. Then, the hamburger-like meat was coated, using the immersion technique, with the respective chitosan coatings and placed on a sterile support under refrigeration (4 °C) for 7 days, analyzing them on days 2, 5 and 7.

The following treatments were performed: a) Standard product similar to hamburger without coating; b) Standard product similar to hamburger with chitosan coating; c) Standard product similar to hamburger with chitosan coating incorporated with CEO; d) Standard product similar to hamburger with chitosan coating incorporated with CEO and 50% sodium nitrite; and e) Standard product similar to hamburger with 100% sodium nitrite.

### Statistical analysis

Statistical analysis of the results of antimicrobial activity determination was performed by the analysis of variance with the post-hoc Tukey test ( $p < 0.05$ ). The in situ antimicrobial activity analysis was carried out through the analysis of variance (ANOVA) with the post-hoc Fisher's least significant difference test, LSD ( $p < 0.05$ ).

## 3. Results and Discussion

The clove essential oil was composed of 58% of the area referring to the eugenol compound, and 41% of caryophyllene. These results are consistent with those reported by other studies, where eugenol is considered the major compound of clove essential oil, being isolated or in synergism with the other compounds, responsible for the biological potentials of the oil, such as antioxidant, antimicrobial, and antihyperglycemic activity (Radünz et al., 2019; Kalaiselvi et al., 2019; Radünz et al., 2021).

In the disk diffusion method, CEO inhibition halos were detected against *E. coli* and *S. aureus* bacteria (Table 1). According to Arora and Kaur (1999), the presence of halos smaller than 7 mm are considered non-active, they are active when between 7 and 12 mm and have a satisfactory inhibitory effect when they are more than 12 mm. The CEO inhibition zones were 12 mm and 16 mm for *E. coli* and *S. aureus*, respectively. Therefore, they are considered to have a satisfactory inhibitory effect.

**Table 1.** Antimicrobial activity of clove essential oil (CEO) by disk diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Bacteria	Disk diffusion (mm)*	MIC (mg/mL)	MBC (mg/mL)
<i>Escherichia coli</i>	12	3.74	374.33
<i>Staphylococcus aureus</i>	16	3.74	374.33

\*Average of triplicates. Source: Authors.

The results found in this study were comparable to those reported by Santos et al. (2011), which obtained an 11 mm halo for *E. coli* and 15.7 mm for *S. aureus*. In the study by Pereira et al. (2008), the halos showed a variation of 8 mm and 12 mm for *E. coli* and 10 mm to 15 mm for *S. aureus*. The authors Ghabraie et al. (2016) did not detect the CEO antimicrobial action against *E. coli*; however, they point out 19.9 mm inhibition halos for *S. aureus*.

Table 1 also presents the results for MIC and MBC of CEO, which indicated an inhibitory effect up to a concentration of 3.74 mg/mL against *E. coli* and *S. aureus*.

The study by Silvestri et al. (2010) also evaluated CEO and found a MIC value of 0.30 mg/mL for *S. aureus*, while other authors found values of 0.60 mg/mL (Beraldo et al., 2013), 0.64 mg/mL (Ivanovic et al., 2013), 0.50 mg/mL (Santos et al., 2011), 0.80 mg/mL (Lu et al., 2011) and 0.62 mg/mL (Xu et al., 2016). Conversely, among the essential oils evaluated by Santos et al. (2017), CEO was the only one that did not inhibit microbial growth in the analyzed concentrations.

For *E. coli*, a MIC value of 0.307 mg/mL was found, which was higher than that assessed by Santos et al. (2011) and Sienkiewicz et al. (2017), where inhibition values of 0.100 mg/mL were observed. Other studies have found values of 0.600 mg/mL (Beraldo et al., 2013), 0.550 mg/mL (Silva et al., 2015) and 0.027 mg/mL (Göni et al., 2009).

The CEO antimicrobial action is possibly related to the synergistic effect of the compounds present in essential oil, such as eugenol, and caryophyllene, which have hydrophobicity as a characteristic. These compounds can allow the division of lipids present in the bacterial cell membrane, modifying their permeability, promoting cytoplasmic content leakage and resulting in cell death (Devi et al., 2010; Costal et al., 2011; Bakkali et al., 2008).

In the present study, CEO had a bactericidal effect on the concentration of 374.33 mg/mL for both evaluated bacteria (Table 1). The studies previously mentioned on the CIM results, also assessed MBC of CEO. Beraldo et al. (2013) indicate MBC of 1.8 mg/mL for *S. aureus* and Silva et al. (2015) obtained MBC from 1 mg/mL, both lower than that found in this trial. Studies by the authors Silva et al. (2015) and Sienkiewicz et al. (2017) discovered the need for 0.100 mg/mL to inhibit *E. coli*.

The difference in values in the results achieved in the CIM and MBC analyses can be explained by the variations between the evaluation methods employed as well as the solubility of the essential oil or its components (Opalchenova & Obreshkova, 2003).

In any case, the CEO antimicrobial action was found in this study, even if in higher concentrations when compared to other studies, it attributes the potential to be used as a natural antimicrobial agent in foods.

In the test for antimicrobial activity in the micro-atmosphere, CEO was active against the two bacteria examined (Table 2). The CEO application at a 100 µL concentration was able to promote reductions of up to 76% against the tested bacteria.

**Table 2.** Antimicrobial activity of clove essential oil (CEO) in a micro-atmosphere against *Escherichia coli* and *Staphylococcus aureus*.

Essential oil	Volume (µL)	<i>Escherichia coli</i> (%)*	<i>Staphylococcus aureus</i> (%)*
Clove	12.5	15.8	18.9
	25	51.7	31
	50	61.7	34
	100	70	76

\* Percentage reduction in cell count (CFU) compared to a control containing sterile distilled water. Source: Authors.

The lower volume of essential oil added (12.5 µL) also resulted in decreases between 15.8% and 18.9% for *E. coli* and *S. aureus*, respectively. These results indicate that the medium, where the essential oil is applied, directly influences its diffusion and propagation.

The authors Ghabraie et al. (2016) explain that the nonpolar characteristic of essential oils hinders their propagation in water-based media, since the antimicrobial constituents of essential oils come into contact with the microbial cell through volatilization, in the micro-atmosphere.

In the Total Coliforms analysis and at 45 °C (Thermotolerants), it was possible to observe that in all formulations, evaluated over 7 days of refrigerated storage, with the presence of chitosan coating with and without the incorporation of CEO, indicated counts <0.3 MPN / g during the storage period, remaining constant. Concomitantly, there was a reduction in the initial count of 0.94 MPN/g of Total Coliforms in the control formulation.

According to the standards required by Resolution (Resolução de Diretoria Colegiada, RDC) number 12/2001 of the National Health Surveillance Agency (ANVISA), raw, chilled or frozen meat products must have a tolerance of  $5 \times 10^3$  MPN/g for Coliforms at 45 °C (Brazil, 2001). Thus, the obtained results indicated that the analyzed hamburger-like meat product was within the standards established by the current legislation and acceptable for consumption.

The results point to the efficiency of the chitosan coating added with essential oil as a natural antimicrobial in foods, as it can promote the control of microbial proliferation that could allow the multiplication of pathogenic microorganisms. Similar behavior was observed in coatings formed by chitosan (2%) and gelatin (6%) incorporated with 0.75% CEO, which reduced the counts of decaying bacteria in cod fillets (*Gadus morhua*) for 11 days of refrigerated storage (Gómez-Estaca et al., 2010).

The averages of the Coagulase-Positive *Staphylococcus* quantifications present in the formulation of hamburger-like meat product submitted to different treatments can be seen in Table 3.

**Table 3.** Averages of Coagulase-Positive *Staphylococcus* quantifications in hamburger-like meat product submitted to different treatments stored at 4 °C for up to 7 days.

Treatment	Coagulase-Positive <i>Staphylococcus</i> (Log UFC g <sup>-1</sup> )			
	Time 0 (0 days)	Time 1 (2 days)	Time 2 (5 days)	Time 3 (7 days)
<b>T1</b>	4.35 <sup>aA</sup>	-	-	-
<b>T2</b>	-	1.13 <sup>bA</sup>	0.00 <sup>bB</sup>	0.00 <sup>bB</sup>
<b>T3</b>	-	1.13 <sup>bA</sup>	0.00 <sup>bB</sup>	0.00 <sup>bB</sup>
<b>T4</b>	-	0.00 <sup>bA</sup>	0.00 <sup>bB</sup>	0.00 <sup>bB</sup>
<b>T5</b>	-	0.00 <sup>bA</sup>	0.00 <sup>bB</sup>	0.00 <sup>bB</sup>

T1 - Control (Standard product similar to hamburger without coating); T2 - Standard product similar to hamburger with chitosan coating; T3 - Standard product similar to hamburger with chitosan coating incorporated with CEO; T4 - Standard product similar to hamburger with chitosan coating incorporated with CEO and 50% nitrite; T5 - Standard product similar to hamburger with 100% sodium nitrite. Different lower-case letters in the line or different upper-case letters in the column indicate significant differences (Fisher –  $p < 0.05$ ). Source: Authors.

For the Coagulase-Positive *Staphylococcus* microorganism, treatment T3, with the coating application, significantly reduced ( $p < 0.05$ ) the counts from 4.35 to 1.33 log CFU.g<sup>-1</sup> compared to the control treatment (T1), remaining lower until the last analyzed time. However, there was no significant reduction ( $p > 0.05$ ) in the number of viable cells between times after the second day of storage (Time 1). Hamburger-like meat made with chitosan coating met the requirements established by legislation for Coagulase-Positive *Staphylococcus* with counts below  $5 \times 10^3$  MPN/g (Brazil, 2001).

The authors Asbahani et al. (2015) suggest that the antimicrobial activity of essential oils is related to their hydrophobic structure, considering the phospholipids of the cytoplasmic membrane as their main cellular targets, as it would facilitate their diffusion through the membrane structure. This accumulation alters the density of the cell membrane and, consequently, the permeability, causing the gradual loss of vital cellular components to the external environment (Burt, 2004; Rai et al., 2017).

Brazilian legislation recommends the use of potassium nitrite and sodium nitrate at levels of up to 0.015g / 100g and 0.03g / 100g in meat products, respectively (Brasil, 2019). The *in situ* analysis showed potential by incorporating 1% of the CEO to the chitosan coating in meat products similar to hamburgers. Thus, the total or partial replacement of the preservative by CEO becomes a viable alternative to a natural preservative, since nitrite can form nitrosamines that may have carcinogenic

potential.

#### 4. Conclusion

The clove essential oil was mainly composed of eugenol, and through *in vitro* tests, clove essential oil showed a satisfactory effect against the tested bacteria. In the evaluated hamburger-like meat product, the chitosan coatings incorporated with the clove essential oil, as well as its antimicrobial activity *in vitro*, were able to promote the control of the microbial proliferation of Total Coliforms, Coliforms at 45 °C and Coagulase-Positive *Staphylococcus* throughout 7 days of refrigerated storage, making it a promising alternative to control the development of microorganisms of importance in foods.

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