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Evaluation of the antimicrobial activity of sodium alginate films integrated with cinnamon essential oil and citric acid on sliced cooked ham

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Abstract

Bio-based antimicrobial films have been developed using sodium alginate and 6 wt% of cinnamon essential oil (CEO) as a volatile antimicrobial substance and 4 and 6 wt% of citric acid (CA) as a non-volatile antimicrobial substance. Antimicrobial activity of the films was tested in in vitro tests against *Escherichia coli* and *Listeria innocua* by disc diffusion or vapour diffusion assay. Sodium alginate films containing CA exhibited a zone of inhibition between 30.86 ± 2.55 and 45.87 ± 1.90 against *E. coli* and *L. innocua* in the disc diffusion assays. Films containing CEO also showed significant antimicrobial activities in the vapour diffusion assays that resulted in a log reduction of 5.3 for *E. coli* and 3.2 for *L. innocua* after 6 days. Antimicrobial activities of all films were also tested against *L. innocua* on sliced cooked ham. Films containing CEO did not prevent the growth of *L. innocua* inoculated on ham. On the other hand, sodium alginate films with CA fully inhibited the growth of *L. innocua* on ham during storage at 7.5°C for 12 days resulting in a bacterial count below the detection limit after 12 days. The addition of antimicrobial substances in sodium alginate films resulted in a slight colour change (but significant) and reduced the tensile strength of the films significantly. Adding CA to sodium alginate films increased the moisture content (from 24.81% to 35.41–48.02%) as well as the elongation at break (from 11.3% to 22.6–33.2%) of the films.

KEYWORDS
active films, active packaging, antimicrobial packaging, essential oils, food packaging

1 | INTRODUCTION

The prevention of food spoilage due to microorganisms has been of great interest to the food industry since early times and has been well studied.[1] To prevent microbial spoilage, additives have often been incorporated directly into the food product as preservatives. With the recent consumer trend towards more natural and less processed food,

there has also been a push for fewer additives in food products. In response to this trend, antimicrobial packaging systems have been developed to preserve the quality and ensure the safety of food.[2–5] Antimicrobial substances can be integrated into the packaging and released afterwards either directly into the product or into the head-space surrounding the product, extending the microbial shelf life of the product and ensuring its safety. One potential application of these

active packaging systems is packaged ready-to-eat meat products, such as ham, as these are susceptible to microbial spoilage and are often consumed without further heat treatment. Psychrotrophic and facultative anaerobic pathogens such as *Listeria monocytogenes* are of particular concern, since they can grow even at refrigeration temperatures and cause serious illness.[6]

If antimicrobial packaging is to show an antimicrobial activity through the headspace, volatile antimicrobial agents should be incorporated into the packaging.[7,8] Among the volatile antimicrobial substances, essential oils have been extensively studied due to their strong antimicrobial efficacy, natural origin and comparatively harmless effect on human health.[9–11] Various essential oils have been integrated into packaging films or edible coatings as an antimicrobial substance.[8,12–15] Cinnamon essential oil (CEO) is one of the most studied EO, which according to Ojagh et al.[12] has shown a broad spectrum of antimicrobial activity, including combatting food spoilage bacteria such as *L. monocytogenes*, *Pseudomonas fluorescens* and *Escherichia coli*.[12,16–23] For the products where the packaging is in direct contact with the food, organic acids and their derivatives are potential antimicrobial substances to be integrated into the packaging as they have long been used as food preservatives and are incorporated into various food products. Citric acid (CA), as a non-volatile organic acid, is widely used in the food industry and is well known for its antimicrobial effect and unproblematic application in food.[24–26] It has been shown to possess a rather broad antimicrobial spectrum, inhibiting *Staphylococcus aureus*, *E. coli* (O157:H7), *Salmonella enteritidis*, *L. monocytogenes*, *Bacillus cereus*, *Candida albicans* as well as certain *Shigella* species among others.[27–31]

Integration of antimicrobial substances into bio-based films merits consideration in view of the negative environmental impact of conventional plastics.[2,32] Among the bio-based materials, alginate, a naturally occurring polysaccharide produced by various brown seaweeds and bacterial species, is of great interest.[33,34] The biodegradability as well as the superior properties such as water resistance upon inclusion of calcium ions qualifies the material as an excellent candidate for a potential bio-based antimicrobial food packaging.[35–37]

When antimicrobial substances are integrated into packaging materials, their antimicrobial activities should be studied with the appropriate method using direct contact or vapour diffusion assays, depending on the potential application.[38] In addition to the in vitro tests, antimicrobial activities should also be tested with the real food systems, as in vitro activities do not necessarily correlate with food tests.[38–41] In this study, we developed bio-based antimicrobial films, using sodium alginate as a bio-based material and CEO and CA as a volatile and non-volatile natural antimicrobial substances. We evaluated the antimicrobial activity of the films against *E. coli* and *Listeria innocua* in vitro by disc diffusion assay (CA) or vapour diffusion assay (CEO). Furthermore, we tested the efficacy of the antimicrobial films against *L. innocua* in a food test using sliced cooked ham to demonstrate the potential of the active films as a direct or indirect contact antimicrobial films for food packaging applications.

2 | MATERIALS AND METHODS

2.1 | Materials

The material used for film production consisted of sodium alginate, glycerol anhydrous (≥99%), calcium chloride (≥97%) and propionic acid (≥99.5%) purchased from Sigma Aldrich Chemical Co., (Buchs, Switzerland) as well as Tween 80 (Fluka, Buchs, Switzerland). The antimicrobial substances CEO (Ceylon type, St. Louis, Sigma Aldrich) and CA monohydrate from Fluka (Buchs, Switzerland) were used for the incorporation. Sliced cooked ham (M-Budget Hinterschinken) was obtained from Micarna AG, Switzerland.

2.2 | Preparation of sodium alginate films

Films were produced by casting method using 100 g of a sodium alginate film-forming solution (2 wt%). Film-forming solution was prepared by dissolving 2 g of sodium alginate in 64-g H_2O under constant stirring at 600–800 rpm and 60°C for 60 min until a clear solution was obtained. To prevent any loss of liquid, the beaker was sealed with Parafilm.

Further, 0.6-g anhydrous glycerol was then added dropwise to the solution and homogeneously mixed for another 15 min at 600–800 rpm and 60°C. Meanwhile, 0.1 g of $CaCl_2$ was dissolved in 33.3-g H_2O by stirring briefly and in a next step added dropwise to the film-forming solution. After approximately half of the calcium chloride solution had been added, the stirring speed was increased to 1000–1200 rpm for another 30 min. Once a homogeneous thick film-forming solution had been obtained, 45 g of the solution was poured into a Petri dish (diameter 145 mm) for production of the control film. Thereafter, integration of CEO and CA was performed by the slow addition of the antimicrobial substances and stirring for further 30 min at 1000–1200 rpm at 60°C before pouring 25 g of the film-forming solution into a Petri dish (diameter 145 mm). CEO was added at a concentration of 6 wt% (g/100 g) (CEO-6) to the sodium alginate solution whereas CA was added in concentrations of 4 wt% (CA-4) and 6 wt% (CA-6). For a more homogeneous and long-lasting distribution of the oil-based liquid antimicrobial substance CEO in the film-forming solution, the surfactant Tween 80 was added to the CEO in a ratio of 0.2% (w/w CEO) before incorporating the mixture dropwise into the film-forming solution. The Petri dishes containing film-forming solutions were placed uncovered in a drying cabinet at 40°C and 50% relative humidity for 24 h in a horizontal position. The film was then peeled off the Petri dish and conditioned for an additional 24 h at 21°C and 50% relative humidity before use.

2.3 | Physical properties of sodium alginate films

2.3.1 | Film thickness and moisture content

For measurement of the thickness of the sodium alginate films, a digital micrometre calliper was used (Mitutoyo, Germany) at

5 random locations on the film. Mean thickness values for each sample were calculated. The moisture content of the films was calculated using the weight loss after storage of the films at 95°C for 24 h in a heating chamber (Karg Industrietechnik, Germany). Measurements were done in triplicate.

2.3.2 | Mechanical properties

Tensile strength and elongation at break of the sodium alginate films were measured using a ZwickiLine Z0.5 TH texture analyser (Zwick GmbH & Co., Germany) according to DIN EN ISO 527-1 and DIN E ISO 527-3. A load cell of 500 N with an initial measuring distance of 100 mm between the grips and a crosshead test speed of 50 mm/min were used. Film samples were cut with a width of 20 mm and a length of 140 mm and conditioned for at least 48 h prior to testing at 23 ± 2°C and a relative humidity of 50 ± 10%. All measurements were repeated five times for each film sample. Tensile strength (MPa), elongation at break (%) and elastic modulus were calculated using the testing software testXpert II – V3.5.

2.3.3 | Colour

A Chroma Meter CR-410 colorimeter (Konica Minolta Sensing, Germany) was used for colour measurements of the films using the L* a* b* colour system, of which the total colour differences (∆E) were calculated using the control film as standard reference. The colorimeter was calibrated using the standard white plate, and measurements were replicated fivefold.

$$\Delta E = \left(\Delta L^2 + \Delta a^2 + \Delta b^2 \right)^{0.5}$$

where $\Delta L = L_{standard} - L_{sample}, \Delta a = a_{standard} - a_{sample}, \Delta b = b_{standard} - b_{sample}$. Standard values were taken from the pure sodium alginate control film.

2.4 | Preparation of inoculum for in vitro antimicrobial activity tests and food tests

For the in vitro evaluation of antimicrobial activity of the sodium alginate films, *E. coli* (ATCC 8739) and *L. innocua* (ATCC 33090, a surrogate for *L. monocytogenes*) were selected as test microorganisms. For the disc diffusion assay, overnight cultures of *E. coli* were prepared in 10-mL LB broth (Biolife, Italy) and for *L. innocua* in 10 mL of BHI broth (Biolife, Italy) at 37°C for 20 h. For the food tests, cold-adapted *L. innocua* were cultivated in 10 mL of BHI broth for 7 days at 4°C and subsequently pelleted by centrifugation at 4000 rpm for 2 min at room temperature (Sigma 3-18 K) and washed twice with 8 mL of 0.1% peptone water containing 0.85% NaCl before use. The concentration of the cell cultures was adjusted by counting with a Neubauer

improved counting chamber followed by serial dilution using 0.1% peptone water including 0.85% NaCl. For the in vitro tests, a concentration of 10^7 CFU/mL was used, whereas the cultures for the food tests were diluted to 10^3 CFU/mL. To verify the dilution series, the countable dilution steps were plated out on the respective agar plates (BHI agar for *L. innocua* and LB agar for *E. coli*).

2.5 | Disc diffusion assay

For the disc diffusion assay, a BHI agar plate for *L. innocua* and an LB agar plate for *E. coli* were inoculated with 0.1 mL of the respective bacterial overnight culture suspension at a concentration of 10^7 CFU/mL. A round piece of sodium alginate film sample (control film, CEO-6, CA-4 and CA-6) with a diameter of approximately 23 mm was then placed on the air-dried inoculated agar plates. The plates were incubated at 37°C for 24 h. The inhibition zone that formed around the packaging film was subsequently measured using a digital calliper (Wiha Tools Ltd., Germany). The assay was performed in quintuplicates.

2.6 | Vapour diffusion assay

As an inoculum, 1 mL of the bacterial culture of *L. innocua* at a concentration of 10^7 CFU/mL was added to 1 L of sterilised drinking water and stirred briefly to obtain a final bacterial concentration of 10^4 CFU/mL. Of this, 100 mL were filtered through a cellulose nitrate filter (0.45 μm, Sartorius Stedim Biotech GmbH, Germany) using a vacuum pump to transfer 10^6 CFU to this filter. The filter loaded with bacteria was then placed on a Tryptone Soya Agar (TSA, Oxoid, UK) plate with a diameter of 60 mm (Eppendorf, Germany). The sodium alginate film sample containing CEO as well as the sodium alginate control film with a diameter of 50 mm was attached to the lid of the Petri dish containing the inoculum with double-sided adhesive tape, preventing direct contact with the bacteria. In case of positive controls, a sterile filter paper discs (diameter 47 mm) loaded with 237 μL of CEO or 100 μL of propionic acid were attached to the lid of the Petri dish. The headspace of the Petri dish measured approximately 14.5 cm^3. The Petri dishes were sealed airtight with a rubber gasket as well as a Parafilm. Additionally, they were individually packed in a PET AlOx/PE high barrier sealed pouch (Wipf AG, Switzerland). The volume in the pouch amounted to a maximum of 160 cm^3. Incubation lasted 1 or 6 days at a temperature of 37°C. After incubation, the cellulose nitrate filters were placed in a test tube containing 10 mL of nutrient broth (BHI for *L. innocua* and LB for *E. coli*) and vortexed for 15 min at room temperature until all bacteria were in solution. A dilution series was prepared from this bacterial solution, and appropriate dilution steps were plated out on the respective agar plates using the spread-plate method (BHI for *L. innocua* and LB for *E. coli*). After 24 h of incubation at 37°C, microbial counting of the agar plates was performed. The assay was conducted in quintuplicates.

2.7 | Food tests on sliced cooked ham

Sliced cooked ham (pork, nitrite salting mix [table salt, preservative: E 250], seasoning mix, maltodextrin, glucose, yeast extract, stabiliser: E 451, antioxidant: E 301, aroma; a_w: 0.981, pH: 6.04) (M-Budget Hinterschinken, Micarna SA, Switzerland) with a diameter of 60 mm and an approximate weight of 3 g was placed in a sterile Petri dish (60 mm diameter, Eppendorf, Germany) and inoculated with 100 μL of the cold-adapted *L. innocua* at a concentration of 10^3 CFU/mL. Thereafter, the sodium alginate film samples control film, CEO-6, CA-4 and CA-6 were cut to 55 mm in diameter and either placed directly on the ham (control film, CA-4, CA-6) or attached to the lid of the Petri dish with double-sided adhesive tape to prevent direct contact (CEO-6). The Petri dishes were sealed airtight with a rubber seal and Parafilm and packed in a PET AlOx/PE high barrier bag (Wipf AG, Switzerland) with an approximate head volume of 160 cm^3. The samples were stored at 7.5 ± 0.4°C 12 days, which corresponds to the standard shelf life for cooked ham in Switzerland. The initial *L. innocua* load and the recovery rate of inoculated bacteria were measured 1 h after sampling preparation (t0). Additionally, the initial
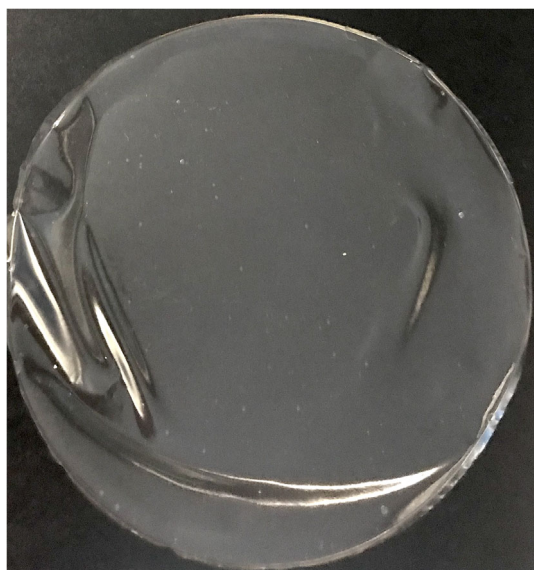
bacterial load of *L. innocua* was determined in non-inoculated samples. Microbial analyses were carried out after 6 and 12 days of storage. For this purpose, the sliced cooked ham samples were diluted 1:10 with Half Fraser Broth (Biokar Diagnostics, France) and homogenised with a stomacher (Seward Stomacher 400 circulator) at 300 rpm for 2 min. Microbial counts were obtained by serial plating on Agar Listeria acc. to Ottaviani & Agosti (ALOA) (Biolife, Italy) with an incubation period of 24 h at 37°C. All samples were tested fivefold.

2.8 | Statistical analyses

For all microbiological analyses, the results are given as mean value ± standard deviation. The data were analysed by means of one-factorial analysis of variance (ANOVA) and unpaired two-sample *t* test using the data analysis software OriginPro (by OriginLab Corporation), provided that the data showed a normal distribution. For data with a deviation from the normal distribution, the Kruskal–Wallis test and the Mann–Whitney *U* test were used. To test data sets for normal distribution, the Shapiro–Wilk test was performed. Unequal variances of two data groups were detected with the Levene test (*F* test). Statistically significant differences were assumed if *p* < 0.05. All tests were calculated with a data set of at least *n* = 3.

3 | RESULTS AND DISCUSSION

3.1 | Characteristics of sodium alginate films

Initially, sodium alginate films with and without antimicrobial substances were prepared, and their physical and mechanical characteristics were evaluated. The sodium alginate films (control film) appeared to be clear, glossy and colourless (Figure 1). Due to the incorporation of calcium ions into the film-forming solution, the films revealed no contraction on exposure to moisture. The thickness, moisture content and density of the control film and the active films measured are listed in Table 1. Control films had a thickness of 74 ± 5 μm, density of 1.03 ± 0.20 g/cm^3 and a moisture content of 24.81 ± 4.31%. Integration of 6 wt% of CEO increased the thickness of the films to 127 ± 11 μm but did not have any significant influence on the density and moisture content of the films. The addition of CA also resulted in

FIGURE 1 Image of a dried 2 wt% sodium alginate film (control film) with an amount of film-forming solution of 0.27 g/cm^2.

TABLE 1 Effect of cinnamon essential oil (CEO) and citric acid (CA) integration on the physical properties of sodium alginate films.

Film	Thickness [μm]	Density [g/cm^3]	Moisture content [%]	Elongation at break [%]	Tensile strength [MPa]	Elastic modulus [MPa]
Control film	74 ± 5[a]	1.03 ± 0.20[a]	24.81 ± 4.31[ab]	11.3 ± 2.4[a]	40.9 ± 3.3[a]	1090.0 ± 180.9[a]
CEO-6	127 ± 11[b]	0.94 ± 0.16[a]	24.04 ± 2.53[a]	4.8 ± 2.3[b]	6.8 ± 1.9[bc]	207.0 ± 32.2[a]
CA-4	111 ± 8[c]	1.13 ± 0.44[a]	35.41 ± 0.24[bc]	22.6 ± 7.4[c]	11.0 ± 3.9[b]	37.2 ± 6.4[b]
CA-6	175 ± 12[d]	1.77 ± 0.03[b]	48.02 ± 4.40[c]	33.2 ± 8.2[c]	4.5 ± 1.1[c]	3.5 ± 1.5[b]

Note: Values are expressed as mean ± standard deviation (*n* = 3, moisture content, density; *n* = 5, thickness, elongation at break, tensile strength, elastic modulus). Different letters in superscript refer to being statistically significantly different in each column (*p* ≤ 0.05).

a significant increase in thickness (111 ± 8 μm for 4 wt% and 175 ± 12 μm for 6 wt%) compared to the control samples. However, only alginate films with 6 wt% CA showed a significant increase in density (1.77 ± 0.03 g/cm^3) and moisture content (48.02%).

The incorporation of CA and CEO resulted in significantly different thicknesses of the films. The integration of the antimicrobial substances into the sodium alginate film might have increased the thickness of the dried films due to a higher content of solid components in the film-forming solution.[42] Results showed that the density of the films correlated with their corresponding moisture content. Specifically, the film CA-6 showed increased values that might be caused by the solubility of CA and its water binding capacity, resulting in a significantly higher moisture content.[25] This increase in moisture content may have led to an increased thickness of the active packaging film. A change in moisture content compared to the control film was not observable for the CEO-6 film. This may be due to the hydrophobic nature of the integrated CEO that seemed to reduce the moisture content and density of the film somewhat. This finding might be explained by the lack of water binding behaviour of CEO in the film. The increase in the thickness of the CEO-6 film must therefore only be due to the increased proportion of solid substance in the film-forming solution.

3.2 | Mechanical properties of sodium alginate films

Table 1 reports the values of the important parameters elongation at break, tensile strength and elastic modulus of the control film as well as the three active films, describing their mechanical properties. Integration of CA into the sodium alginate film increased the elongation at break significantly from 11.3 ± 2.4% to 22.6 ± 7.4% with 4 wt% CA and 33.2 ± 8.2% with 6 wt% CA and decreased the tensile strength from 40.9 ± 3.3 MPa to 11.0 ± 3.9 MPa with 4 wt% CA and 4.5 ± 1.1 MPa with 6 wt% CA. The decrease in tensile strength was also accompanied by a significant decrease in the elastic modulus of the sodium alginate film from 1090.0 ± 180.9 MPa to 37.2 ± 6.4 MPa (4 wt% CA) and 3.5 ± 1.5 MPa (6 wt% CA). The addition of CEO, however, significantly reduced both the elongation at break and the tensile strength to 4.8 ± 2.3% and 6.8 ± 1.9 MPa, respectively. However, no significant change in the modulus of elasticity was observed (207.0 ± 32.2 MPa.). A similar result where rosemary extract

decreased the tensile strength and the elongation at break of starch-alginate composite films was also reported by Yan et al.[43] Mahcene et al. also showed that addition of various essential oils in sodium alginate films decreased the tensile strength of the films significantly.[44] Decrease in the tensile strength of the sodium alginate films with the addition of CEO could be due to the uneven dispersion of the CEO in the polymer matrix. The decrease in the tensile strength, the elongation at break and the elastic modulus for the CEO-6 film might be due to its heterogeneity and its rather porous structure of the polymer network with an oil-based liquid integrated, as proposed by Yan et al. and Ribeiro-Santos et al.[43,45] The decrease in the tensile strength with the addition CA could be due to the weakening of the bonding between the molecules of the polymer. This phenomenon was similarly observed in a study with a packaging film made of linear low-density polyethylene (LLDPE) and corn starch containing different amounts of CA.[46]

3.3 | Colour of sodium alginate films

The appearance, including the colour, of a packaging material influences consumer purchase behaviour through visual acceptance.[47] The evaluation of the colour measurements of the active packaging films developed sometimes revealed significant differences between the three film types tested (Table 2), suggesting that the integration of antimicrobial substances as well as their concentration in the film affect the optical properties of the packaging film. Delta E, a reference for the total colour distance of objects, showed significant differences between the control film and the three films CEO-6, CA-4 and CA-6. Further, visually assessed data showed a decrease in transparency of the films with an increase of the concentration of antimicrobial substances. This is in accordance with other studies reported by Ojagh et al., Zhang et al. and Pranoto et al.[12,22,48]

CEO-6 film displayed a tendency towards a yellow tint, which might be due to the yellow colour of the essential oil, in this case CEO.[42,48] This colour deviation appeared to be the most prominent of all the films and resulted in the largest delta E value of the active films at 3.17. The change in colour observed between CA-4 and CA-6 suggests that the concentration of CA has a detectable effect on the optical properties of the film, with an increase in CA of 2 wt% causing a change in delta E value of 1.43 and a visible decrease in transparency.

TABLE 2 Colour attributes of sodium alginate films integrated with cinnamon essential oil (CEO) and citric acid (CA).

Film	L*	a*	b*	ΔE
Control film	90.90 ± 0.36[a]	1.57 ± 0.08[a]	−0.15 ± 0.44[a]	0.00 ± 0.00[a]
CEO-6	87.80 ± 0.13[b]	1.32 ± 0.05[b]	0.36 ± 0.29[a]	3.17 ± 0.17[b]
CA-4	89.97 ± 0.13[c]	1.66 ± 0.02[a]	−0.77 ± 0.14[b]	1.13 ± 0.14[c]
CA-6	88.84 ± 0.22[d]	1.26 ± 0.06[b]	1.33 ± 0.27[c]	2.56 ± 0.34[d]

Note: Values are expressed as mean ± standard deviation measured in quintuplicates. Different letters in superscript refer to being statistically significantly different in each column at $p \leq 0.05$.
Abbreviation: ΔE, total colour differences.

3.4 | In vitro antimicrobial activity of sodium alginate films
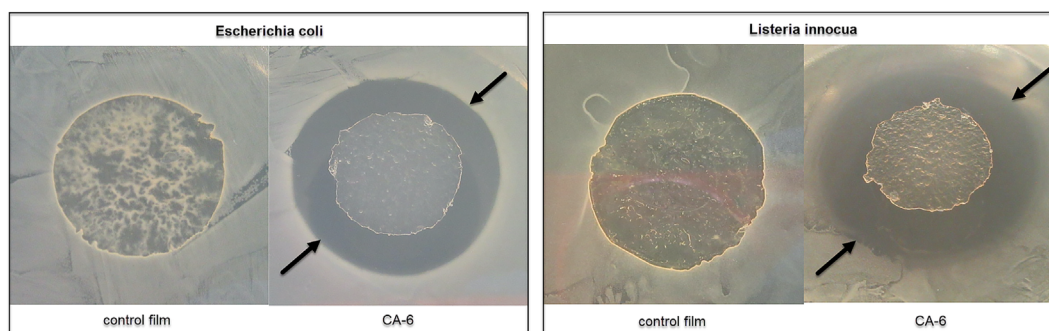
3.4.1 | Disc diffusion assay

The control film did not show any inhibition zone with both *E. coli* and *L. Innocua* showing that sodium alginate films do not have an intrinsic antimicrobial activity against these microorganisms. This is in line with the findings of Mahcene et al. with the pure sodium alginate films.[44] All three active packaging films tested in the disc diffusion assay demonstrated antimicrobial activity against gram-negative *E. coli* as well as gram-positive *L. innocua* (Table 3). Both microorganisms seemed to be more sensitive to CA than to CEO, as the inhibition zones formed around the CA-6 films were significantly larger than the CEO films (containing same amount of antimicrobial substance). With the increase in CA concentration in the film from 4 wt% to 6 wt%, the inhibition zone and so the antimicrobial effect increased significantly. Highest zone of inhibitions was obtained with the CA-6 films 40.10 ± 1.00 mm for *E. coli* and 45.87 ± 1.90 mm for *L. innocua* (Figure 2), respectively.

In general, it may be concluded that *L. innocua* reacts more sensitively to the substance CA than *E. coli*, whereas *E. coli* seems to react more sensitively to CEO than *L. innocua*. The difference in the sensitivity of the two bacteria towards the antimicrobial substances could be due to the differences in the cell wall structure of the gram-negative *E. coli* and gram-positive *L. innocua* as well as the different mode of action of CA and CEO.[49–51]

3.4.2 | Vapour diffusion assay

In order to evaluate the antimicrobial effect of the volatile substance in the CEO-6 film via gas phase, a vapour diffusion assay was performed. The control film with an initial load of 5.9 log CFU/filter, used as a negative control, showed no inhibition of *E. coli* after 1 and 6 days as the load increased to 10.6 and 9.4 log CFU/filter, respectively (Figure 3). With the CEO-6 film, microbial load increased to 9.6 log CFU/filter after 1 day, which remained significantly lower than the control, and decreased afterwards to 4.1 log CFU/filter after 6 days. This resulted in a significant antimicrobial activity with a log reduction of 5.3 CFU/filter compared to the control film. Pure CEO as well as propionic acid was used as positive controls in the assay. Both substances exhibited a strong antimicrobial effect, inhibiting *E. coli* below the detection limit after 24 h and 6 days of incubation.

In the vapour diffusion assay using *L. innocua* and the control film, initial microbial load of 4.9 log CFU/filter increased to 9.3 log CFU/filter after 1 day and to 9.4 log CFU/filter after 6 days (Figure 4). No significant difference in bacterial counts was detected between the control sample and the CEO-6 film after 1 day, suggesting that the CEO-6 film did not show any antimicrobial activity after 1 day of incubation. However, the bacterial load decreased significantly from 9.3 to 6.2 log CFU/filter after 6 days with the CEO-6 film. Pure CEO as a positive control sample also revealed no significant reduction in bacterial counts and thus no antimicrobial activity against *L. innocua* after 1 day (similar to CEO-6 films), whereas all bacteria were successfully inhibited after 6 days. Lower antimicrobial activity of the CEO-6 films compared to the samples with pure CEO after 6 days could be due to the lower release rate of cinnamon EO from the alginate films and so the late formation of an equilibrium in the gas phase. Similar

TABLE 3 Direct contact antimicrobial activity of sodium alginate films with cinnamon essential oil (CEO) and citric acid (CA) integrated.

Film	Diameter of inhibition zone [mm]	
	E. coli	*L. innocua*
Control film	00.00 ± 0.00[a]	00.00 ± 0.00[a]
CEO-6	36.76 ± 0.64[b]	30.86 ± 2.55[b]
CA-4	36.20 ± 0.93[b]	40.86 ± 2.45[c]
CA-6	40.10 ± 1.00[c]	45.87 ± 1.90[d]

Note: Values depicted as mean ± standard deviation ($n = 5$). Different letters in superscript refer to being statistically significantly different in each column ($p \leq 0.05$).
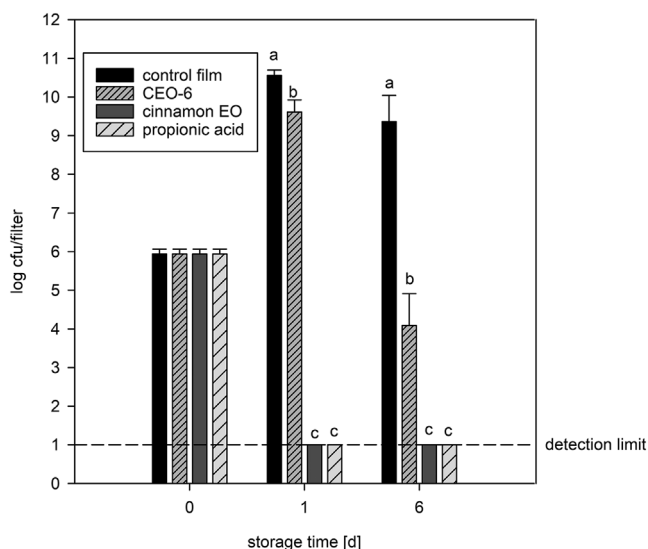

FIGURE 2 Images of the inhibition zone formation of the in vitro disc diffusion assay using sodium alginate film integrated with 0 wt% and 6 wt% of citric acid (CA) with *E. coli* (left) as well as *L. innocua* (right) as inoculum tested at 37°C. Distance between the arrows shows the diameter of inhibition zone.

FIGURE 3 Indirect contact in vitro antimicrobial activity of sodium alginate film with 6 wt% of cinnamon essential oil (CEO) using _E. coli_ as inoculum at 37°C. Represented as mean ± standard deviation ($n = 5$). Identical letters within a time point for each sample indicate that the outcomes are not significantly different ($p \geq 0.05$).
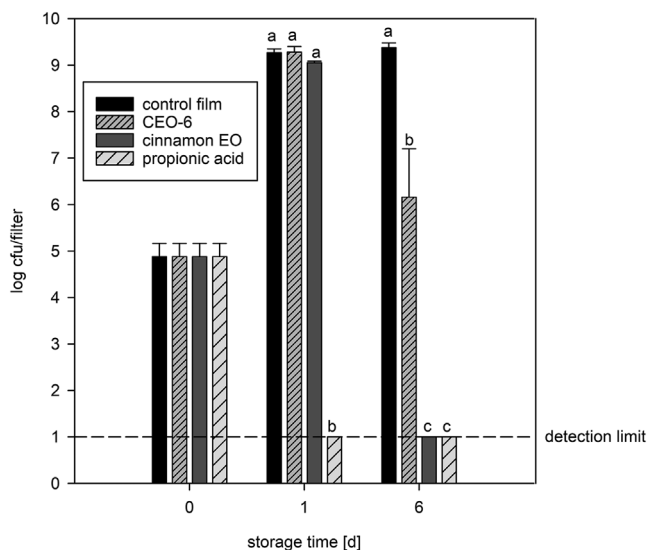
FIGURE 4 Indirect in vitro antimicrobial activity of sodium alginate film with 6 wt% of cinnamon essential oil (CEO) using _L. innocua_ as inoculum at 37°C. Data are depicted as mean ± standard deviation ($n = 5$). Identical letters within a time point for each sample indicate that the outcomes are not statistically significantly different ($p \geq 0.05$).

results have also been shown by Chu et al in pullulan and Xu et al. in chitosan films.[18,20] Propionic acid, the second positive control sample, fully inhibited bacterial growth after 1 and 6 days with a microbial load below the detection limit, similar to the experiment with _E. coli_.

3.5 | Direct and indirect contact antimicrobial activity in food tests

As pointed out in previous studies, although in vitro antimicrobial activity tests are very useful to develop and optimise the antimicrobial films to achieve high antimicrobial activities against target microorganisms, the results of such tests could be significantly different to the antimicrobial activities in food tests using the same films, due to the influence of the food matrix.[38,39,52] In order to assess the antimicrobial efficacy with regard to possible food packaging applications, the antimicrobial activity of the sodium alginate films containing 6 wt% CEO as well as 4 wt% CA or 6 wt% CA was evaluated against _L. innocua_ on cooked sliced ham. Each ham sample was inoculated with 0.1 mL of the inoculum containing 10^3 CFU/mL _L. innocua_ (to simulate the possible natural low contamination). Since many food products prone to microbiological contamination are stored at refrigerator temperatures, the food test in this study was carried out at 7°C. The antimicrobial activity of the active films containing CEO was tested through the gas phase, whereas the antimicrobial activity of the films containing CA was tested through the direct contact of the film with the inoculated surface of the ham samples. Control film (sodium alginate film with no antimicrobial substance) was used as a control for films containing CA. No _L. innocua_ were detected on the untreated and uninoculated ham samples.

As shown in Figure 5, the bacterial concentration of the inoculated samples on day zero was below the detection limit (1.0 log CFU/g) due to the low inoculation concentration. When CEO-6 films (indirect contact) were used, the growth of _L. innocua_ could not be inhibited, and the levels of _L. innocua_ increased to 3.6 ± 0.1 log CFU/g after 6 days and 4.8 ± 0.2 log CFU/g after 12 days. On the other hand, use of CA-4 and CA-6 films completely inhibited the growth of _L. innocua_ over the 12 days, showing a strong antimicrobial activity compared to the control films, which resulted in levels of _L. innocua_ below the detection limit after both 6 and 12 days.

The decrease in or lack of antimicrobial activity of the volatile antimicrobial substances in food tests compared to in vitro tests has also been reported in previous studies.[11] Rüegg et al. demonstrated that there is indeed a difference between in vitro and food tests, strongly depending on the essential oil applied.[38] According to their findings, selected essential oils such as, for instance, rosemary essential oil are able to exhibit antimicrobial activity against _L. innocua_ on sliced cooked chicken breast meat through a vapour phase, which could be proven in this study using sliced cooked ham as well. Other studies found similar results, where selected essential oils exhibited strong antimicrobial activity in vitro, which could not be replicated and confirmed in tests with real food products.[40,53,54] Burt demonstrated a need for a higher concentration of essential oils in food tests compared to in vitro tests in order to achieve similar antimicrobial effects.[53] Otero et al. observed antimicrobial activity of oregano essential oil in vitro against _E. coli_ O157:H7 in direct contact as well as through vapour phase, whereas they detected no effective inhibition
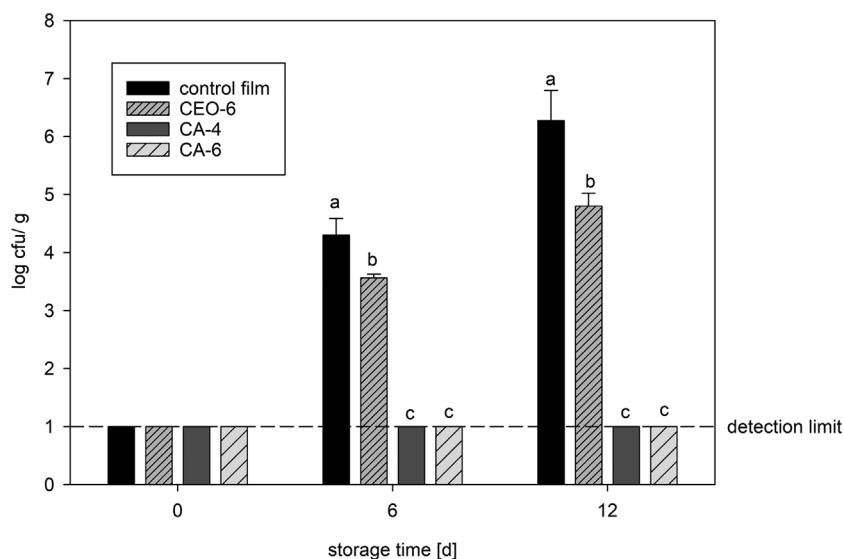
FIGURE 5 Antimicrobial activity of sodium alginate films integrated with cinnamon essential oil (CEO) and citric acid (CA) in a food test using sliced cooked ham with *L. innocua* as inoculum at 6.9 ± 0.5°C. Data are depicted as mean ± standard deviation ($n = 3$). Identical letters within a time point for each sample indicate that the outcomes are not statistically significantly different ($p ≥ 0.05$). *Note*: CEO-6, indirect contact; CA-4 and CA-6, direct contact; control film, direct contact.

when tested on raw milk cheese (Zamorano) as a food product at cold storage.[54]

The findings of the CEO-6 film in the vapour diffusion assay as well as the food test suggest that the volatilisation rate of the CEO is slowed down by its incorporation into the sodium alginate film. This could be due to the interactions between the biopolymer and the substances of the cinnamon EO, as reported for other EOs in chitosan films by Sánchez-González et al.[55] Further, it has been demonstrated by Kuorwel et al. that the release rate of volatile antimicrobial substances from essential oils can be influenced by specific environmental factors such as relative humidity and temperature.[56] We performed the food tests at 7°C compared to 37°C in vitro tests which probably decreased the release rate of CEO from alginate films and therefore also had a negative effect on the antimicrobial activity.

Due to its antimicrobial activity, CA as a prominently used food preservative can be found in products such as jams, ice creams, carbonated beverages, fruit and vegetable juices, salad dressings and canned food products.[57] The addition of CA to foods as a preservative is favourable not only because of its strong antimicrobial activity but also because of its stability and persistence in effectiveness of bacterial growth inhibition.[58] Similarly, in the case of packaging films, CA appears capable of stable integration while improving the antimicrobial properties of packaging films.[59,60] In this study, alginate films with 4 wt% and 6 wt% CA exhibited strong antimicrobial activity in direct contact in vitro as well as in food tests with sliced cooked ham. Therefore, CA integrated films seem to have high potential for packaging rapidly perishable food products.

4 | CONCLUSION

In this study, sodium alginate films including 6 wt% CEO as volatile and 4 wt% as well as 6 wt% CA as non-volatile antimicrobial substances were developed to evaluate their potential to be used as antimicrobial films for sliced cooked ham. It has been shown that the

integration of the antimicrobial substances changes the optical, physical and mechanical properties of the alginate films, which has to be taken into consideration if these films are to be used for food packaging.

The sodium alginate films developed with CEO and CA exhibited a significant antimicrobial effect in in vitro tests against the pathogen surrogates *E. coli* and *L. innocua*. In refrigerated food tests using sliced cooked ham, both alginate films with CEO and CA showed significant antimicrobial activity against *L. innocua*. However, only alginate films with CA inhibited the growth. The results indicate that with regard to industrial applications, the antimicrobial activity of such films should be tested with the targeted food product under real application and storage conditions. In addition, essential oils released from antimicrobial films, such as CEO used in this study, may result in changes in sensorial properties of food. In this respect, use of antimicrobial agents such as CA can be more conducive to consumer acceptance.

DATA AVAILABILITY STATEMENT
Research data are not shared.

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