

Antimicrobial bilayer starch film containing zein electrospun fibers encapsulating thyme essential oil for cherry tomato packaging

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Abstract

Background: This study evaluated the antimicrobial activity of thyme essential oil (TEO) encapsulated in electrospun zein fibers for use in bilayer films as active packaging for cherry tomatoes. The antimicrobial activity of TEO and bilayer films with 20%, 40%, and 60% TEO was assessed with agar diffusion and microatmosphere assays. Films containing 60% TEO were tested with viable cell count against microorganisms and applied as bioactive packaging for cherry tomatoes. Weight loss, decay rate, firmness, total soluble solids, titratable acidity, pH, and microbiological quality were analyzed over a 14 day storage period.

Results: The free TEO exhibited antimicrobial activity against all tested bacteria and fungi, with the strongest antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus* and complete fungal inhibition in the microatmosphere assay. The 60% TEO bilayer films completely inhibited *S. aureus* and *Penicillium crustosum*. They reduced *Escherichia coli* growth by 71.5% and *Aspergillus flavus* growth by 71.3%. As active packaging for cherry tomatoes, after 14 days of storage, the bilayer films reduced weight loss from 14.7% to 6.8% and decay incidence from 83.3% to 33.3% compared with the control, while maintaining total soluble solids (5.0 °Brix), titratable acidity, and pH. The bilayer films also reduced *E. coli* by approximately 92% and decreased aerobic mesophilic bacteria by approximately 1.8 log colony-forming units (CFU) g⁻¹ and yeasts and molds by approximately 1.5 log CFU g⁻¹.

Conclusion: The results confirm TEO's antimicrobial effectiveness in bilayer films and potential for active packaging, offering a sustainable alternative to extend the shelf life of perishables and a promising technology for industrial fresh produce preservation.

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INTRODUCTION

Fresh fruits and vegetables play a vital role in a healthy diet.¹ However, their high water content, rapid metabolism, and susceptibility to microbial activity make them highly perishable.² Postharvest diseases caused by bacteria and fungi, leading to decay, are a major cause of losses in vegetables, particularly fruits.³ Food waste due to improper storage, microbial contamination, and logistical failures represents a major challenge in the postharvest chain of fresh fruits and vegetables.^{4,5} Among these products, climacteric fruits such as cherry tomatoes are especially susceptible to deterioration due to their structural fragility and intense postharvest enzymatic activity.⁶

Despite potential health risks, the food industry still relies on chemical preservatives and pesticides to extend the shelf life of fresh produce. It also makes extensive use of petroleum-based packaging materials, which are environmentally harmful and relatively ineffective in preventing microbiological spoilage.⁷ These

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packaging systems are passive, lacking active functionality to control microbial growth or preserve the sensory quality of packaged products.⁸ As a result, biodegradable polymers with active properties, such as antimicrobial activity and controlled release of volatile compounds, have gained attention as promising alternatives to conventional packaging. These active systems contribute to the extension of the shelf life of fresh and minimally processed foods, such as fruits, by directly inhibiting microbial growth and stabilizing quality attributes.^{9,10}

In recent years, biodegradable active packaging systems have been investigated extensively as sustainable alternatives for preserving fresh fruits and vegetables.^{9,11} Films based on polysaccharides, proteins, and composite materials incorporating natural antimicrobial compounds have demonstrated promising results in delaying microbial growth, reducing quality losses, and extending shelf life.^{12,13} However, several of these systems still face limitations associated with the rapid release and volatilization of active compounds, reducing their long-term antimicrobial effectiveness. Developing strategies capable of protecting bioactive compounds while enabling their controlled release therefore remains an important challenge in the development of active food packaging.¹⁴

In this context, the incorporation of essential oils (EOs) into natural polymer matrices has emerged as an effective strategy for extending the shelf life of perishable foods, particularly fruits and vegetables.¹⁵ Rich in volatile phenolic compounds such as thymol and carvacrol, EOs exhibit strong antimicrobial and antioxidant activity, contributing to the preservation of food quality and safety.¹⁶ Thus, combining different materials in bilayer films creates more efficient functional systems for use in active packaging applications.^{17,18} Polysaccharides such as starch stand out as promising alternative materials for film production due to their transparency, abundance, low cost, flexibility, and biodegradability.¹⁹ Starch can serve as the structural layer in multilayer systems, providing mechanical strength and barrier properties.²⁰ However, because of its high hydrophilicity, starch exhibits poor moisture resistance, requiring the addition of complementary hydrophobic materials.²¹ To develop materials with bioactive properties, the incorporation of a second functional layer is also necessary.²²

In this context, zein, a hydrophobic protein extracted from corn, has been used widely in the production of electrospun fibers, enabling the formation of porous structures with a high surface area. This feature facilitates the incorporation of antimicrobial agents, such as EOs, protecting them from thermal and oxidative degradation and allowing for their sustained release over time.²³ In this bilayer system, the outer film layer, made of sweet potato starch, acts as a physical barrier that prevents the migration of the antimicrobial agent into the external environment, directing its release exclusively toward the food. Meanwhile, the inner layer, composed of zein, regulates the controlled release of these bioactive compounds into the food matrix. This unidirectional release architecture is essential to maximize antimicrobial efficacy, reduce losses due to volatilization, and promote a prolonged effect on the packaged food.

Thyme essential oil (*Thymus vulgaris*) (TEO) is rich in bioactive compounds, primarily thymol and carvacrol, which act synergistically as antimicrobial agents.²⁴ Its incorporation into polymeric packaging has demonstrated effectiveness against a wide range of microorganisms, including fungi and pathogenic bacteria, making it particularly useful for food-related applications.^{25,26} However, due to the high volatility and sensitivity of TEO components to light, oxygen, and heat, technological strategies

such as encapsulation in electrospun fibers are necessary to ensure protection against degradation and to enable controlled release, maintaining its effectiveness over time.^{27,28}

In a previous study,²⁹ a bilayer system composed of sweet potato starch films and zein electrospun fibers containing TEO was developed and characterized. The material exhibited improved physicochemical, mechanical, and barrier properties, in addition to antioxidant activity and the potential for controlled release of bioactive compounds. These results highlighted the potential of the matrix as a functional and sustainable material for application in active packaging. Despite these advances, few studies have evaluated bilayer systems combining starch films with electrospun zein fibers encapsulating essential oils under food storage conditions. Moreover, evidence regarding their antimicrobial performance against both foodborne and spoilage microorganisms and their effectiveness in preserving fresh produce remains limited. Building upon these results, the present study investigates, for the first time, the antimicrobial performance of the previously developed bilayer films through *in vitro* assays against foodborne and spoilage microorganisms and evaluates their effectiveness as active packaging for preserving the physicochemical and microbiological quality of cherry tomatoes during storage.

This study hypothesizes that bioactive films containing encapsulated TEO exhibit effective antimicrobial activity against bacteria and fungi, making them suitable for use as active packaging to extend the shelf life of foods such as cherry tomatoes. In addition to their antimicrobial potential, bilayer films based on biopolymers align with the principles of the circular economy by promoting sustainable and safe packaging solutions. The study therefore investigates the antimicrobial activity of TEO and its application as a bioactive compound in bilayer films containing zein/TEO fibers for use in active packaging. The sustained action of TEO over time is evaluated, enabled by its controlled release through encapsulation. The antimicrobial efficacy of TEO and the bilayer films was tested against gram-positive and gram-negative bacteria as well as fungi. The practical applicability in preventing food spoilage was assessed through the use of these films in packaging cherry tomatoes, with the analysis of physicochemical and microbiological parameters over 14 days of storage.

MATERIAL AND METHODS

Material

The yellow sweet potato (*Ipomoea batatas* L. 'Amelia') was acquired from the local market in Pelotas, Rio Grande do Sul, Brazil, for starch extraction. The starch extraction procedure was performed according to the methodology previously described in an earlier study.²⁹ Glycerol (CAS registry number 56-81-5; Synth, Diadema, Brazil) was used to produce biodegradable films.

Thyme (*Thymus vulgaris*) essential oil (TEO) was purchased from Ferquima - Indústria e Comércio de Óleos Essenciais (Vargem Grande Paulista, Brazil) in an amber bottle with a total volume of 100 mL. The chemical composition of the TEO used in this study was previously characterized by gas chromatography-mass spectrometry (GC-MS), identifying *p*-cymene (36.46%), thymol (29.48%), carvacrol (5.78%), γ -terpinene (5.34%), and linalool (4.63%) as the major constituents.²⁹ Commercial zein (97% purity, CAS registry number CAS 9010-66-6) was obtained from Sigma-Aldrich, Barueri, Brazil, and ethanol ($\geq 99.8\%$ purity, CAS registry number CAS 64-17-5) from Éxodo Científica, Sumare, Brazil. The ethanol was used as the solvent for electrospun fiber formation.

The MecLab MegaPurity system (MecLab, Jacarei, Brazil) provided ultrapure water (Milli-Q, Merck KGaA, Darmstadt, Germany).

The following culture media were used: potato dextrose agar (PDA) (KASVI, CAT: K25-1022, Pinhais, Brazil), Mueller–Hinton agar (MH) (IonLab, IO263, Araucaria, Brazil), Baird–Parker agar (BP) (IonLab, IO013), xylose lysine desoxycholate agar (XLD) (Kasvi, K25-1274), polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar (Merck, 1.11755), mannitol egg yolk polymyxin (MYP) agar (Merck, 1.05267), eosin methylene blue (EMB) agar (Kasvi, K25-1050), *Pseudomonas* Isolation Agar (M406; HiMedia, Thane, India), brain heart infusion (BHI) broth (M210; HiMedia), Mueller–Hinton broth (Biokar Diagnostics, Allonne, France), RPMI 1640 with L-glutamine without bicarbonate (INLAB, Sao Paulo, Brazil), and the reagents 3-(N-morpholino)propanesulfonic acid (MOPS) (CAS 1132-61-2; INLAB) and anhydrous D-glucose (CAS 50-99-7; Synth).

Standard strains of the following bacterial species were used: gram-positive (*Staphylococcus aureus* ATCC 10832, *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, and methicillin-resistant *S. aureus* [MRSA]), and gram-negative (*Escherichia coli* ATCC 43895, *Salmonella* Typhimurium ATCC 13311, and *Pseudomonas aeruginosa* ATCC 10145). The MRSA strain was obtained from Instituto Portugues de Oncologia do Porto. The fungi *Penicillium crustosum* and *Aspergillus flavus*, previously isolated from cheese samples and characterized,³⁰ as well as *Fusarium oxysporum* ATCC 48112, were used. These microorganisms were selected as representative foodborne pathogens and spoilage fungi to evaluate the broad-spectrum antimicrobial performance of the bilayer films before their application as active packaging for cherry tomatoes.

Preparation of bilayer films

The casting technique, chosen for its simplicity and ability to form homogeneous starch-based films, was used to produce starch films as previously described.³¹ Sweet potato starch was selected based on preliminary tests with different starch sources because it exhibited superior film-forming properties, producing homogeneous, continuous, and non-brittle films under the experimental conditions adopted in this study. The film-forming suspension was prepared by dispersing 40 g L⁻¹ starch in 100 mL of distilled water. Glycerol (1.20 g) was added as a plasticizer at a plasticizer-to-starch ratio of 0.30 g g⁻¹. Complete starch gelatinization was achieved by heating the suspension at 90 °C for 30 min on a hot plate with magnetic stirring (model 752A; Fisatom, Sao Paulo, Brazil). The suspension was then cooled to 50 °C, and 55.55 g was poured into acrylic plates (Ø 15 cm). The films were dried in a forced-air oven (Ethik Technology, Vargem Grande Paulista, Brazil) at 30 °C for approximately 16 h.

The electrospun fibers were prepared with zein as a polymer at a concentration of 300 g L⁻¹, dissolved in 700 mL L⁻¹ ethanol (in Milli-Q water) as the solvent. The solutions were stirred for 30 min using a magnetic stirrer at room temperature (22 ± 2 °C) to ensure complete homogenization. Thyme essential oil was subsequently incorporated into the zein solution at concentrations of 0.20, 0.40, and 0.60 mL g⁻¹ (v/w, relative to the zein mass), and the formulations were stirred for 15 min in the dark. A solution without TEO was prepared for use as a control.³²

The parameters used for electrospun fiber production followed a previously described method,²⁸ with modifications. The polymer solution was pumped at a flow rate of 0.9 mL h⁻¹ using an infusion pump (Model 100, KD Scientific, USA), under a system voltage of 20 kV (model INSES-HV30, Instor, Viamao, Brazil), with

controlled temperature (18 ± 2 °C) and relative humidity (40 ± 2%). A 10 mL disposable syringe equipped with a needle with an internal diameter of 0.8 mm was used, positioned 20 cm from the fiber collector. The electrospun fibers were directly deposited onto starch films previously fixed at the center of the collector target, thus forming a bilayer film, following the method described by Vitoria *et al.*²⁹

The zein/TEO electrospun fibers and corresponding bilayer films used in this study were previously developed and comprehensively characterized in terms of morphology by scanning electron microscopy (SEM), fiber diameter distribution, Fourier transform infrared (FTIR) profile, and thermal, physicochemical, mechanical, antioxidant, and release properties, as described in a previous study.²⁹

Antimicrobial analysis

Preparation of bacterial inoculum

The bacterial strains used in the experiment were preserved frozen in BHI broth with glycerol (propane-1,2,3-triol) at a 3:1 (v/v) ratio. For reactivation, a loopful of the frozen culture was transferred to BHI broth and incubated at 37 °C for 24 h. Subsequently, a loopful of this culture was streaked onto Petri dishes containing selective media and incubated at 37 °C for 24 h to isolate colonies. All incubations were carried out under standard conditions recommended for each microbial group.

From the bacterial growth on the Petri dishes, a loopful was extracted and resuspended in saline solution (8.5 g L⁻¹ NaCl, Synth), which was standardized to 0.5 on the McFarland scale (1.5 × 10⁸ colony-forming units (CFU) mL⁻¹). Subsequently, serial dilutions were performed to adjust the inoculum to the concentration required for each antimicrobial assay. Finally, 0.1 mL of the standardized bacterial suspension was uniformly distributed on the surface of MH agar plates with a diameter of 90 mm.

Preparation of fungal inoculum

Cultures of the fungi previously stored in PDA under refrigeration were transferred to a plate containing the same culture medium and incubated at 25 °C for 5 days in a germination chamber (model SSGFa, SolidSteel, Piracicaba, Brazil). The fungal inoculum was prepared following the methodologies described by Gurgel *et al.*³³ After the incubation period, 2 mL of sterile saline solution (8.5 g L⁻¹ NaCl) was added to the surface of the cultures. The resulting suspension was collected carefully, ensuring that no culture medium fragments were removed, and transferred to sterile tubes. It was then diluted in sterile saline solution (8.5 g L⁻¹ NaCl, Synth) and standardized to a McFarland scale of 0.5 (1.5 × 10⁸ CFU mL⁻¹). Subsequently, serial dilutions were performed to adjust the inoculum to the concentration required for each antimicrobial assay. Finally, 0.1 mL of the standardized fungal suspension was distributed evenly on the surface of PDA plates with a diameter of 90 mm.

In vitro antimicrobial activity of thyme essential oil

Agar diffusion test. The agar diffusion analysis was conducted against the bacteria *S. aureus*, *L. monocytogenes*, *E. coli*, and *Salmonella* Typhimurium, and the fungi *P. crustosum*, *A. flavus*, and *F. oxysporum*, following the method described by the Clinical and Laboratory Standards Institute³⁴ with adaptations. The bacterial and fungal inocula were adjusted to approximately 1 × 10⁸ CFU mL⁻¹ (McFarland 0.5 standard). Three sterile filter paper discs (4 mm in diameter) were placed equidistantly on the surface of the culture medium in each plate. For the negative

control, sterile distilled water was applied to one of the discs, and the other two discs received the same volume of free TEO (2, 5, or 10 µL). Each TEO volume was evaluated using independent plates. After sample application, the Petri dishes were sealed with Parafilm to prevent moisture loss and contamination during incubation. The plates were incubated at 37 °C for bacteria on Mueller–Hinton agar and at 25 °C for fungi on PDA, both placed in a germination chamber (SolidSteel). After incubation periods of 24 h for bacteria and 8 days for fungi, the plates were examined for inhibition zones. All treatments were performed in triplicate.

Microatmosphere test. Microatmosphere analysis, which is particularly suitable for evaluating the antimicrobial activity of volatile compounds in confined systems, was performed against the bacteria *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. Typhimurium* and the fungi *P. crustosum*, *A. flavus*, and *F. oxysporum*, following the method proposed by Ghabraie et al.,³⁵ with some modifications. The bacterial and fungal inocula were adjusted to approximately 1×10^5 CFU mL⁻¹. Volumes of 2, 5, 10, and 15 µL of free TEO were applied to sterile filter paper discs (85 mm in diameter) affixed to the inner surfaces of the Petri dish lids. To ensure uniform and homogeneous distribution on the filter paper, the essential oil was pre-emulsified in a sterile solution of distilled water and 10 mL L⁻¹ Tween 80 (CAS Registry Number 9005-65-6; Synth). A sterile saline solution (8.5 g L⁻¹ NaCl) was used as a positive growth control.

The plates were sealed with Parafilm and incubated in an inverted position (lid facing downward) at 37 °C for 24 h for bacteria and at 25 °C for 3 days for fungi in a germination chamber. After the incubation period, the colony-forming units (CFU) were counted. All treatments were performed in triplicate. The results were expressed as a percentage reduction in microbial growth and calculated according to Eqn (1), where 'C_c' represents the growth in the control (without TEO) and 'C_t' represents the growth in the treatment with TEO:

$$\text{inhibition (\%)} = \left(\frac{C_c - C_t}{C_c} \right) \times 100 \quad (1)$$

In vitro antimicrobial activity of bilayer film

Agar diffusion test. The agar diffusion analysis was conducted against the bacteria *S. aureus* and *E. coli* and the fungi *P. crustosum* and *A. flavus*, following the methodology described by the Clinical and Laboratory Standards Institute,³⁴ with adaptations. For this assay, the bacterial and fungal inocula were adjusted to approximately 1×10^8 CFU mL⁻¹ (McFarland 0.5 standard). Starch bilayer films with zein electrospun fibers were cut into 4 mm diameter discs and placed equidistantly on the surface of the culture medium in plates. In each plate, one disc prepared without TEO encapsulated in the zein electrospun fibers served as the negative control, and the other two discs contained the same TEO concentration (20%, 40%, or 60%). Each concentration was evaluated using independent plates. The plates were sealed with Parafilm to prevent the dispersion of volatiles and incubated at 37 °C for bacteria and 25 °C for fungi in a germination chamber (SolidSteel). After incubation periods of 24 h for bacteria or 8 days for fungi, the plates were examined for inhibition zones, which were measured in millimeters using a digital caliper (model LWJ-308, Luatex, Sao Paulo, Brazil). All treatments were performed in triplicate.

Microatmosphere test. The microatmosphere analysis was conducted against the bacteria *S. aureus* and *E. coli* and the fungi *P. crustosum* and *A. flavus*, following the method proposed by Ghabraie et al.,³⁵ with some modifications. For this assay, the bacterial and fungal inocula were adjusted to approximately 1×10^5 CFU mL⁻¹. Starch bilayer films with zein electrospun fibers loaded with TEO at concentrations of 20%, 40%, and 60% were cut (85 mm in diameter) and evaluated based on the action of volatiles. The films were fixed to the inner side of the plate lids. Films without TEO encapsulated in the zein electrospun fibers were used as controls. Subsequently, the plates were sealed with Parafilm and incubated in an inverted position (lid facing downward) at 37 °C for 24 h for bacteria and at 25 °C for 3 days for fungi in a germination chamber (SolidSteel). After the incubation period, CFUs were counted. All treatments were performed in triplicate. The results were expressed as percentage reductions in microbial growth and calculated according to Eqn (1).

Viable cell count assay. Based on the results obtained from the agar diffusion and microatmosphere analyses, starch bilayer films with zein electrospun fibers loaded with 60% TEO were selected for viable cell count analysis and application as bioactive food packaging.

The protocol for the viable cell count assay was conducted against the bacteria *S. aureus*, *L. monocytogenes*, *B. cereus*, MRSA, *E. coli*, and *P. aeruginosa*, following the method described by Campos et al.,³⁶ with modifications. The same methodology was adapted for the fungi *P. crustosum*, *A. flavus*, and *F. oxysporum*, with adjustments in the culture media and incubation times. The bilayer films, with and without TEO (control), were cut into 1 cm diameter discs, sterilized by ultraviolet (UV) light exposure for 15 min on each side, and placed in sterile test tubes.

For the bacterial assays, 100 µL of the inoculum at a concentration of 10^5 CFU mL⁻¹ was added onto each disc. The discs were incubated with the bacterial inoculum in BHI broth at 37 °C for different time intervals (0, 1, 2, 5, and 8 h). After each incubation time, 900 µL of peptone water was added to each tube, and the resulting suspension was homogenized and subjected to serial dilutions at appropriate dilution factors. Aliquots of 50 µL from each dilution were plated onto Mueller–Hinton agar and incubated at 37 °C for 24 h.

For the fungal assays, 150 µL of the inoculum at a concentration of 10^6 CFU mL⁻¹ was applied to each disc. The discs were incubated with the fungal inoculum in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 20 g L⁻¹ glucose and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (0.165 mol L⁻¹) at 25 °C for different time intervals (0, 12, 24, 48, and 72 h). After each incubation time, 1.35 mL of peptone water was added to each tube, and the resulting suspension was homogenized and serially diluted at appropriate dilution factors. Aliquots of 100 µL from each dilution were plated onto PDA and incubated at 25 °C for 3 days. The resulting colonies were then counted and the results were expressed as log CFU. Two replicates of each film type were used for each time point.

In situ activity of the film

Bilayer films containing 60% TEO were selected for *in situ* tests using cherry tomatoes as model fruits, aiming to evaluate the effect of the films as packaging on fruit preservation, acting through the volatilization of bioactive compounds. The experimental conditions were defined based on previous results obtained from agar diffusion and microatmosphere assays.

Simulation of bioactive food packaging. Cherry tomatoes at the same ripening stage (stage 6 maturity), of uniform size, consistent red coloration, and free from mechanical damage or lesions, were selected, washed with sterile distilled water, and placed in sterile containers, with three tomatoes per container. Three treatments were prepared for each sampling time: control (open container), polyvinyl chloride (PVC) film, commonly used in commercial packaging for fruit storage, and bilayer film (sweet potato starch films with zein fibers loaded with TEO) (Fig. 1). A total of 24 tomatoes (eight containers with three tomatoes each) were used for each formulation.

The experiment was based on the method described by Xiang *et al.*,³⁷ with some modifications. The films were arranged to wrap the samples completely, promoting effective contact between the volatilized bioactive compounds and the surface of the tomatoes. The bilayer film or PVC covered the opening of the container, while Parafilm was applied only around the edges to secure the films in place and minimize leakage between the film and the recipient. The packaged samples were stored in a biochemical oxygen demand (BOD) incubator (model EL131/4; Eletrolab, Sao Paulo, Brazil) under controlled conditions at $24 \pm 1^\circ\text{C}$ and approximately 65% relative humidity for 14 days.

Storage study of tomatoes. Tomato quality parameters, including weight loss rate, decay rate, firmness (N), total soluble solids ($^\circ\text{Brix}$), titratable acidity, and pH, were evaluated on days 0, 3, 7, 10, and 14 of storage. Baseline analyses (day 0) were performed using untreated tomatoes before packaging. On day 14, microbiological analyses were performed for the detection of *Salmonella* spp. and *E. coli*, following the standards established by Normative Instruction No. 161/2022 of the Agência Nacional de Vigilância Sanitária (ANVISA).³⁸ Counts of aerobic mesophilic bacteria, molds, and yeasts were undertaken. As microbiological analyses required destructive sampling of 25 g of tomato samples; therefore, they were performed only at the end of the storage period (day 14). All analyses were conducted at least in triplicate using a completely randomized design with two factors: treatment and storage time.

Weight loss ratio. Tomato weight loss was determined using a digital scale (model BL-1200AS, Bioscale, Sao Paulo, Brazil), according to the method described by Lian *et al.*³⁹ The initial weight (w_0) was recorded on day 0, and subsequent weights (w_x) were measured on the designated storage days. Weight loss was calculated using Eqn (2).

$$\text{Weight loss (\%)} = \left(\frac{W_0 - W_x}{W_0} \right) \times 100 \quad (2)$$

Overall deterioration rate. At each storage interval, the number of fruits displaying deterioration was recorded, including those showing visible signs of decay and those exhibiting severe shriveling. The total deterioration rate was calculated as described by Lian *et al.*,³⁹ using Eqn (3), where T_x represents the total number of deteriorated fruits and T_0 the initial number of fruits.

$$\text{decay rate (\%)} = \left(\frac{T_x}{T_0} \right) \times 100 \quad (3)$$

Firmness. Tomato firmness was determined using a penetrometer (HOMIS, model 3020, Brazil). The tomatoes were punctured with a 3 mm diameter probe, and the results were expressed as peak force in N, as described by Li *et al.*⁴⁰ Approximately 12 measurements were performed for each treatment at each storage interval.

Total soluble solids (TSS), titratable acidity, and pH. Tomato samples were centrifuged at $2300 \times g$ for 5 min. Soluble solids content was determined using a digital portable refractometer (Model MA871, Milwaukee Instruments, Rocky Mount, NC, USA), and results were expressed in $^\circ\text{Brix}$. Titratable acidity was assessed by titrating 5 mL of the juice with 0.1 mol L^{-1} sodium hydroxide until reaching pH 8.1. This procedure followed the method described in AOAC 942.15⁴¹ and the results were expressed as the percentage of citric acid. The pH measurements were performed using a

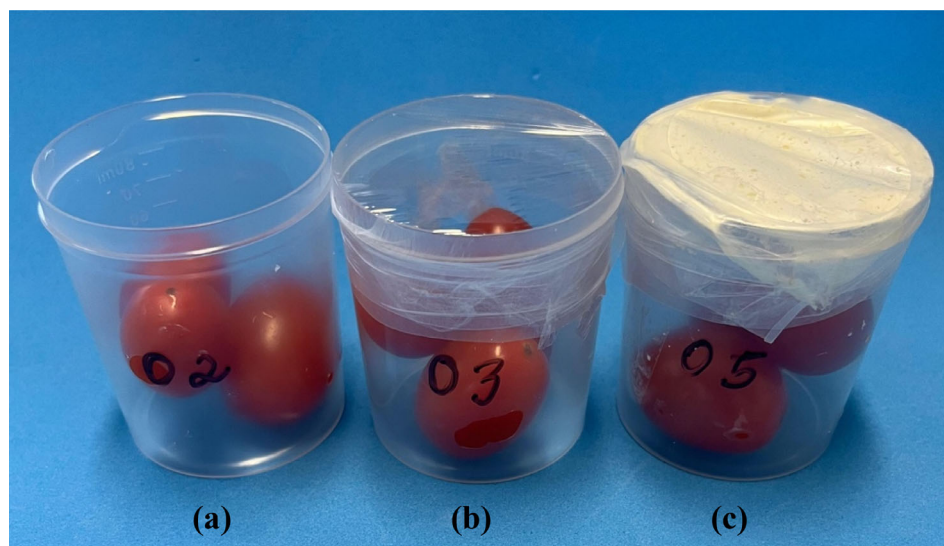


Figure 1. Active food packaging simulation: (a) control, (b) PVC film, and (c) bilayer film with zein/TEO fibers. PVC, polyvinyl chloride; TEO, thyme essential oil.

digital potentiometer (model DM-20; Digimed, Sao Paulo, Brazil). All analyses were performed in triplicate.

Microbiological quality. For the microbiological quality analyses, 25 g of tomatoes was weighed on day 0 and 25 g from each treatment was weighed on day 14 of storage. The samples were homogenized in 225 mL of sterile buffered peptone water (initial dilution) and then subjected to microbiological analyses following the recommendations of the Bacteriological Analytical Manual of the Food and Drug Administration (FDA)⁴² and the American Public Health Association (APHA).⁴³

For the detection of *Salmonella* spp., a pre-enrichment step was performed in sterile buffered peptone water, followed by incubation at 37 °C for 24 h. Subsequently, selective enrichment was carried out in tetrathionate and Rappaport–Vassiliadis broths, with the former incubated at 35 °C and the latter at 42 °C (in a water bath), both for 24 h. After enrichment, aliquots from the broths were streaked onto selective media: Hektoen enteric (HE) agar (IondLab, IO203) and XLD Agar (Kasvi, K24-1056), followed by incubation at 37 °C for 24 h. Colonies showing typical morphology on HE and XLD media were subjected to biochemical tests on triple sugar iron (TSI) agar (Sigma-Aldrich), lysine iron agar (LIA) (Kasvi), and the urease test, as well as serological confirmation using polyvalent anti-*Salmonella* serum (Sigma-Aldrich).

For the quantification of *E. coli*, decimal dilutions (10^{-2} and 10^{-3}) were prepared from the diluted sample, and the most probable number (MPN) technique was used, with three tubes per dilution. One milliliter of each dilution was inoculated into tubes containing lauryl sulfate tryptose (LST) broth (Sigma-Aldrich) with fermentation tubes (Durham tubes) and incubated at 35 °C for 48 h. From the tubes showing turbidity and gas production, a loopful was transferred to tubes containing *E. coli* (EC) broth with Durham tubes, followed by incubation at 45 °C for 48 h. Positive EC broth tubes were then streaked onto EMB agar plates using the exhaustion method, in which the inoculum was progressively streaked across the agar surface to obtain isolated colonies characteristic colonies, with subsequent incubation at 37 °C for 24 h. The MPN was determined based on the number of positive tubes at each dilution and expressed as MPN g⁻¹.

For the enumeration of aerobic mesophilic bacteria, 0.1 mL aliquots from serial decimal dilutions were inoculated onto Petri dishes containing plate count agar (PCA) (Kasvi, K25-1056) using the surface spread technique. The plates were incubated at 35 °C for 48 h. After the incubation period, characteristic colonies were counted, and the results were expressed as CFU g⁻¹.

For the enumeration of molds and yeasts, 0.1 mL aliquots from serial decimal dilutions were inoculated onto PDA plates using the surface spread technique. The plates were incubated at 25 °C for 72 h. After the incubation period, characteristic colonies were counted and the results were expressed as CFU g⁻¹.

Statistical analysis

The storage experiment was conducted using a completely randomized design with two factors (treatment and storage time). All analytical determinations were conducted in at least triplicate, and the results are expressed as means $\pm$ standard deviations. Data were subjected to analysis of variance (ANOVA), followed by Tukey's multiple comparison test using Statistica version 13 (StatSoft Inc., Tulsa, OK, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

In vitro antimicrobial activity of thyme essential oil

The antimicrobial activity of TEO was evaluated using agar diffusion and microatmosphere assays. These tests simulate the effectiveness of the antimicrobial agent under controlled conditions, allowing for an initial screening of its inhibitory potential against target microorganisms. The selection of bacterial species was based on their representation of gram-positive (*S. aureus* and *L. monocytogenes*) and gram-negative (*E. coli* and *S. Typhimurium*) bacteria, which are commonly associated with food contamination. These microorganisms are recognized as priority pathogens in ready-to-eat products and are widely used as reference strains in antimicrobial efficacy studies. Their inclusion also enables the evaluation of TEO's performance against different cell wall structures, contributing to a more comprehensive comparative analysis. The fungal strains (*P. crustosum*, *A. flavus*, and *F. oxysporum*) were selected due to their relevance in food spoilage and contamination contexts, as they are frequently isolated from stored and marketed fruits and are known for their resistance to conventional antimicrobial agents.³

In the agar diffusion assay, the presence and diameter of inhibition zones are directly related to the sensitivity of the microorganisms to the tested EO, although the low diffusion of hydrophobic compounds in solid media may underestimate the actual antimicrobial potential of TEO. Inhibition zones larger than 12 mm indicate satisfactory inhibitory activity, whereas those smaller than 7 mm are considered to indicate low activity.⁴⁴ In the agar diffusion test, TEO exhibited greater antibacterial activity against *L. monocytogenes* at all tested concentrations (Table 1). Inhibition halos measuring 46.2 ± 2.3 mm were observed at the 2 μ L treatment, with a significant increase in diameter as the TEO concentration increased, reaching complete inhibition across the plate at 10 μ L. A similar trend was observed against *S. aureus*, although with lower antimicrobial intensity, with inhibition zones ranging from 40.7 ± 2 mm at 2 μ L to complete inhibition at 10 μ L of TEO.

Thyme essential oil exhibited lower antimicrobial activity against *E. coli* and *S. Typhimurium*, with inhibition zones ranging from 31.6 ± 1.5 mm and 30.5 ± 1.1 mm, respectively, with the 2 μ L treatment, to 58.9 ± 1.9 mm and 67.9 ± 1.5 mm with the 10 μ L treatment. Inhibition zones ≥ 28 mm, as observed in this study, indicate strong inhibitory activity of TEO against the tested bacteria.⁴⁵ However, in cases where complete or nearly complete inhibition was observed, the antimicrobial effect may have resulted not only from diffusion through the agar but also from the vapor-phase activity of volatile TEO compounds, particularly thymol and carvacrol, since the Petri dishes were sealed with Parafilm during incubation. The inhibition zones should therefore be interpreted as a qualitative indication of the antimicrobial activity of free TEO rather than as a direct quantitative measure of compound diffusion through the agar. Nevertheless, the difference in efficacy between gram-positive and gram-negative bacteria, especially at TEO concentrations of 2 and 10 μ L, may be attributed to differences in cell wall structure. Gram-positive bacteria have a thick peptidoglycan layer associated with hydrophobic components such as proteins and teichoic acids, which may facilitate the penetration of hydrophobic compounds like those found in essential oils. In contrast, gram-negative bacteria, such as *E. coli* and *S. Typhimurium*, possess a more complex cell envelope consisting of an outer membrane rich in proteins and lipopolysaccharides, which acts as an additional barrier, hindering the entry of hydrophobic substances.⁴⁶

Table 1. *In vitro* evaluation of the antibacterial activity of thyme essential oil (TEO) by agar diffusion and microatmosphere techniques.

TEO (μ L)	<i>Staphylococcus aureus</i>	<i>Listeria monocytogenes</i>	<i>Escherichia coli</i>	<i>Salmonella Typhimurium</i>
Inhibition zones (mm)				
2	40.7 $\pm$ 2.0 ^{c,B}	46.2 $\pm$ 2.3 ^{c,A}	31.6 $\pm$ 1.5 ^{c,C}	30.5 $\pm$ 1.1 ^{c,C}
5	49.1 $\pm$ 4.4 ^{b,AB}	56.9 $\pm$ 3.9 ^{b,A}	36.8 $\pm$ 2.4 ^{b,C}	41.0 $\pm$ 5.1 ^{b,BC}
10	Ti ^{a,A}	Ti ^{a,A}	58.9 $\pm$ 1.9 ^{a,C}	67.9 $\pm$ 1.5 ^{a,B}
Inhibition percentage (%)				
2	35.6 $\pm$ 6.4 ^{b,A}	12.1 $\pm$ 4.5 ^{c,B}	3.2 $\pm$ 2.7 ^{d,C}	8.3 $\pm$ 2.4 ^{d,BC}
5	100.0 $\pm$ 0.0 ^{a,A}	18.2 $\pm$ 5.9 ^{c,C}	16.3 $\pm$ 4.4 ^{c,C}	29.6 $\pm$ 5.9 ^{c,B}
10	100.0 $\pm$ 0.0 ^{a,A}	36.2 $\pm$ 2.5 ^{b,C}	42.8 $\pm$ 4.8 ^{b,C}	55.5 $\pm$ 6.0 ^{b,B}
15	100.0 $\pm$ 0.0 ^{a,A}	88.8 $\pm$ 9.8 ^{a,B}	76.5 $\pm$ 3.6 ^{a,B}	100.0 $\pm$ 0.0 ^{a,A}

Note: The results are expressed as means of three replicates ($n = 3$) $\pm$ standard deviation. Values with different lower case letters in the same column differ significantly ($P < 0.05$). Values with different upper case letters in the same row differ significantly ($P < 0.05$). TEO, thyme essential oil; Ti, total inhibition (no bacterial growth observed across the plate surface).

A previous study reported that the use of 40 μ L of TEO in the agar diffusion test against *B. cereus*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. Typhimurium* resulted in the highest inhibitory activity among the EOs tested (parsley, lovage, basil, and thyme), with inhibition zones ranging from 12.1 to 36.4 mm.⁴⁷ The antibacterial activity of pink pepper EO was evaluated against gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and gram-negative bacteria (*S. Typhimurium* and *E. coli*). In the agar diffusion test, pink pepper EO showed antimicrobial activity only against the two gram-positive bacteria tested. The inhibition zones using 10 μ L of pink pepper EO were 24.3 mm and 23.9 mm against *S. aureus* and *L. monocytogenes*, respectively.⁴⁸

In the microatmosphere test, the antimicrobial activity of TEO was evaluated by exposing the microorganisms to its vapors (gaseous contact), allowing assessment of TEO's potential in modified atmosphere systems or sealed packaging. Antibacterial activity significantly increased with the concentration of TEO for all tested bacteria (Table 1). Unlike the results obtained from the agar diffusion assay, TEO showed greater activity against *S. aureus* and *S. Typhimurium*, reaching complete inhibition with only 5 μ L and 15 μ L, respectively. Against *L. monocytogenes* and *E. coli*, although inhibition was lower, the results were still satisfactory, achieving 88.8 $\pm$ 9.8% and 76.5 $\pm$ 3.6%, respectively. In this assay, no correlation was observed between cell wall type and TEO efficacy, possibly due to differences in the antimicrobial mechanisms

involved in the two tests. In the microatmosphere assay, the antimicrobial compounds in EO volatilize, forming a vapor phase that directly contacts bacterial cells without the need to diffuse through the solid medium (agar) in which the bacteria are present. This suggests that the gaseous phase enables direct interaction with the plasma membrane, even in gram-negative bacteria.³⁵

Lages *et al.*⁴⁹ evaluated the action of TEO using the microatmosphere test and observed reductions in the viable cell count of *E. coli* of 62.5% at 10 μ L, 99.1% at 20 μ L, and 100% at both 45 and 90 μ L. For *S. aureus*, reductions of 100% were recorded at concentrations of 20, 45, and 90 μ L, while the lowest concentration tested (10 μ L) showed no significant effect. These results differ from those obtained in the present study, in which TEO exhibited a greater inhibitory effect, possibly due to variations in the oil's composition, which may be influenced by factors such as botanical origin, cultivation conditions, extraction method, and storage. This highlights the importance of standardizing the chemical composition and concentration of essential oils in microbiological assays.⁵⁰

Table 2 shows the strong antifungal activity of TEO. In the agar diffusion test, no significant differences were observed in the inhibition zone sizes among the tested fungi. Likewise, no significant differences were found between treatments with 2 and 5 μ L, with inhibition zones ranging from 29.7 $\pm$ 5.7 mm (2 μ L against

Table 2. *In vitro* evaluation of the antifungal activity of thyme essential oil (TEO) by agar diffusion and microatmosphere techniques.

TEO (μ L)	<i>Penicillium crustosum</i>	<i>Aspergillus flavus</i>	<i>Fusarium oxysporum</i>
Inhibition zones (mm)			
2	30.0 $\pm$ 1.4 ^{b,A}	30.9 $\pm$ 4.4 ^{b,A}	29.7 $\pm$ 5.7 ^{b,A}
5	34.2 $\pm$ 3.0 ^{b,A}	34.7 $\pm$ 2.4 ^{b,A}	34.5 $\pm$ 5.8 ^{b,A}
10	Ti ^{a,A}	Ti ^{a,A}	Ti ^{a,A}
Inhibition percentage (%)			
2	17.6 $\pm$ 6.7 ^{c,A}	17.3 $\pm$ 4.2 ^{c,A}	26.0 $\pm$ 7.8 ^{c,A}
5	35.3 $\pm$ 5.5 ^{b,A}	23.8 $\pm$ 6.7 ^{c,A}	30.0 $\pm$ 7.2 ^{c,A}
10	100.0 $\pm$ 0.0 ^{a,A}	60.1 $\pm$ 7.2 ^{b,B}	63.5 $\pm$ 9.5 ^{b,B}
15	100.0 $\pm$ 0.0 ^{a,A}	90.3 $\pm$ 8.4 ^{a,B}	100.0 $\pm$ 0.0 ^{a,A}

Note: The results are expressed as means of three replicates ($n = 3$) $\pm$ standard deviations. Values with different lower case letters in the same column differ significantly ($P < 0.05$). Values with different upper case letters in the same row differ significantly ($P < 0.05$). TEO, thyme essential oil; Ti, total inhibition (no fungal growth observed across the plate surface).

F. oxysporum) to 34.7 ± 2.4 mm (5 μ L against *A. flavus*). In the 10 μ L TEO treatment, complete inhibition of all three tested fungi was observed, with no measurable halo due to the absence of fungal growth across the entire surface of the plate. This result suggests a high potential for using TEO as a preventive system for application on fresh fruits, considering that the fungi evaluated represent a significant threat to the postharvest distribution chain of horticultural products.

Previous studies have highlighted the effectiveness of TEO against a variety of fungal species.^{51,52} Bounar et al.⁵³ investigated the antifungal activity of TEO against different *Fusarium* species and observed inhibition zone diameters ranging from 20.43 ± 0.79 mm to 45.13 ± 2.13 mm. Among the evaluated species, *F. oxysporum* showed the highest resistance, with the smallest inhibition zone recorded (20.43 ± 0.79 mm). In the microatmosphere test, a significant increase in fungal inhibition was observed as TEO concentration increased (Table 2). The antimicrobial effectiveness of EOs in the vapor phase depends on the volatility of their components and their corresponding antimicrobial activity.⁵⁴ Thus, TEO demonstrated the highest inhibitory effect against *P. crustosum*, ranging from $17.6 \pm 6.7\%$ at 2 μ L to complete inhibition at 10 μ L. At a concentration of 15 μ L, 100% inhibition was achieved against *F. oxysporum* and $90.3 \pm 8.4\%$ against *A. flavus*, both of which showed similar responses across all tested concentrations.

The antifungal activity of 10 μ L of orange peel essential oil against *P. crustosum* and *A. flavus* resulted in lower inhibition than that found in the present study, with inhibition rates of $67.2 \pm 5.9\%$ and $81.2 \pm 5.2\%$, respectively.¹² In another study, vapors generated by TEO were able to control the growth of fungal strains isolated from stored wheat grains (*A. nomius*, *E. hirayamae*, *P. cinnamomipureum*, and *P. viridicatum*).⁵⁴

As with their antibacterial activity, the antifungal activity of EOs is largely attributed to the action of their lipophilic constituents on the fungal cell membrane. These compounds disrupt the lipid bilayer, increase membrane permeability, and cause the leakage of metabolites essential for cell survival. They can also impair cellular homeostasis, inhibit key enzymes involved in fungal metabolism, and induce the formation of reactive oxygen species, ultimately causing structural and functional damage that results in cell death.⁵⁵

In vitro antimicrobial activity of bilayer film

Agar diffusion and microatmosphere assays

The antimicrobial activity of bilayer films containing different concentrations of TEO encapsulated in zein fibers was evaluated using agar diffusion and microatmosphere assays, with the aim of determining the most effective concentration of the EO against the tested microorganisms (Table 3). These methods allow for the assessment of the efficacy of active compounds released from the polymeric structures, simulating their performance under different storage conditions.

In the agar diffusion test, the bilayer films showed no inhibition zones against the tested microorganisms, regardless of the concentration of encapsulated TEO. In this type of assay, the diffusion of compounds from the solid matrix is limited. Thus, this result may be attributed to the direct contact of the films with the culture medium, which likely restricted the diffusion of TEO compounds encapsulated in zein fibers, the primary contributors to its antimicrobial activity. This compromised the antimicrobial efficacy of TEO with this evaluation method. Microatmosphere assays and viable cell counts were therefore conducted to investigate

the antimicrobial action of TEO, as these approaches allow for better simulation of the real conditions under which active packaging interacts with the surrounding food atmosphere.

Table 3 highlights the high efficacy of the bilayer films, attributed to the controlled release of volatile compounds from the encapsulated TEO. Antimicrobial activity progressively increased with increasing TEO concentration, with complete inhibition achieved in some cases and significant effects observed against all tested microorganisms, confirming the importance of antimicrobial agent concentration in determining the effectiveness of the system. As observed with free TEO, inhibition of *S. aureus* (gram-positive bacteria) was significantly higher at a TEO concentration of 60%, reaching 100%. This result is consistent with the findings of Lages et al.,⁴⁹ who also reported complete inhibition of *S. aureus* with high EO concentrations in a microatmosphere. In contrast, inhibition of *E. coli* (gram-negative bacteria) at the same concentration was $71.5 \pm 8\%$. The antifungal action of the bilayer film containing 60% TEO was significantly greater against *P. crustosum* ($100 \pm 0\%$) than against *A. flavus* ($71.3 \pm 3.6\%$), possibly due to a higher affinity of the volatile compounds for the fungal membrane of this genus.

Reyes-Jurado et al.⁵⁴ highlighted that several studies have reported greater efficacy of EOs when applied in vapor form compared with direct-contact applications, whether in aqueous solutions or solid media, indicating that the vapor mode of action enables more effective interaction with exposed cellular structures. This enhanced effect was particularly evident against fungi, which tend to develop and cause spoilage on food surfaces. This outcome is attributed to the action of volatile compounds present in EOs, which exhibit greater diffusion capacity in the gaseous state. Pinto et al.⁵¹ investigated the effect of red TEO on the shelf life and spoilage of oranges caused by *Penicillium* during refrigerated storage. The application of red TEO vapors was effective in reducing the percentage of infected wounds, the area of external mycelial growth, and spore production significantly.

The application of EOs in the gaseous phase also offers distinct advantages, such as the possibility of using lower doses to achieve effective antimicrobial effects and a reduced risk of chemical irritation. In this form, the volatile compounds of EOs have less impact on the sensory characteristics of treated foods than direct liquid application, which is particularly desirable for fresh fruits intended for direct consumption, such as cherry tomatoes.⁵¹ The results of the present study indicate that the use of TEO in vapor form as part of an active packaging system represents a promising strategy for the food industry, aligning with sustainability principles and the reduction of synthetic inputs in the food supply chain. This approach has the potential to extend the shelf life of fresh produce, contributing to the reduction of postharvest losses and the maintenance of microbiological quality.

Viable cell counting

Given the promising results of the microatmosphere tests, the bilayer films containing 60% of TEO encapsulated in zein fibers were selected for more systematic investigation of their antimicrobial properties through viable cell count analysis. These analyses aimed to evaluate the effectiveness of the prolonged release of TEO from the bilayer film in microbial inhibition over time, allowing the monitoring of microorganism survival and growth throughout the storage period.

Thus, the antibacterial effectiveness of the bilayer film containing 60% encapsulated TEO was evaluated over time against both

Table 3. *In vitro* evaluation of the antimicrobial activity of bilayer film by agar diffusion and microatmosphere techniques.

Bilayer films*	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Penicillium crustosum</i>	<i>Aspergillus flavus</i>
Inhibition zones (mm)				
20% TEO	Nd	Nd	Nd	Nd
40% TEO	Nd	Nd	Nd	Nd
60% TEO	Nd	Nd	Nd	Nd
Inhibition percentage (%)				
20% TEO	10.2 ± 4.9 ^{c,A}	14.8 ± 7.5 ^{c,A}	11.0 ± 3.5 ^{c,A}	5.7 ± 4.2 ^{c,A}
40% TEO	41.1 ± 3.8 ^{b,A}	38.3 ± 3.7 ^{b,A}	52.5 ± 6.6 ^{b,A}	31.0 ± 3.0 ^{b,B}
60% TEO	100.0 ± 0.0 ^{a,A}	71.5 ± 8.0 ^{a,B}	100.0 ± 0.0 ^{a,A}	71.3 ± 3.6 ^{a,B}

Note: The results are expressed as means of three replicates ($n = 3$) ± standard deviations. Values with different lower case letters in the same column differ significantly ($P < 0.05$). Values with different upper case letters in the same row differ significantly ($P < 0.05$). Nd, not detected (no inhibitory effect on the microorganism).

*Bilayer films with 20%, 40%, or 60% (v/w) of thyme essential oil (TEO).

gram-positive and gram-negative bacteria (Fig. 2), using the bilayer film without TEO as a control. Overall, the bilayer film containing 60% TEO showed a progressive reduction in the viable counts of all tested bacteria, with complete inhibition observed at different times depending on the microorganism. *Pseudomonas aeruginosa* exhibited the shortest inhibition time, with complete inhibition after only 2 h of contact with the film, which may be attributed to the high permeability of its outer membrane to TEO. *Bacillus cereus*, MRSA, and *E. coli* were inhibited after 5 h of storage, indicating that contact time is a critical factor for the system's effectiveness. In this analysis, *S. aureus* and *L. monocytogenes* required up to 8 h to reach complete inhibition under these experimental conditions.

As Fig. 3 shows, the bilayer film containing TEO demonstrated the greatest inhibitory effect against *P. crustosum*, achieving complete inhibition within 72 h – an important result considering the relevance of this fungus in the spoilage of stored fruits and vegetables. For the other fungi, complete inhibition was not observed during the evaluated period. However, viable cell counts decreased over time compared with the control film, particularly for *F. oxysporum* and, to a lesser extent, *A. flavus*. These results indicate a progressive inhibitory effect of the bilayer film, consistent with the expected behavior of a controlled-release system. Although complete inhibition was not achieved for all fungal species, the reduction in viable counts suggests potential for reducing fungal growth and contributing to food preservation.

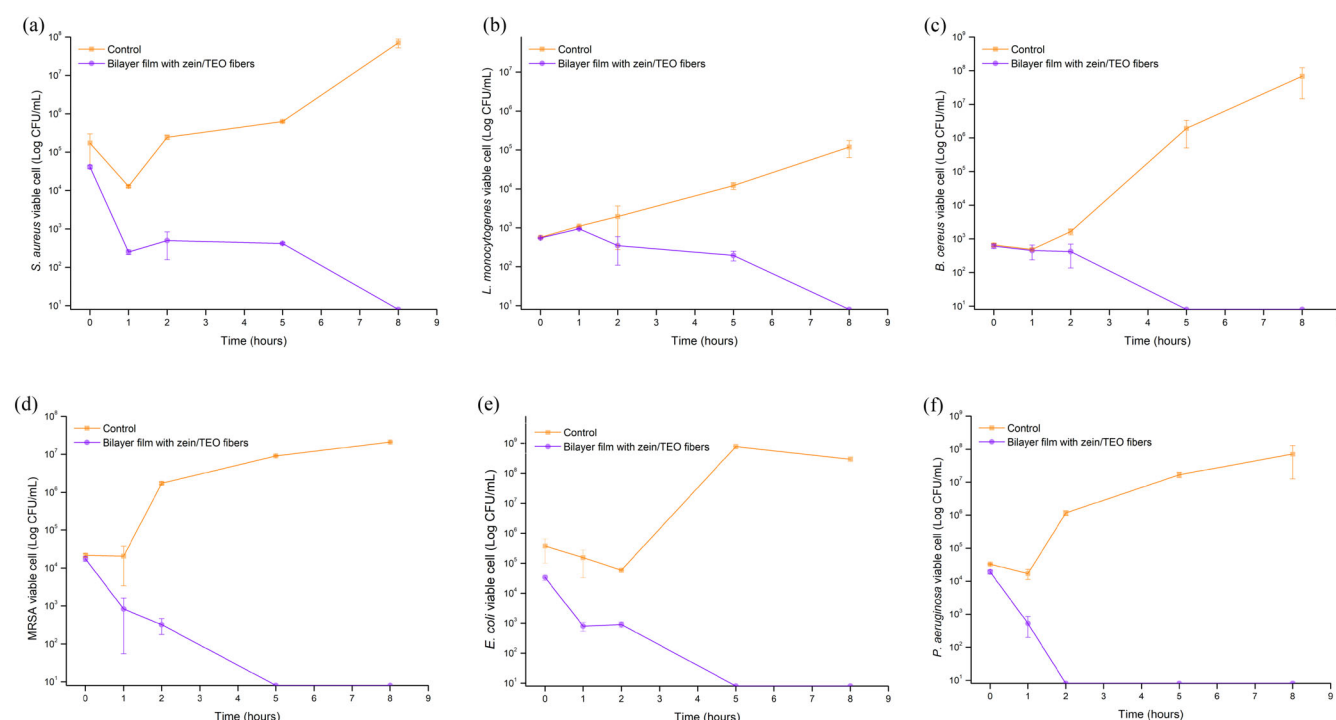


Figure 2. Growth inhibition curves of selected gram-positive bacteria – (a) *Staphylococcus aureus*, (b) *Listeria monocytogenes*, (c) *Bacillus cereus*, and (d) methicillin-resistant *Staphylococcus aureus*. Inhibition curves of selected gram-negative bacteria: (e) *Escherichia coli* and (f) *Pseudomonas aeruginosa* – in contact with bilayer films with zein/TEO fibers and control films (without TEO). The apparent asymmetry of the range bars results from the logarithmic scale used on the y-axis, which affects their visual appearance but does not alter the actual range values. TEO, thyme essential oil.

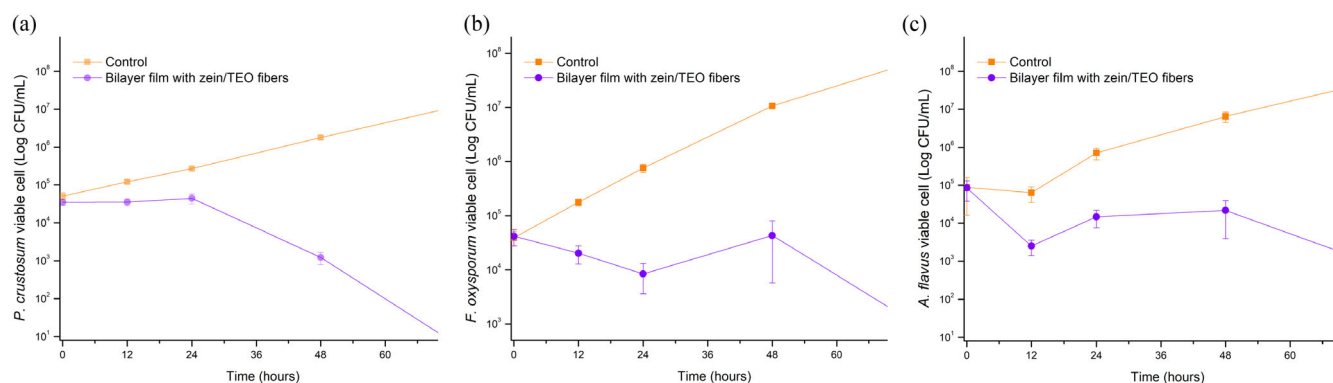


Figure 3. Growth inhibition curves of selected fungi – (a) *Penicillium crustosum*, (b) *Fusarium oxysporum*, and (c) *Aspergillus flavus* – in contact with bilayer films with zein/TEO fibers and control films (without TEO). The apparent asymmetry of the range bars results from the logarithmic scale used on the y-axis, which affects their visual appearance but does not alter the actual range values. TEO, thyme essential oil.

Encapsulation is a technology that involves trapping an active compound (core) within a matrix or wall material, forming structures known as encapsulates. When applied to active packaging systems with EOs, this process helps mitigate limitations such as strong flavor and odor, low stability, and high sensitivity to oxidation, in addition to enabling controlled release.¹⁴ In the viable cell count test, it is possible to monitor how the gradual release of TEO acts on different microorganisms over time and to assess the dynamic and prolonged effectiveness of the antimicrobial agents. This type of analysis allows for the observation not only of the inhibition capacity at a specific time point but also of the response, adaptation, or resistance of the microorganisms to the treatment during the storage period. This provides data for the development of more effective and safer active packaging systems with the potential to extend shelf life and improve the microbiological safety of food products.

Simulation of bilayer films as bioactive food packaging for cherry tomato conservation

Cherry tomatoes are climacteric fruits with a relatively short post-harvest shelf life, making the use of appropriate packaging a key strategy to extend their preservation.⁵⁶ Bioactive packaging incorporating antimicrobial and antioxidant compounds represents an innovative approach to prolong shelf life and maintain the sensory quality of fresh fruits. In this context, cherry tomatoes were used to evaluate the application potential of bilayer films containing encapsulated TEO as bioactive packaging. Visual changes in the fruits over 14 days of storage were used as an indicator of deterioration and are shown in Fig. 4. The effects of different types of packaging were evaluated: control (open container), PVC film, and bilayer film with zein fibers containing 60% encapsulated TEO.

Weight loss rate, decay rate, and firmness

The weight loss rate of tomatoes stored in an open container (control) showed a continuous increase over the 14-day storage period, reaching significantly higher losses, with a final value of 14.7%, compared with the other treatments, whose maximum loss was approximately 7% (Fig. 5a). Weight loss is a direct indicator of dehydration and physiological deterioration of the fruit, reflecting changes in the integrity and quality of the plant tissue. When comparing fruits packaged with PVC film and those with the bilayer film containing TEO, a slight difference was observed

on days 3 and 10, with greater weight loss in tomatoes packaged with the bilayer film. However, by the end of the storage period, no significant difference was found between these two packaging types, indicating comparable performance between the bilayer film and PVC in terms of moisture retention.

Torres-Vargas et al.⁵⁷ reported a maximum weight loss of 5.80% in fruits packaged with corn starch-based films reinforced with 8% cellulose nanocrystals extracted from corn husks; the control group showed a significantly higher loss of 14.80%. Another study⁵⁸ reported that cherry tomatoes packaged with control films exhibited a weight loss of 15.98%, whereas those packaged with active films showed only a 10.5% loss during the first 10 days of storage.

The incorporation of zein fibers with encapsulated TEO plays a fundamental role in the permeability of bilayer films due to their hydrophobic nature, contributing to reduced water transfer through the film.²⁹ Other factors related to the bilayer structure, such as increased thickness, lower solubility, and reduced moisture content, can further limit its permeability in comparison with other types of films. These aspects highlight the advantages of bilayer films in improving barrier properties, reinforcing their potential for applications where moisture sensitivity is a critical concern, such as in food packaging.

In line with the weight-loss data, the decay rate was significantly higher in the control group, exceeding 80% by the tenth day of storage, likely due to the lack of a barrier against the action of spoilage fungi such as *Penicillium* spp. However, when comparing the effectiveness of the PVC film with that of the bilayer film containing encapsulated TEO, it was observed that at all evaluated time points, tomatoes packaged with the bilayer film showed lower decay rates (Fig. 5b). Even after the electrospinning process, the TEO-loaded fibers exhibited significant antioxidant activity due to the high concentration of encapsulated TEO (60%) and the stability of the encapsulation method, which preserves the integrity of the bioactive compounds.²⁹ This stability is essential to ensure the controlled release of volatile compounds with antioxidant action, which can slow oxidative processes in packaged foods. From this perspective, a suitable internal atmosphere within the packaging may reduce the respiratory rate of the fruits, contributing to the delay in their deterioration process.⁵⁹ Overall, although the fruits packaged with the bilayer film tended to maintain higher firmness throughout the storage period, no statistically significant differences were observed among the treatments (Fig. 5c).

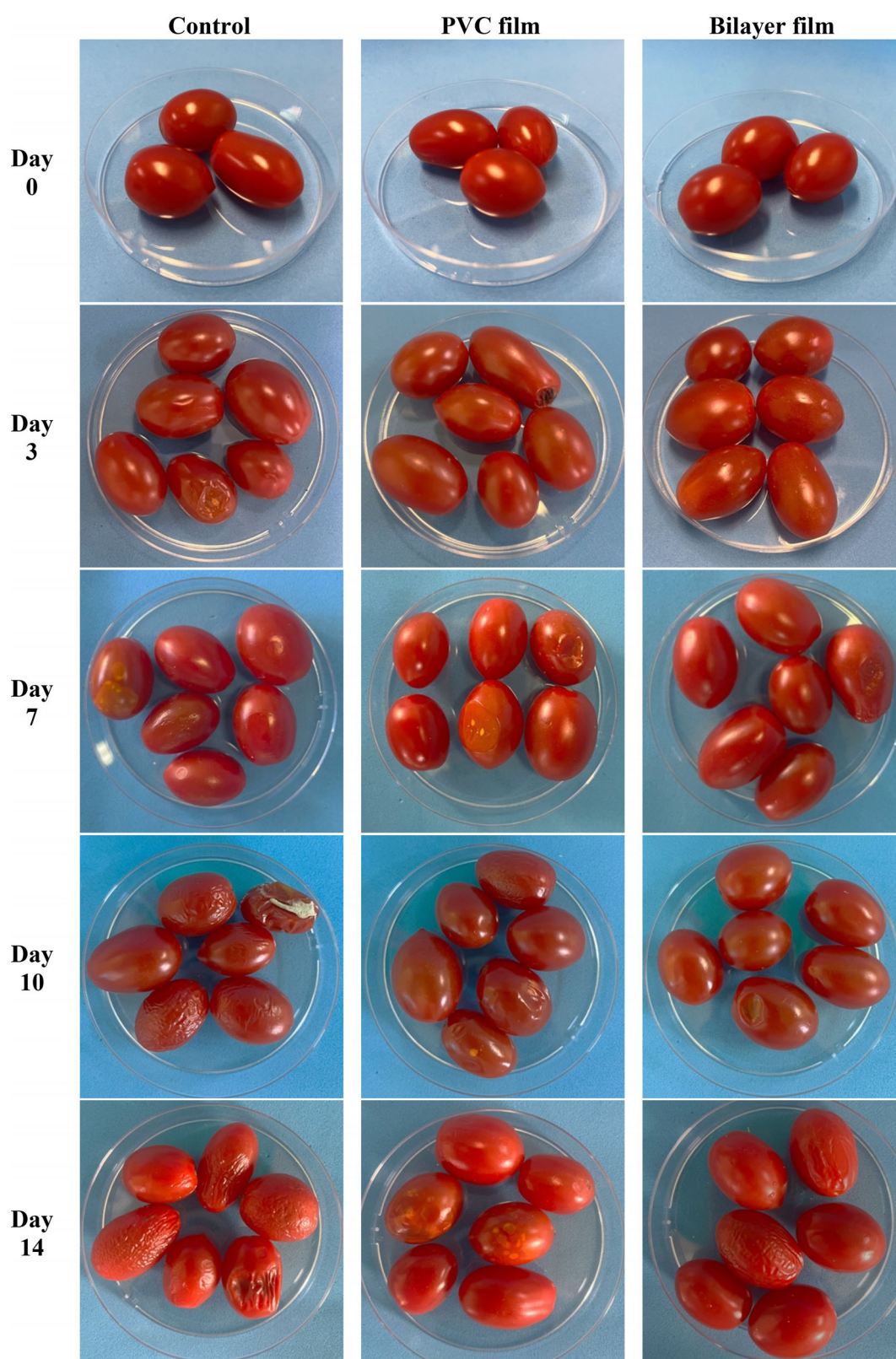


Figure 4. Physical changes in cherry tomatoes under different storage conditions: control, PVC film, and bilayer film with zein/TEO fibers, evaluated on days 3, 7, 10, and 14 at 24 ± 1 °C and $65 \pm 1\%$ relative humidity. PVC, polyvinyl chloride; TEO, thyme essential oil.

Polyvinyl chloride film is known for its superior moisture retention capacity, forming a more efficient barrier against water loss in fruits. In contrast, bilayer films tend to be more hydrophilic

and less effective at retaining moisture, which may lead to slightly greater evaporation during the initial days. Although zein is a hydrophobic protein, the bilayer film structure can exhibit

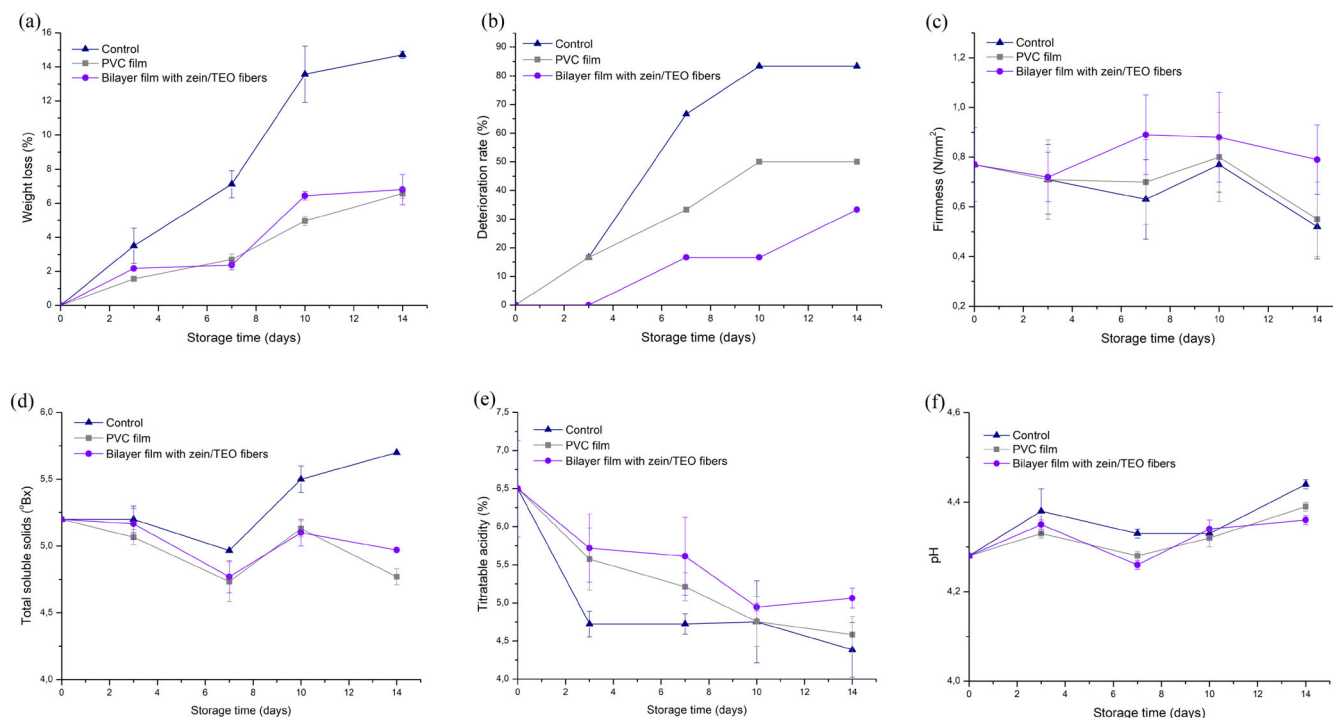


Figure 5. (a) Weight loss, (b) decay rate, (c) firmness, (d) total soluble solids, (e) titratable acidity, and (f) pH of cherry tomatoes during storage.

porosity that facilitates water vapor permeation. However, over time, the antimicrobial effect of the encapsulated TEO present in the bilayer film may have reduced the fruit's metabolic activity, such as respiration and transpiration, in addition to limiting the action of spoilage microorganisms through the sustained release of volatile compounds. This could have offset the lower moisture barrier, resulting in weight loss similar to that of PVC and a lower decay rate by the end of the storage period. These findings highlight the potential of the bilayer film as a bioactive packaging material, not only by serving as a physical barrier but also by enabling the controlled release of antimicrobial and antioxidant compounds, which helps delay the proliferation of spoilage microorganisms and represents a promising alternative for packaging fresh or minimally processed fruits.

Total soluble solids, titratable acidity, and pH

The total soluble solids (TSS) content showed a significantly higher increase in the tomatoes from the control group during storage than the other treatments, especially after day 10, reaching 5.7 ± 0 °Brix at the end of the period (Fig. 5d). In contrast, fruits packaged with PVC film and the bilayer film containing TEO exhibited similar values throughout most of the storage period, differing only on the 14th day, when they reached 4.8 ± 0.1 and 5.0 ± 0 °Brix, respectively. This result indicates that the bilayer film with TEO was effective in delaying the ripening of cherry tomatoes during storage, possibly due to a reduction in the respiration rate and metabolic activity of the fruit. This effect helped slow physiological processes such as the hydrolysis of polysaccharides and the consequent accumulation of soluble sugars. The difference observed between the PVC and bilayer film on day 14, although small, may reflect the additional effect of encapsulated TEO in modulating the internal atmosphere of the package or its antimicrobial action, contributing to more effective

preservation with less variation in flavor, texture, and appearance attributes.

These results are consistent with previous findings reporting a less pronounced increase in TSS in oranges and tomatoes when packaged with active films compared with uncoated samples.^{60,61}

The smaller variation in TSS is an indication of a better preservative effect of the packaging,⁶² as this parameter tends to increase during fruit ripening and senescence, mainly due to the hydrolysis of structural polysaccharides into simple monosaccharides.⁶³

The titratable acidity of tomatoes subjected to different treatments showed a gradual reduction throughout the storage period (Fig. 5e), which may directly affect the characteristic acidic flavor of the fruit. On days 3 and 7, the control group fruits, without any coating, exhibited a significantly more pronounced decrease, with citric acid levels reaching $0.30 \pm 0.01\%$, whereas tomatoes coated with PVC film showed $0.36 \pm 0.03\%$ and $0.32 \pm 0.01\%$, and those with the bilayer film recorded $0.37 \pm 0.04\%$ and $0.36 \pm 0.03\%$, respectively. By day 10, the differences among treatments became less pronounced, with titratable acidity values approaching 0.30% citric acid. These results suggest that, during the initial days of storage, both the PVC and bilayer films were effective in delaying the loss of acidity. By the end of the experimental period (14 days of storage), tomatoes coated with the bilayer film maintained higher acidity levels ($0.33 \pm 0.01\%$ citric acid) than the control fruits ($0.28 \pm 0.01\%$) and those coated with PVC ($0.29 \pm 0.03\%$), indicating a greater ability of the bilayer film to preserve organic acids during storage. This preservation may contribute to maintaining the characteristic acidic taste of tomatoes.

The reduction in titratable acidity during storage is an expected behavior in tomatoes, as organic acids, primarily citric acid, are metabolized as an energy source during respiratory activity throughout the ripening process.⁶⁴ The greater decrease in acidity observed in the control group during the initial days indicates a

faster progression of respiratory metabolism and, consequently, fruit ripening. This result is consistent with previous studies reporting that the absence of physical barriers leads to greater postharvest quality loss.⁶⁵ In contrast, fruits coated with PVC film, and especially those with the bilayer film, showed a smaller reduction in acidity, highlighting the effect of packaging on gas exchange modulation and the reduction of the respiration rate of the fruits. The TEO present in the bilayer films, which contains carvacrol and thymol – compounds with recognized antimicrobial activity – may have inhibited the metabolic activity of microorganisms present on the tomatoes, contributing to the maintenance of acidity.⁶⁶

Figure 5f shows an increasing trend in pH values across all samples during storage, corroborating the data for titratable acidity. This behavior may be related to the fruit's respiratory process, in which organic acids are used as substrates, resulting in decreased acidity and, consequently, an increase in pH, because the consumption of these acids leads to progressive alkalization of plant tissue.³⁷ On the final day of storage, the control group presented the highest pH value (4.4 ± 0.0), likely due to the absence of a physical barrier that facilitated greater gas exchange. Tomatoes packaged with PVC film, although somewhat protected, showed a pH of 4.39 ± 0.0 , possibly due to increased fungal growth. Finally, fruits packaged with the bilayer film showed the smallest variation, with a pH of 4.36 ± 0.0 , indicating greater effectiveness in preserving the chemical characteristics of the tomatoes during storage.

Microbiological quality

The microbiological quality of the tomatoes was analyzed to compare the different types of packaging (Table 4). On day 0, before the storage period and the application of the packaging, the tomato samples showed no presence of *Salmonella* spp., had 23 MPN g⁻¹ of *E. coli*, 1.35×10^5 CFU g⁻¹ of aerobic mesophilic bacteria, and 4×10^4 CFU g⁻¹ of molds and yeasts. At the end of the storage period (day 14), differences in microbial counts were observed among the treatments. As expected, the absence of *Salmonella* spp. was maintained in all samples.

Regarding the other tested microorganisms, the unpackaged fruits (control) showed a general increase, with the highest counts for all groups. When comparing the PVC film with the

bilayer film containing encapsulated TEO, the fruits packaged with the bilayer film exhibited lower microbial counts. On day 14, the *E. coli* count was 23 MPN g⁻¹ for PVC and 3.6 MPN g⁻¹ for the bilayer film; aerobic mesophilic bacteria reached 2.86×10^6 CFU g⁻¹ in the PVC group and 1.17×10^5 CFU g⁻¹ in the bilayer group; molds and yeasts reached 2.39×10^6 CFU g⁻¹ in fruits with PVC and 9.5×10^4 CFU g⁻¹ in those packaged with the bilayer film.

According to the standards established by ANVISA Normative Instruction No. 161/2022,³⁸ the microbiological limits for fresh tomatoes require that *Salmonella* spp. are not detected in a 25 g sample and specify a maximum concentration of 100 CFU g⁻¹ for *E. coli*. All samples met the legal requirement for *Salmonella* spp. at both sampling times. For *E. coli*, the results obtained in this study, expressed as MPN g⁻¹, indicated that all treatments remained below the limit of 100 CFU g⁻¹ throughout storage, including the control. The observed values suggest that this count could reach unsafe levels over a longer storage period, especially in the absence of a physical barrier or an active antimicrobial agent. However, fruits packaged with the bilayer film showed lower *E. coli* counts at the end of storage, indicating that this technology was effective in delaying the growth of this bacterium.

Although Brazilian legislation does not establish a specific legal limit for the count of mesophilic aerobic bacteria, molds, and yeasts in fresh tomatoes, these parameters are widely used as indicators of hygienic-sanitary quality in international regulations. High values may indicate inadequate hygiene conditions during postharvest handling, storage, or transportation, as these microbial groups reflect both the conservation status of the product and the onset of spoilage processes, which can compromise the safety, appearance, and sensory acceptance of the product.⁶⁷

Mesophilic aerobic bacteria represent a broad group that includes both spoilage microorganisms and potential opportunistic pathogens, such as *Pseudomonas* spp., *Bacillus* spp., and *Enterobacter* spp. High counts of this group can also accelerate the sensory degradation of fruit, affecting characteristics such as odor, flavor, and texture.⁶⁸ Molds and yeasts are particularly important in foods with high water activity and near-neutral pH, such as tomatoes. Examples include molds like *Penicillium*, *Aspergillus*,

Table 4. Microbiological quality parameters of cherry tomatoes packaged with different materials over 14 days of storage.

	Treatment	Day 0	Day 14
<i>Salmonella</i> spp. (presence 25 g ⁻¹)	Control	Absent	Absent
	PVC film		Absent
	Bilayer film		Absent
<i>Escherichia coli</i> (MPN g ⁻¹)	Control	23.00	43.00
	PVC film		23.00
	Bilayer film		3.60
Aerobic mesophilic bacteria (CFU g ⁻¹)	Control	1.35×10^5	7.03×10^6
	PVC film		2.86×10^6
	Bilayer film		1.17×10^5
Yeast and mold (CFU g ⁻¹)	Control	4.00×10^4	3.29×10^6
	PVC film		2.39×10^6
	Bilayer film		9.50×10^4

Note: The results are expressed as means of three replicates ($n = 3$).

Abbreviations: CFU, colony forming units; MPN, most probable number; PVC, polyvinyl chloride.

and *Fusarium*, and yeasts such as *Candida* and *Saccharomyces*. These microorganisms are responsible for visible changes, such as the development of fungal colonies on the surface and undesirable fermentation. Some fungal species are also associated with the production of mycotoxins, such as ochratoxin A, aflatoxins, and fumonisins, produced by *Aspergillus* and *Fusarium* species.^{67,69} As tomatoes are often consumed raw, controlling the load of these microorganisms is essential to ensure that the product reaches the consumer with acceptable microbiological quality within the projected shelf life, while preventing health risks.⁶⁷

Kapetanakou et al.⁷⁰ demonstrated that the application of volatile compounds with antimicrobial activity, such as carvacrol, incorporated through sachets in active packaging, was effective in reducing spoilage and weight loss in cherry tomatoes during storage. The treatment significantly decreased the spoilage rate, reduced firmness loss, and controlled microbial growth over the storage period, particularly by lowering counts of mesophilic aerobic bacteria and filamentous fungi compared with the control, reinforcing the concept of active packaging with the release of volatile antimicrobial compounds. Another study showed that incorporating TEO into zein fibers by electrospinning was effective in preserving the quality of strawberries during refrigerated storage. The authors observed significant antimicrobial activity inhibiting the growth of *B. cereus* and *E. coli*, reducing weight loss, maintaining firmness, and preserving total soluble solids of the fruit over 15 days.⁶ These findings highlight the combined physicochemical and microbiological action of encapsulated delivery systems.

In addition to their antimicrobial activity, the bilayer films demonstrated antioxidant activity, as reported in an earlier study.²⁹ The bilayer films containing TEO encapsulated in zein electrospun fibers exhibited 43.3% and 33.8% inhibition of ABTS and DPPH radicals, respectively. This antioxidant capacity is mainly attributed to the phenolic constituents of TEO, particularly thymol and carvacrol, which are able to scavenge free radicals and delay oxidative reactions. These previously reported findings further support the multifunctional potential of the developed bilayer films for active food packaging and are consistent with the improved preservation of cherry tomatoes observed during storage.

Thus, the differences observed in microbial counts among the treatments in this study highlight the crucial role of packaging in controlling the microbiological quality of fresh tomatoes during storage. As demonstrated by the physicochemical analyses, the absence of a physical barrier increased the fruits' exposure to oxygen and pH fluctuations, creating favorable conditions for microbial growth. Although the PVC film provided some protection by reducing direct exposure to the external environment, microbial counts in this treatment were still higher than those observed in tomatoes packaged with the bilayer film containing encapsulated TEO. These findings suggest that the combined effect of reduced gas exchange and the antimicrobial activity of the active compound may have contributed to the formation of a modified atmosphere within the packaging, limiting the growth of aerobic microorganisms such as mesophilic bacteria, molds, and yeasts, as shown in microatmosphere testing. However, these results were obtained under controlled laboratory conditions, and further studies under commercial retail conditions are required to confirm the performance of the developed packaging. This demonstrates the potential of bilayer films as a promising and sustainable solution for preserving fresh fruits throughout the supply chain.

CONCLUSION

The results confirm the initial hypothesis by demonstrating the antimicrobial activity of thyme essential oil (TEO), supporting its application in biodegradable and active bilayer films composed of sweet potato starch and zein fibers containing encapsulated TEO. Films containing 60% encapsulated TEO exhibited significant antimicrobial activity through the release of volatile compounds, with notable effectiveness against *S. aureus*, *E. coli*, and *P. crustosum*. The encapsulation of TEO in zein fibers also proved effective in providing controlled release, extending its antimicrobial action over time. This was supported by the porous structure and high surface area of the electrospun zein fibers.

The practical application of the bilayer films in the packaging of cherry tomatoes highlighted their potential as active packaging, resulting in reduced deterioration, lower weight loss, and greater stability of physicochemical parameters such as soluble solids content, titratable acidity, and pH over 14 days of storage, in comparison with the control group and PVC film. Microbiological quality also improved at the end of the period, with lower *E. coli* counts, reduced aerobic mesophilic bacteria, and effective control of mold and yeast growth, compared with the control treatment. These results reinforce the feasibility of the proposed technology as a sustainable and functional alternative for developing food packaging with antimicrobial properties, focusing on food safety and quality, especially for minimally processed fruits and vegetables, with potential application in the fresh produce supply chain. Further studies are recommended to apply these bilayer films to other perishable food matrices and to assess sensory parameters, stability at different temperatures, and the toxicological safety of the released volatile compounds.

AUTHOR CONTRIBUTIONS

Jéssica Silveira Vitoria: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization. Tamires Soares Schug: conceptualization, methodology, formal analysis, investigation. Laura Martins Fonseca: conceptualization, methodology, data curation, writing – review and editing. Débora A. Campos: methodology, resources, validation, writing – review and editing, supervision. Manuela Pintado: resources, validation, supervision, funding acquisition. Tatiane Kuka Valente Gandra: validation, writing – review and editing, supervision. Eleessandra da Rosa Zavareze: conceptualization, resources, writing – review and editing, supervision, funding acquisition. Eliezer Avila Gandra: conceptualization, resources, writing – review and editing, supervision, project administration, funding acquisition.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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