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EDITED BY
Milena Corredig,
Aarhus University, Denmark

REVIEWED BY
Maria Simona Chi,
University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania
T. Anukiruthika,
University of Manitoba, Canada

*CORRESPONDENCE
Maria D. Antunes
✉ mantunes@ualg.pt

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# Antimicrobial activity of different nanocellulose films embedded with thyme, cinnamon, and oregano essential oils for active packaging application on raspberries

Sara Casalini[1], Marco G. Baschetti[1], Martina Cappelletti[2], Adriana C. Guerreiro[3], Custódia M. Gago[4], Silvia Nici[2] and Maria D. Antunes[4]*

[1]Department of Civil, Chemical, Environmental and Materials Engineering-DICAM, University of Bologna, Bologna, Italy, [2]Department of Pharmacy and Biotechnology (FABIT), University of Bologna, Bologna, Italy, [3]CEOT –Centre for Electronics, Optoelectronics, and Telecommunications, FCT, Universidade do Algarve, Faro, Portugal, [4]MED – Mediterranean Institute for Agriculture, Environment and Development and CHANGE - Global Change and Sustainability Institute, FCT, Universidade do Algarve, Faro, Portugal

The study focuses on the antimicrobial activity of nanocellulose films (NFC) embedded with thyme, cinnamon, and oregano essential oils for active packaging application. The activity against model pathogenic bacteria was first tested to obtain each oil's minimum inhibitory concentration (MIC). The tests showed that the surface area of the films and the quantity of essential oil strongly influenced the antimicrobial effect. Then, the different active packaging systems were tested directly on packed raspberries to study their actual commercial packaging conditions. Through 12 days of storage at 1°C, it was observed that thyme and oregano essential oils were more effective in maintaining the firmness and reducing the weight loss of the fruits compared to cinnamon essential oil or the control; no significant effect was observed on the other quality parameters that were considered. The essential oils independently proved effective in preventing fungal growth. However, the combined impact of thyme+oregano (NAP_TO) demonstrated the best performance.

KEYWORDS

**nanocellulose, biopolymers, essential oils, antimicrobial activity, active packaging**

## 1. Introduction

Recently, there has been a strong push toward studying more sustainable materials for active packaging applications to reduce the usage of oil-based plastics while decreasing food spoilage. Active packaging systems are among the most advanced solutions to increase the shelf-life of various foods (Wyrwa and Barska, 2017). They are based on matrix films embedded with different antimicrobial and antioxidant agents that are released inside the package to perform their activity.

Due to the strong need for more sustainable industrial development, active packaging systems that include biodegradable and biobased materials enhanced with natural agents are a crucial topic of study. Several biopolymers have been studied to comply with the standards

required for packaging technologies, such as cellulose, chitosan, poly-lactic acid (PLA), alginate, agar, and others (Casalini and Baschetti, 2022). For example, Subbuvel and Kavan prepared and characterized a PLA film embedded with curcumin and fenugreek essential oils to improve the functional properties of the composite (Subbuvel and Kavan, 2022). The PLA-based film showed good antibacterial and antioxidant properties, suitable for commercial strawberry packaging. Likewise, ginger and curcumin essential oils were used by Mohan and Panneerselvam to fabricate PLA functional films. They were tested on bread slices, resulting in a suitable option for prolonging the shelf-life of fresh foods using this packaging (Mohan and Panneerselvam, 2022).

Of the various biopolymers in the field, cellulose is one of the most studied and used. This is due to its high availability, biocompatibility, and adaptability to different systems and chemical environments (Ahankari et al., 2021; Casalini and Baschetti, 2022). In recent years, significant efforts have gone into the study of nanocellulose, which can produce thin transparent films made from cellulose nanofibers combining the properties of cellulose with the advantages of nanoscale, and its use as reinforcement filler in nanocomposite materials or as matrix itself (Trache et al., 2020). These films have several unique characteristics, such as high strength and toughness, low thermal expansion, and excellent gas barrier properties. They are also biodegradable and renewable, making them an eco-friendly alternative to traditional plastic films. In the field of active packaging, nanocellulose has been used as a carrier for active substances like antioxidants and antimicrobials agents, oxygen scavengers, and moisture absorbers (Khan et al., 2014; Azeredo et al., 2017; Casalini and Baschetti, 2022). These functionalities can help to reduce the growth of microorganisms, prevent oxidation, and maintain the freshness and quality of the packaged product. However, while active packaging systems based on nanocellulose films offer several advantages over commercial ones, their feasibility depends on various factors specific to the application and the regulatory environment in which they are used.

Among the various active agents available in the market, essential oils attract considerable attention due to their well-known properties and natural origin (Falleh et al., 2020). Essential oils from aromatic plants present natural antimicrobial and antioxidant activities. They have long been used as additives in various fields, such as food and beverage, packaging, and cosmetics (Atarés and Chiralt, 2016). Each essential oil has specific characteristics depending on the plant from which it is produced and the extraction method. Also, they interact in different ways with different bio-matrices in which they are incorporated, leading to very diverse behaviors (Sharma et al., 2021; Wang et al., 2021). Their presence in the matrix, therefore, not only enhances the resistance against spoilage and degradation by pathogens but may also help improve the films' mechanical properties (Asgher et al., 2020).

Essential oils have been proven to have antibacterial activity against both Gram-negative and Gram-positive bacteria (Bouhdid et al., 2009). At the same time, because of their chemical complexity, their toxicity needs to be appropriately evaluated to guarantee their safe utilization (Llana-Ruiz-Cabello et al., 2015). The antibacterial activity of essential oils appears to be related mainly to their lipophilic nature, which induces their accumulation in cellular membranes, impacting membrane permeability and composition (Burt, 2004). This, in turn, leads to cell lysis and interference with different cell processes, such as the electron transport chain, membrane potential, and protein translocation (di Pasqua et al., 2007; Bouhdid et al., 2009). Several studies have shown different antibacterial activity depending on the strains under analysis, generally in association with the differences characterizing the cell envelope of Gram-negative and Gram-positive bacterial strains (Bouhdid et al., 2009).

Among the many existing essential oils, those obtained from thyme, cinnamon, and oregano have the highest antimicrobial and antioxidant activity and are among the most studied in active packaging applications. Cinnamon essential oil effect has been studied in various bio-matrices, such as nanocellulose (Montero et al., 2021; Souza et al., 2021), chitosan (Hu et al., 2015), and sodium alginate (Han et al., 2018). Similarly, thyme and oregano contain common active agents for active packaging applications (Yemiş and Candogan, 2017; Hossain et al., 2019). For instance, Yemiş and Candogan (2017) demonstrated that the application of soy-protein edible coatings infused with 3% thyme or oregano essential oil could have an effective antimicrobial activity against pathogenic bacteria such as *E. coli*, *Listeria monocytogenes*, and *S. aureus*, enhancing color stability of fresh beef while maintaining acceptable sensory characteristics (Yemiş and Candogan, 2017). Similarly, Hossain et al. observed that bioactive chitosan films loaded with thyme and oregano essential oils produced a 2-log reduction in fungal growth in inoculated rice; the sensorial evaluation showed no significant change from the untreated rice (Hossain et al., 2019).

Raspberry (*Rubus idaeus* L.) is a high-value fruit crop with increasing production worldwide (FAO, 2021). Fresh raspberries, however, are highly perishable owing to their fragile structure, high respiration rates, and susceptibility to fungal infections, leading to substantial post-harvest losses. Due to their fast post-harvest ripening and degradation, small fruits like raspberries and berries are frequently used to study active packaging applications. Different methods based on the direct contact activity of the oils have been applied and monitored to select the best type and quantity of essential oil/plant extract for this purpose (Pandey et al., 2022). For example, Cefola et al. (2022) studied incorporating green tea and rosemary ethanolic extract into chitosan (Cefola et al., 2022). Moreno et al. (2020) examined antifungal edible coatings for raspberries containing propolis extract (Moreno et al., 2020). Likewise, Gabrieli De Souza et al. (2021) monitored the influence of edible coatings enriched with citral and eugenol (Guerreiro et al., 2016).

Interestingly, most of these studies are focused on the effect of the antimicrobial agents when in direct contact with the bacterial strain or the food to be treated while their activity in the vapor phase, which is created from their diffusion from the active film to the headspace of the packaging, is less investigated. To the best of our knowledge, only a few studies can be found in the literature which reports the vapor diffusion assays of various essential oils to estimate their vapor activity (López et al., 2005; Inouye et al., 2006; Mejía-Garibay et al., 2015; Wu et al., 2017).

To partially fill this gap, the aim of the present study was to analyze the antioxidant and antimicrobial activity of nanocellulose

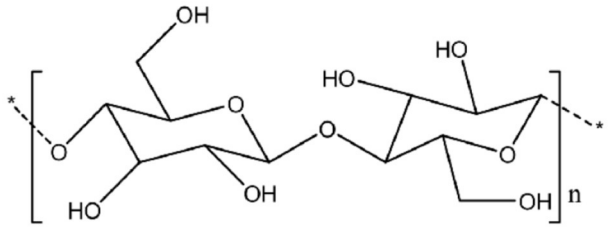


FIGURE 1
Nanocellulose chemical structure (polymerdatabase.com).

films embedded with thyme, cinnamon, and oregano essential oils, which were left to evaporate in the headspace of the packaging. To thoroughly evaluate the system's effectiveness, the experiments were performed *in vitro*, testing the activity against *Staphylococcus aureus* and *Escherichia coli* bacterial strains and directly on packed fresh raspberries. The experiments on the bacterial strains were fundamental to determining the effectiveness of such an innovative system and defining the most relevant parameters for the process, such as the minimum inhibitory concentration (MIC). At the same time, the experiments on freshly packed fruit were fundamental to testing the system in a real-case scenario. For this reason, in the latter tests, a sensory evaluation was performed to have quantitative data on the consumers' perception and their willingness to accept a food product treated with such a technique.

## 2. Materials and methods

### 2.1. Preparation of films

The nanofibrillated cellulose (NFC) utilized in this work was provided by INOFIB (France) in water suspension and was obtained from eucalyptus pulp. The nanofibrils had a final diameter of 80-150 nm and a superficial charge density of 30 μequiv/g (Figure 1).

Cinnamon, Thyme, and Oregano essential oils were provided by Destilerías Muñoz Gálvez, S.A. (Murcia, Spain). They were 100% pure oils, with respectively 74.7 % v/v of *Eugenol,* 55.5 % v/v of *Thymol,* and 71.5 % v/v of *Carvacrol* as major active compounds. Deionized water from the laboratory was used to dilute the solutions during the film preparation procedures.

### 2.2. Preparation of the active nanocellulose films and *in vitro* anti-bacterial activity assays

The nanocellulose films were prepared from water dispersions through a solvent-casting technique, as explained in previous works (Casalini et al., 2022). Once the films were dried, a controlled amount of the essential oil of interest was added to the surface of the films with a micropipette to reach the desired concentration. The reference concentration was related to the maximum oil absorption rate of the nanocellulose, i.e., until the nanocellulose was saturated with the oil. This value was determined through experiments done in previous works (Casalini et al., 2022). The concentration of the oils in the nanocellulose matrix was tuned to be always higher than the saturation value to ensure a constant flux through the film over time and hence prolong the antimicrobial activity (Fan and Singh, 1989). Then, higher concentrations (up to 0.85 mg of oil per mg of the film) were also tested to correlate the concentration effect on the antimicrobial activity. Nanocellulose films of 9 cm and 4 cm in diameter and 30-50 μm thick were used to test the size effect on the antibacterial activity.

A summary of the various systems tested can be found in Table 1, where the quantity of oil used (μL) for each membrane and the ratio between the mass of the oil and the mass of the film are also reported. This ratio remained constant for the 4 cm and 9 cm membranes to analyze the impact of the quantity of oil introduced in the system without modifying its concentration in the nanocellulose films. For the sake of clarity in the text, the samples of nanocellulose active packaging (NAP) in the various experiments were marked with the dimension of the film (9 cm or 4 cm) followed by the name of the oil present in the membrane, and the ratio between the oil quantity and the mass of the film. For example, NAP9_T-0.19 referred to a nanocellulose membrane that was 9 cm in diameter and embedded with thyme essential oil in a ratio of 0.19 (mg) per mass of the film (mg).

After preparation, the active film was attached to an aluminum foil, ensuring no oil leaked from the edges. In particular, the active surface was directly in contact with the aluminum foil, while the edges of the film were sealed with hot glue. In this way, oil could only diffuse through the nanocellulose film before being dispersed in the headspace of the testing system (Petri dish). Thus, uncontrolled releases before the nanocellulose film was correctly placed inside the system for the test were significantly reduced.

The bacterial strains used for the tests were the Gram-positive *Staphylococcus aureus* ATCC6538P and the Gram-negative *Escherichia coli* ATCC8739. A volume of each bacterial suspension was taken from 5 mL cultures grown overnight up to a concentration of $10^6$ – $10^8$ CFU/mL and spread onto LB agar plates. Immediately after that, the active film (4 cm or 9 cm) was attached to the inside cover of the Petri dish lid to test the antimicrobial activity associated with the oil volatilization. The agar plates containing films activated with the same oil were incubated at 37°C in static conditions after insertion into a humid box. Aluminum foils with only nanocellulose without the oils were used as control. Bacterial growth was detected and enumerated as CFU/mL counting after incubation at 24 h and 168 h (i.e., one week). These CFU numbers were compared with those counted on agar plates without films, which were spread with the same inoculum and incubated in the same box with the same conditions (controls). The 4 cm films activated with thyme oil were also tested in Petri dishes sealed with parafilm to limit the external dispersion of the essential oil and to evaluate its effect on the observed antibacterial activity. This condition was indicated with an asterisk (NAP4_T-0.38*). All the tests were performed in duplicate.

### 2.3. Real case application: raspberry case-study system

The raspberry fruits (*Rubus idaeus* L.) cv. Eros, used for the actual conditions tests, were purchased from a local producer

TABLE 1 Nanocellulose-oil systems used for *in vitro* tests.

| Sample | Diameter (cm) | Oil | Quantity (μL) | m,oil (mg)/m,film(mg) | V,oil (μL)/liter of air |
|---|---|---|---|---|---|
| NAP9 | 9 | No oil | 0 | 0 | 0 |
| NAP9_T-0.19 | 9 | Thyme | 25 | 0.19 | 393 |
| NAP9_T-0.37 | 9 | Thyme | 50 | 0.37 | 786 |
| NAP9_T-0.60 | 9 | Thyme | 80 | 0.60 | 1258 |
| NAP9_T-0.75 | 9 | Thyme | 100 | 0.75 | 1572 |
| NAP9_C-0.68 | 9 | Cinnamon | 80 | 0.68 | 1258 |
| NAP9_C-0.85 | 9 | Cinnamon | 100 | 0.85 | 1572 |
| NAP9_O-0.62 | 9 | Oregano | 80 | 0.62 | 1258 |
| NAP4_T-0.19 | 4 | Thyme | 5 | 0.19 | 79 |
| NAP4_T-0.38 | 4 | Thyme | 10 | 0.38 | 157 |
| NAP4_T-0.38* | 4 | Thyme* | 10 | 0.38 | 157 |
| NAP4_T-0.57 | 4 | Thyme | 15 | 0.57 | 236 |
| NAP4_C-0.65 | 4 | Cinnamon | 15 | 0.65 | 236 |

The dimension of the films, the type of oils, and their quantity and concentration are reported in the table. The "NAP4_T-0.38*" test represents the sample sealed with Parafilm.

and immediately transported to the post-harvest laboratory at the University of Algarve, where they were selected for the experiments. The media used for antimicrobial analyses on fruit were Plate Count Agar (PCA), Buffered Peptone Water (Oxoid), and Dichloran Rose Bengale, purchased from Biokar Diagnostics.

The fruits were put in commercial clamshell-type PET boxes (125g) with 13.5x11x3 cm size. Then the essential oils were placed on the active nanocellulose films (9 cm diameter), which were then attached with hot glue directly to the inner surface of the lid of the raspberry PET boxes5. Four replications were done for each oil used, in a total of four treatments plus the control. The fruits were stored in separate boxes for each treatment/replication at 1°C. The measurements were performed every 6 days until 12 days of total storage.

Two sets of experiments were performed to optimize the quantity of the oils and select the most effective treatment. In the first test, the quantity of the oils was chosen based on the MIC resulting from the tests performed directly on bacterial strains. In the second experiment, thyme and oregano essential oils were used separately and in combination with a higher concentration (five times higher than the MIC) to further test the antimicrobial activity related to their quantity and to identify the possible synergetic effects. For both experimental sets, the control was considered the fruit box without any film or essential oil. Table 2 shows the quantity of oils used in the experiments (μL) and the ratio between the mass of the oils used and the mass of the nanocellulose films. The ratio between the volume of the oil used, and the headspace of the packaging is also reported in Table 2 as a reference for future applications that may be applied to different fruit/packaging conditions.

### 2.3.1. General quality parameters

Quality parameters were analyzed on ten fruits per box/replication. The color of the raspberries was measured using a Minolta Chroma meter CR-300 (EC Minolta, Japan) using the CIELab scale (L*, hue* and Chroma) (McGuire, 1992).

Firmness is another crucial parameter to define the quality attributes of the fruits. The tissue softening not only affects the visual appeal but also reduces the fruit's shelf-life. The firmness of the fruit was determined by compression with a Chatillon TCD200 and a Digital Force Gauge DFIS 50 (Jonh Chatillon and Sons, Inc., USA) fitted with a cylindrical plate pressing on the fruit at a maximum depth of 10 mm. To determine the total soluble solids content (SSC), the °Brix was measured, in a drop of juice, by a digital refractometer HI 96801 (Hanna instruments). Weight loss was measured through an electronic balance with a precision of 0.001g and expressed as a percentage of the initial weight.

### 2.3.2. Evaluation of the antimicrobial activity of the nanocellulose films applied to a real setting

Microbial counts were determined for each treatment, starting from the fruit obtained from the cultivar (no inoculum had been applied in this phase). The microbiological parameters considered included counts of aerobic mesophilic and psychrophilic bacteria and molds and yeasts and were determined according to Guerreiro et al. (2015). Experiments were done in duplicate. Results were expressed as $Log_{10}$ CFU (Colony Forming Unit) per gram of fresh weight.

### 2.3.3. Total phenolic content

Phenolic compounds are phytochemicals in fruits and vegetables that have a nutritional and sensory role (Yousuf et al., 2021). Total phenolic content was determined according to the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965) modified for microplates (Guerreiro et al., 2016).

TABLE 2 Essential oils and their quantity used for the two experiments.

| Experiment | Essential oil | Treatment name | Quantity (μL) | m, oil (mg)/m, film(mg) | V, oil (μL)/liter of air |
|---|---|---|---|---|---|
| Test 1 | No oil, nanocellulose film | NAP | 0 | 0 | 0 |
| | Cinnamon | NAP_C | 80 | 0.68 | 359 |
| | Thyme | NAP_T | 50 | 0.37 | 224 |
| | Oregano | NAP_O | 80 | 0.62 | 359 |
| Test 2 | Thyme | NAP_T | 250 | 1.87 | 1122 |
| | Oregano | NAP_O | 400 | 3.09 | 1796 |
| | Thyme + Oregano | NAP_TO | 325 | 2.48 | 1459 |

### 2.3.4. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity and anthocyanins

The capacity for scavenging DPPH free radicals was evaluated by the method described by Miguel et al. (2018).

Total anthocyanins were determined using a modified pH differential method (Lee et al., 2005; Guerreiro et al., 2016). The absorbance of anthocyanins at 520 nm and 700 nm in different pH buffers (pH 1.0 and 4.5) was measured, respectively. Absorbance readings were converted to total mg of cyanidin 3-glucoside per 100 mL fruit juice.

### 2.3.5. Sensory evaluation

Appearance is the main factor determining the consumer's intention to buy fruit. A group of 21 people, drawn from faculty, students, and staff, who usually consume raspberries, were recruited to evaluate the fruit after 12 days of storage for test 2. They were asked to evaluate the fruit appearance for all the treatments based on a 7-point hedonic scale: 1 – dislike very much; 2 – dislike; 3 – dislike slightly; 4 – either like or dislike; 5 – like slightly; 6 – like; 7 – like very much.

## 2.4. Statistical analysis

The experimental design was a completely randomized block design. Statistical analysis was performed using the SPSS 24.0 software (IBM, Inc., Armonk, N.Y., USA). Two-way analysis of variance (ANOVA) was done using treatments and storage time as factors with four replications (each box was a replicate). ANOVA is one of the most widely used statistical methods for hypothesis testing in which data sets are compared and measured to determine their significance (Sthle and Wold, 1989). Duncan's multiple-range tests ($p < 0.05$) for means comparison were undertaken. This least significant range increases with the number of samples means in the subset. If the range of the subset exceeds the least significant range, then the population means can be considered significantly different (Duncan, 1955). In our case, the significant range was set at a value of $P \leq 0.05$.

# 3. Results and discussion

## 3.1. Anti-bacterial properties of the activated nanocellulose films

Figure 2 displays representative agar plates that show the antibacterial activity results obtained after exposing *E. coli* and *S. aureus* cells (spread on solid agar cultures) to the nanocellulose films embedded with cinnamon essential oil. The images of the tests relating to thyme and oregano essential oils can be found in the supporting information (Figures SI1 a-d). From Figure 2, it is possible to observe the bacterial growth in the control (inoculated plate with only aluminum foil), the plate with pure nanocellulose (NAP9), and the one with the cinnamon essential oil (NAP9_C-0.68). The antibacterial effect could be noticed by the reduction or absence of microbial growth on plates exposed to activated films. It was visible in all samples except the control, where it was removed to visualize the colonies better. From Figure 2, it is possible to observe that pure nanocellulose did not have any antimicrobial activity since the colonies were present all over the plate. In contrast, the film containing the cinnamon essential oil clearly had an antimicrobial effect on *S. aureus* since there were no visible colonies. On *E. coli*, the colonies were present only on the borders of the petri dish.

It can be noticed that when the bacterial colonies were present, as in the case of NAP_C with *E. coli*, they were mostly concentrated in the external area of the Petri dish, near the edges. Without being sealed, some of the essential oil vapors could escape from the edges of the Petri dish, decreasing their local concentration and thereby reducing their antibacterial effect on the edges.

A specific test was performed to confirm this hypothesis. The inoculated agar plates were sealed with parafilm to limit the diffusion of essential oil vapors from escaping to the external environment (indicated with the code NAP4_T*-0.38). As a result, when the parafilm was used, microbial growth was absent on the edges of the plate. Although parafilm helped confirm the diffusion effect of the essential oil on the edges of the plates, this experimental setting was not used further because it did not represent real packaging conditions. On the contrary, real fruit packaging conditions are characterized by boxes that have parts

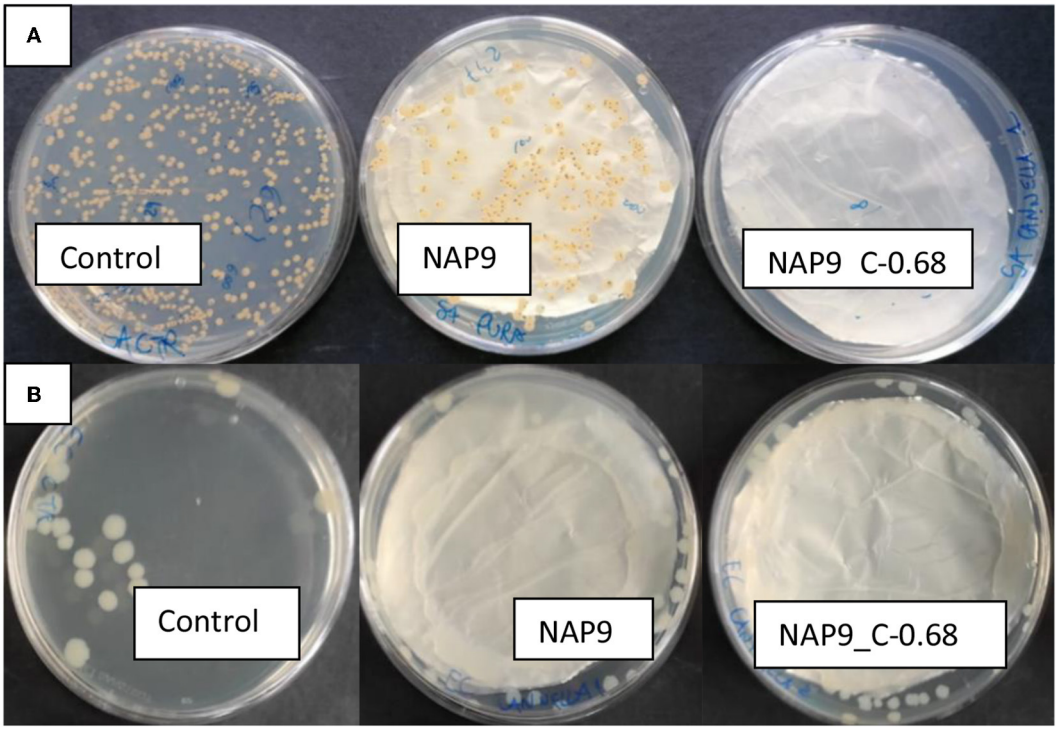


FIGURE 2
The Petri dishes after the test with **(A)** S. aureus ATCC6538P and **(B)** *E. coli* ATCC8739. The figure shows the control, the pure nanocellulose without any oil (NAP9), and the nanocellulose with cinnamon essential oil [0.68 m, oil (mg)/m, film(mg)] (NAP9_C-0.68).

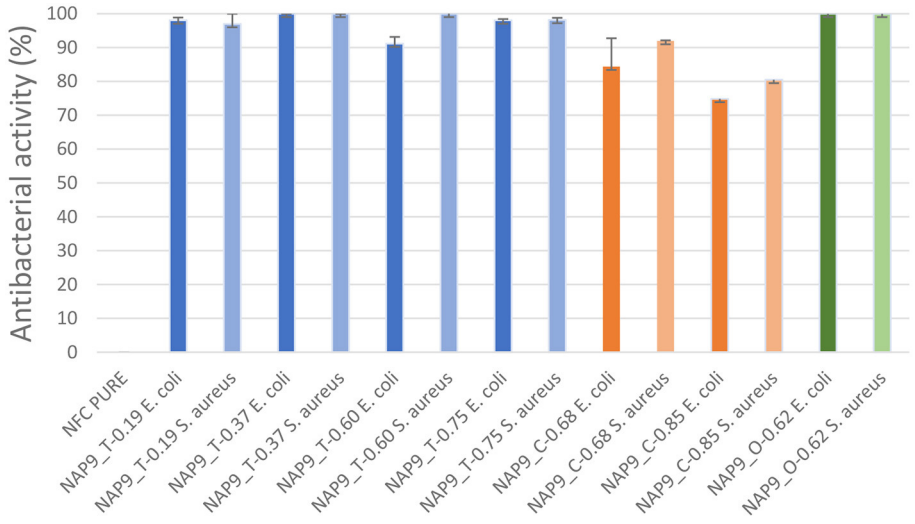


FIGURE 3
Antimicrobial activity against *E. coli* (darkest color) and S. aureus (lighter color) expressed through CFU counting from the nanocellulose samples of 9 cm films embedded with thyme (blue), cinnamon (orange), and oregano (green) essential oils. Values are means of 3 replicates ± standard deviation.

open to the external environment to prevent problems related to the fermentation processes.

The overall quantitative results of the experimental setup are presented in Figures 3, 4. They show the antimicrobial activity of the different samples. Figure 3 refers to the 9 cm diameter nanocellulose films, while Figure 4 refers to samples 4 cm in diameter. In both cases, the results obtained with different concentrations of essential oil were compared with the control and with pure nanocellulose, which always showed no effect on bacterial growth.

Concerning the 9 cm films with the same amount of essential oil, seen in Figure 3, it can be noticed that the oils were consistently

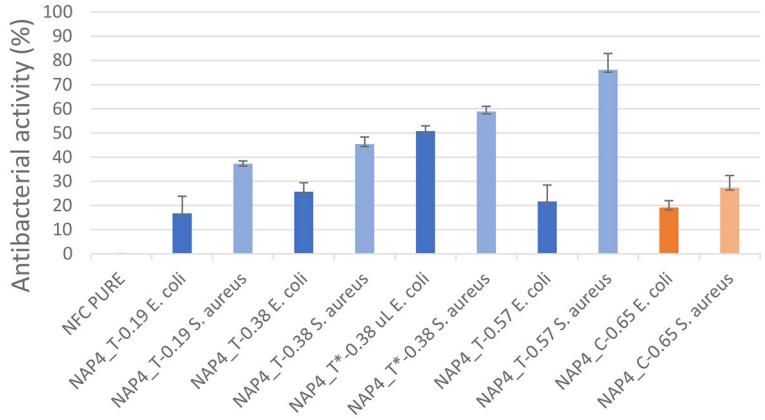


FIGURE 4
Antimicrobial activity against E. coli (darkest color) and S. aureus (lighter color) expressed through CFU counting from the nanocellulose samples of 4 cm films embedded with thyme (blue) and cinnamon (orange) essential oils. Thyme10* indicates the sample that has been tested with parafilm to completely seal the Petri dish and observe the border effect. Values are means of 3 replicates ± standard deviation.

more effective against *S. aureus* rather than *E. coli* (even if the difference never exceeded 10% and in general was not statistically significant). Only in the case of NAP9_T-0.19, the system had a higher antimicrobial effect against *E. coli*, but still very similar to the case of *S. aureus*, with values of 98% and 97%, respectively.

Thyme essential oil appeared to have the highest antimicrobial activity since it was the only one that was 100% effective against both pathogens, with a quantity of 0.37 mg/mg. However, a slight decrease in antimicrobial activity was noticed with an increase in the concentration of thyme essential oil, but the values did not differ significantly. The efficiency of Thyme was followed by oregano and, finally, cinnamon, which had the lowest effect at this concentration. This could also be because cinnamon strongly interacted with the nanocellulose matrix, showing, in general, a higher diffusion coefficient compared to both thyme and oregano essential oils. Thus, cinnamon essential oil could more rapidly diffuse in the vapor phase and quickly disperse in the external environment than the other oils, leading to a lower antimicrobial efficiency over time (Casalini et al., 2022).

The exact concentration of essential oil (mg) per mass of nanocellulose membrane was used on smaller films of 4 cm in diameter to test the amount of oil vapor released in the headspace and its effect on antimicrobial activity. As seen in Table 1, at the same release rate, these films released about one-fifth of the oil compared to the 9 cm samples, thus allowing us to better discriminate between the activity of the different oils.

Observing the results of the 4 cm films, it was possible to notice an increasing trend of antimicrobial activity with the increase of the essential oil quantity. Although, in this case, the difference in the antibacterial effect was more evident. It showed stronger activity against *S. aureus* for all oils at all concentrations, thus confirming what was observed with the 9 cm films. In the case of the Petri dish (NAP4*_T-0.38) sealed with parafilm, a higher antimicrobial effect was observed against both pathogens compared to the normal setup (NAP4_T-0.38) without parafilm sealing. It showed an antimicrobial effect almost double for *E. coli* (51% versus 26% of antimicrobial activity for NAP4*_T-0.38 and NAP4_T-0.38, respectively) and one-third higher for *S. aureus* (59% versus 45% of antimicrobial activity for NAP4*_T-0.38 and NAP4_T-0.38, respectively). In addition, in this case, microbial growth was absent on the edges, unlike all the other Petri dishes that were not sealed (Figure 2). Moreover, 0.57 mg/mg of thyme essential oil was more effective against Gram-positive bacteria than cinnamon, with a concentration of 0.65 mg/mg. However, they had almost the same efficiency against Gram-negative bacteria (22% and 19%, respectively).

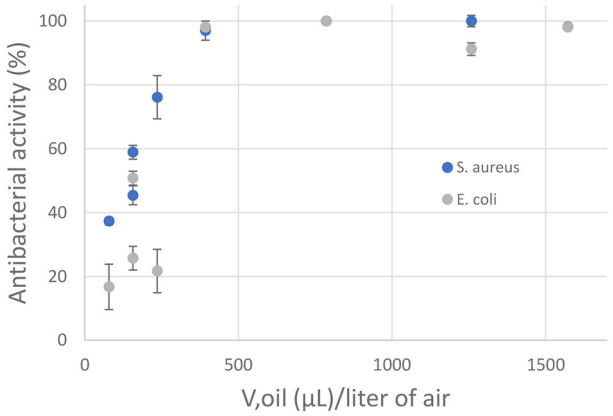


FIGURE 5
Antimicrobial activity (%) of thyme essential oil in nanocellulose films as a function of the Petri dish headspace (V, oil (µL)/lL of air). The blue dots represent the antimicrobial activity (%) against *S. aureus*, while the grey ones represent the antimicrobial activity against *E. coli*.

This fact again points to the importance of the release kinetics and the oil concentration in the headspace to determine the overall activity of the different essential oils. It becomes even clearer when results are compared for 4 cm and 9 cm films. Larger diameter films, characterized by higher oil concentration in the headspace, always showed higher antimicrobial activity, between 75 and 100%, for all the oils. On the other hand, the smaller films never reached antimicrobial activities higher than 80% (the highest value of 76%


FIGURE 6
Raspberry active packaging systems with pure nanocellulose discs (NAP) of 9 cm diameter for the four repetitions of the treatment. The picture was taken after 12 days of storage at 1°C.

was recorded for NAP4_T-0.57 against *S. aureus*). This behavior can be explained by considering the concentration in the headspace of the different specimens, which correlates with the results of the antimicrobial activity. This fact is particularly evident in the case of thyme essential oil (Figure 5). A clear trend was evident starting from sample NAP4_T-0.19 which had the lowest antibacterial effect (37% and 17% of bacterial growth inhibition for *E. coli* and *S. aureus*, respectively) to samples NAP9_T-0.19 and NAP9_T-0.37, where the MIC was obtained.

These results confirm that the oil quantity per volume of headspace determines the antimicrobial effect more than the oil quantity on the nanocellulose film. Since this system works based on the volatile part of the oils and their effect on the edges of the dish, it is essential to calibrate the quantity of oil applied to the film to the headspace of the specific packaging used. For this reason, 9 cm films were used as a reference for the MIC calculation, and oregano essential oil was not tested in the 4 cm membranes. In the 9 cm films, the MIC was 0.37 mg/mg (786 $L_{oil}/L_{air}$) and 0.62 mg/mg (1258 $L_{oil}/L_{air}$) for thyme and oregano essential oils, respectively, and 0.68 mg/mg (1258 $L_{oil}/L_{air}$) for cinnamon. These concentrations were considered as a base for the test on packed raspberries, which will be discussed in the next section.

## 3.2. Real condition application: raspberry case-study

Figure 6 shows how the active packaging systems were marked on the raspberry package lids. The nanocellulose films were attached to the inner side of the packaging lid after being infused with the selected quantity of essential oil. The image shows the appearance of the fruits after 12 days of storage with the treatment applied. It can be noticed that for all tests, the qualitative appearance of the fruit appeared comparable to the regular fruit without treatment. At the same time, significant differences were observed for other quality parameters, as detailed below.

The quantity of oil considered for each test is presented in Table 2. Different tests were run controlling the oil/film mass ratio to values below (first test) and above (second test) the MIC results obtained in the analysis of the microorganisms. In calculating this parameter, the package leakage and the possible diffusion of the essential oils into the plastic constituting the packaging box (r-PET) were neglected. Even in the absence of the aluminum screen, the diffusion process should be negligible; Licciardello et al. (2013) reported a diffusion coefficient of the various oils in PET to be in the range of $0.03 - 6.40 \times 10^{-11} cm^2/s$. These values are two times higher than those obtained for absorbing essential oils in pure nanocellulose matrices (Casalini et al., 2022). Therefore, the oils can be expected to diffuse more rapidly through the nanocellulose film compared to the plastic lid of the package. However, no leakage of oils or solubilization of the lid were observed after 12 days of storage.

### 3.2.1. General and nutritional quality parameters

Figure 7 shows the weight loss (%) of the fruit during storage at 1°C, which is an essential indicator of fruit freshness. Values up to 4-5% were considered to not significantly affect the fruit attributes and consumer acceptance (Nunes, 2015). After 12 days of storage, all the treatments presented a weight loss variation under 5%, with the cinnamon treatment at the acceptance limit of 5.0%; NAP and NAP_C had higher weight loss than the control—4.3%

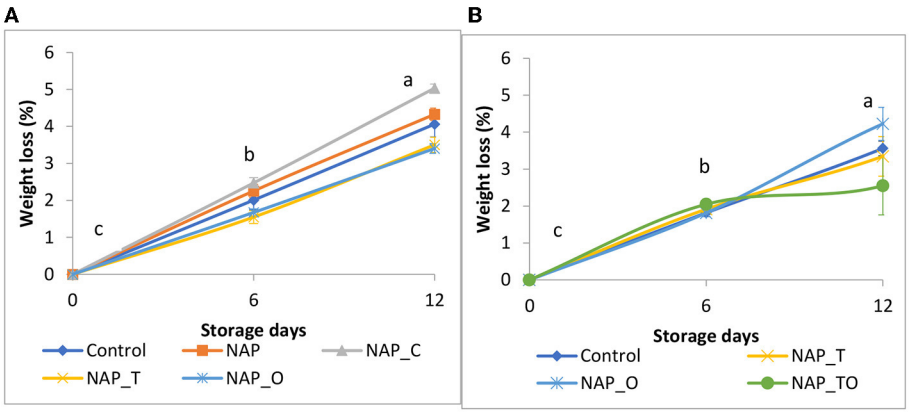


FIGURE 7
Weight loss (%) of the fruit during storage for **(A)** test 1 and **(B)** test 2. The colors represent respectively: the control (blue), the pure nanocellulose without essential oils (orange), the nanocellulose with cinnamon essential oil (grey), the nanocellulose with thyme essential oil (yellow), the nanocellulose with oregano essential oil (light blue), and the nanocellulose with thyme+oregno essential oils (green). Values with different lower case letter are significantly different over time by Duncan's multiple range test ($P < 0.05$). No statistical differences were present for the different treatments for each time (0, 6th, and 12th days).

and 5.0% compared to 4.1% of the control (Figure 7A). In contrast, NAP_T and NAP_O had the lowest weight loss, with 3.5% and 3.4%, respectively. However, in test 2 (Figure 7B), NAP_O had higher weight loss compared to the control (4.2% versus 3.6%), while NAP_TO had the best treatment overall, with weight loss below 3% (2.5%). In both test 1 and test 2, none of the treatments had statistically different values at 6 days or 12 days. Nonetheless, each weight loss for each oil was statistically different, comparing the measurements of the same treatment over time (at 6 days compared to 12 days).

The color parameters—lightness (L*), hue, and chroma for tests 1 and 2 were studied, and results are presented in Tables 3, 4. For test 1, the lightness had a significant decrease in the first 6 days, which was lesser for NAP_O (-7.0%) and higher for NAP_C and NAP_T (-11.1%) compared to the control and NAP (-9.2%). Thereafter, it maintained at the same level until 12 days of treatment without significant difference between each treatment. The same trend was observed in test 1 for hue and chroma. This could be explained by the fact that in the initial phase, the quantity of oil released was higher, causing a higher concentration in the headspace, which then slightly decreased over the remaining days of the test.

In test 2, the results were more scattered than in test 1, and the data for different oils followed different trends. Apart from NAP_TO, no clear sign of parameter stabilization was observed after day 6. In general, lightness was better maintained by the control in the first 6 days, with a significant difference between the NAP_O and NAP_TO; the control registered a decrease of only 0.8% compared to the initial value of 36.9, while in the treatments, the drop was 4.6% and 7.8%, respectively. After 12 days of storage, the control significantly differed from the treatments NAP_O and NAP_TO (35.8 versus 34.0 and 34.5, respectively). The same trend was observed for hue angle (h°), with a statistical difference only between the control and the NAP_O treatment (29.5 versus 28.5), indicating an increase in redness and ripening of the fruits (McGuire, 1992). While for chroma (C*), the values of all the treatments increased in the first 6 days and then decreased until 12 days of treatment without statistically significant differences.

Table 3 (data from test 1) and Table 4 (data from test 2) also report other quality parameters analyzed during the experiments, such as firmness and soluble solids concentration (SSC). In addition, Tables 3, 4 also provide details on the total phenols content, the antioxidant activity calculated through the DPPH method, and the anthocyanins concentration.

Overall, the soluble solid concentration (SSC) first increased (after 6 days) to then slightly decrease over the 12 days of test 1. In test 2, on the other hand, it showed no significant differences. The results are in line with expectations, as raspberries are non-climacteric fruits, which usually show only slight changes in SSC (Guerreiro et al., 2015).

In test 1, the variations of the initial time values of 8.85 °Brix were consistently lower than 4.8%, except for NAP and NAP_O, which, after 6 days, registered a statistically significant increase (+6.21% and +9.60%, respectively). At 12 days, the control had the highest SSC of 9.10 °Brix, while there was no difference between the other treatments. Regarding test 2, none of the treatments had significant variations throughout the test. While comparing the treatments, at 12 days, NAP_T had the highest value of 7.70 °Brix (7.69% increase from the beginning), which meant this treatment resulted in the faster ripening of the fruit.

The firmness of the packed fruit considerably decreased at 12 days of storage for all treatments. In the first test, NAP_C had the highest decrease from 4.75 N to 0.95 N at 12 days, followed by NAP_O and NAP_T (1.51 N and 1.60 N, respectively). However, the latter showed fruit firmness values similar to those of the control and NAP (1.61 N and 1.88 N, respectively), displaying no statistical difference among the treatments. In the second test, the initial firmness values (including the control) were equal to 8.77 N, but the decrease over time was more pronounced than in the first test. After 6 days, NAP_TO had the highest drop to 4.64 N (-47%), while NAP_T and NAP_O decreased significantly compared to the control, with a difference of 37 and 34%, respectively. At the end of the two tests, all the treatments were lower than the control (2.67 N), with NAP_TO having the lowest value of 1.32 N. The fruit softening during the post-harvest period is related to the hydrolysis of starch to sugar and the degradation of pectin in the fruit cell

TABLE 3 Soluble solid content (SSC), firmness, total phenolic content, DPPH, and anthocyanins of raspberries treated with cinnamon (C), thyme (T), and oregano (O) essential oils during storage at 1°C.

| | Days | Control | | | | NAP | | | | NAP_C | | | | NAP_T | | | | NAP_O | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | | SD | | Mean | | SD | | Mean | | SD | | Mean | | SD | | Mean | | SD | |
| Lightness (L*) | 0 | 32.11 | ± | 1.01 | aA | 32.11 | ± | 1.01 | aA | 32.11 | ± | 1.01 | aA | 32.11 | ± | 1.01 | aA | 32.11 | ± | 1.01 | aA |
| | 6 | 29.15 | ± | 0.42 | bAB | 29.16 | ± | 0.58 | bAB | 28.55 | ± | 0.59 | bB | 28.53 | ± | 2.59 | bA | 29.86 | ± | 0.87 | bA |
| | 12 | 28.95 | ± | 0.23 | bA | 28.57 | ± | 0.26 | bcA | 28.53 | ± | 0.78 | bA | 28.74 | ± | 0.20 | bA | 28.48 | ± | 0.79 | cA |
| Hue angle (h°) | 0 | 24.03 | ± | 1.45 | aA | 24.03 | ± | 1.45 | aA | 24.03 | ± | 1.45 | aA | 24.03 | ± | 1.45 | aA | 24.03 | ± | 1.45 | aA |
| | 6 | 19.35 | ± | 1.23 | bB | 20.13 | ± | 1.22 | bAB | 19.14 | ± | 1.14 | bB | 19.97 | ± | 0.78 | bAB | 20.66 | ± | 0.62 | bA |
| | 12 | 19.49 | ± | 0.92 | bA | 19.58 | ± | 2.00 | bcA | 18.64 | ± | 1.60 | bA | 18.45 | ± | 0.60 | cA | 19.28 | ± | 0.54 | cA |
| Chroma | 0 | 31.21 | ± | 2.44 | aA | 31.21 | ± | 2.44 | aA | 31.21 | ± | 2.44 | aA | 31.21 | ± | 2.44 | aA | 31.21 | ± | 2.44 | aA |
| | 6 | 24.51 | ± | 1.18 | bBC | 24.83 | ± | 0.99 | bcB | 23.00 | ± | 1.65 | bC | 23.70 | ± | 1.25 | bBC | 26.87 | ± | 1.30 | bA |
| | 12 | 24.09 | ± | 0.68 | bB | 25.76 | ± | 0.99 | bA | 23.33 | ± | 2.71 | bB | 23.67 | ± | 0.58 | cB | 24.14 | ± | 1.01 | cB |
| SSC (°Brix) | 0 | 8.85 | ± | 0.13 | aA | 8.85 | ± | 0.13 | bA | 8.85 | ± | 0.13 | abA | 8.85 | ± | 0.13 | abA | 8.85 | ± | 0.13 | bA |
| | 6 | 9.18 | ± | 0.17 | aB | 9.40 | ± | 0.16 | aAB | 9.28 | ± | 0.42 | aB | 9.10 | ± | 0.28 | aB | 9.70 | ± | 0.18 | aA |
| | 12 | 9.10 | ± | 0.29 | aA | 8.70 | ± | 0.14 | bAB | 8.98 | ± | 0.39 | abAB | 8.48 | ± | 0.55 | bB | 8.75 | ± | 0.31 | bAB |
| Firmness (N) | 0 | 4.75 | ± | 0.92 | aA | 4.75 | ± | 0.92 | aA | 4.75 | ± | 0.92 | aA | 4.75 | ± | 0.92 | aA | 4.75 | ± | 0.92 | aA |
| | 6 | 4.18 | ± | 0.93 | aA | 3.22 | ± | 1.20 | bB | 3.24 | ± | 0.56 | bB | 3.76 | ± | 0.79 | bAB | 3.02 | ± | 0.20 | bB |
| | 12 | 1.61 | ± | 0.25 | bAB | 1.88 | ± | 0.33 | cA | 0.95 | ± | 0.81 | cB | 1.60 | ± | 0.71 | cAB | 1.51 | ± | 0.23 | cAB |
| Total phenolic content | 0 | 92.42 | ± | 18.68 | abA | 92.42 | ± | 18.68 | aA | 92.42 | ± | 18.68 | aA | 92.42 | ± | 18.68 | aA | 92.42 | ± | 18.68 | aA |
| (mg GAE/100g) | 6 | 97.73 | ± | 15.32 | abA | 95.71 | ± | 5.96 | aA | 81.59 | ± | 8.89 | aA | 98.87 | ± | 28.09 | aA | 87.26 | ± | 6.53 | aA |
| | 12 | 114.7 | ± | 28.61 | aA | 100.0 | ± | 9.24 | aAB | 96.54 | ± | 12.23 | aAB | 107.7 | ± | 9.87 | aA | 79.09 | ± | 8.01 | aB |
| DPPH | 0 | 3071 | ± | 100.7 | aA | 3071 | ± | 100.7 | aA | 3071 | ± | 100.7 | aA | 3071 | ± | 100.7 | aA | 3071 | ± | 100.7 | aA |
| (μM Trolox/g) | 6 | 3206 | ± | 200.4 | aA | 2901 | ± | 232.2 | abAB | 2697 | ± | 789.9 | aABC | 2023 | ± | 121.5 | bC | 2358 | ± | 647.6 | bBC |
| | 12 | 2854 | ± | 1062 | aA | 2527 | ± | 323.0 | bA | 2883 | ± | 507.6 | aA | 2764 | ± | 444.5 | aA | 2444 | ± | 339.1 | abA |
| Anthocyanins | 0 | 47.12 | ± | 9.04 | cA | 47.12 | ± | 9.04 | bA | 47.12 | ± | 9.04 | cA | 47.12 | ± | 9.04 | bA | 47.12 | ± | 9.04 | bA |
| (mg/ml) | 6 | 63.82 | ± | 8.95 | bA | 79.10 | ± | 13.45 | aA | 62.23 | ± | 6.50 | bA | 81.16 | ± | 17.07 | aA | 77.15 | ± | 11.98 | aA |
| | 12 | 70.05 | ± | 9.88 | bB | 85.89 | ± | 1.49 | aA | 76.54 | ± | 5.58 | aAB | 60.95 | ± | 13.23 | abB | 61.90 | ± | 5.05 | abB |

NAP indicates the presence of the nanocellulose film without any oil. Values in the same column followed by different lower-case letters and in the same row followed by different upper-case letters for each parameter are significantly different by Duncan's multiple range test ($P < 0.05$).



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TABLE 4 Soluble solid content (SSC), firmness, total phenolic content, DPPH, and anthocyanins of raspberries treated with thyme (T) and oregano (O) essential oils and their combination (TO) during storage at 1°C.

| | Days | Control | | | | NAP_T | | | | NAP_O | | | | NAP_TO | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | | SD | | Mean | | SD | | Mean | | SD | | Mean | | SD | |
| Lightness (L*) | 0 | 36.86 | ± | 0.50 | aA | 36.86 | ± | 0.50 | aA | 36.86 | ± | 0.50 | aA | 36.86 | ± | 0.50 | aA |
| | 6 | 36.57 | ± | 0.71 | aA | 35.31 | ± | 0.45 | bAB | 35.17 | ± | 0.91 | bB | 33.99 | ± | 1.01 | bB |
| | 12 | 35.84 | ± | 1.51 | aA | 35.09 | ± | 0.85 | bAB | 34.03 | ± | 0.88 | bB | 34.51 | ± | 0.45 | bB |
| Hue angle (h°) | 0 | 30.62 | ± | 0.28 | aA | 30.62 | ± | 0.28 | aA | 30.62 | ± | 0.28 | aA | 30.62 | ± | 0.28 | aA |
| | 6 | 30.37 | ± | 0.55 | aA | 29.44 | ± | 0.27 | bAB | 29.91 | ± | 1.10 | aA | 28.70 | ± | 0.73 | bB |
| | 12 | 29.49 | ± | 1.16 | aA | 28.94 | ± | 1.06 | bA | 28.49 | ± | 0.87 | bA | 28.62 | ± | 0.77 | bA |
| Chroma | 0 | 36.14 | ± | 2.01 | bA | 36.14 | ± | 2.01 | bA | 36.14 | ± | 2.01 | bA | 36.14 | ± | 2.01 | abA |
| | 6 | 38.83 | ± | 0.72 | aA | 38.06 | ± | 0.61 | aA | 38.11 | ± | 0.82 | aA | 37.48 | ± | 1.46 | aA |
| | 12 | 35.99 | ± | 1.64 | bA | 36.25 | ± | 1.27 | bA | 34.80 | ± | 1.88 | bA | 35.43 | ± | 0.56 | bA |
| SSC (°Brix) | 0 | 7.15 | ± | 0.59 | aA | 7.15 | ± | 0.59 | aA | 7.15 | ± | 0.59 | aA | 7.15 | ± | 0.59 | aA |
| | 6 | 6.95 | ± | 0.37 | aAB | 7.68 | ± | 0.29 | aA | 7.48 | ± | 0.60 | aAB | 6.78 | ± | 0.56 | aB |
| | 12 | 7.58 | ± | 0.13 | aA | 7.70 | ± | 0.62 | aA | 6.98 | ± | 0.33 | aA | 7.55 | ± | 0.72 | aA |
| Firmness (N) | 0 | 8.77 | ± | 0.33 | aA | 8.77 | ± | 0.33 | aA | 8.77 | ± | 0.33 | aA | 8.77 | ± | 0.33 | aA |
| | 6 | 7.07 | ± | 0.38 | bA | 5.49 | ± | 1.00 | bB | 5.80 | ± | 0.43 | bB | 4.64 | ± | 0.71 | bC |
| | 12 | 2.67 | ± | 1.05 | cA | 2.20 | ± | 0.49 | cAB | 2.06 | ± | 1.33 | cAB | 1.32 | ± | 0.38 | cB |
| Total phenolic content | 0 | 60.25 | ± | 7.41 | bA | 60.25 | ± | 7.41 | bA | 60.25 | ± | 7.41 | dA | 60.25 | ± | 7.41 | cA |
| (mg GAE/100g) | 6 | 59.32 | ± | 19.95 | bC | 99.88 | ± | 26.92 | aB | 107.77 | ± | 15.27 | bB | 130.76 | ± | 7.76 | aA |
| | 12 | 160.50 | ± | 69.97 | aA | 63.63 | ± | 10.43 | bB | 84.02 | ± | 2.64 | cB | 89.54 | ± | 9.36 | bB |
| DPPH | 0 | 4744 | ± | 36 | abA | 4744 | ± | 36 | bA | 4744 | ± | 36 | bA | 4744 | ± | 36 | aA |
| (μM Trolox/g) | 6 | 4347 | ± | 859 | bA | 5039 | ± | 648 | bA | 5065 | ± | 390 | bA | 4979 | ± | 955 | aA |
| | 12 | 4709 | ± | 208 | abA | 4332 | ± | 44 | cA | 4558 | ± | 255 | bA | 4206 | ± | 729 | aA |
| Anthocyanins | 0 | 29.08 | ± | 2.22 | bA | 29.08 | ± | 2.22 | cA | 29.08 | ± | 2.22 | dA | 29.08 | ± | 2.22 | cA |
| (mg/ml) | 6 | 26.25 | ± | 6.20 | bC | 37.80 | ± | 10.13 | bB | 51.27 | ± | 2.52 | bA | 49.62 | ± | 1.80 | aA |
| | 12 | 44.03 | ± | 17.86 | aA | 36.82 | ± | 3.03 | bcA | 34.48 | ± | 6.60 | cA | 35.90 | ± | 2.73 | bA |

Values in the same column followed by different lower-case letters and in the same row followed by different upper-case letters for each parameter are significantly different by Duncan's multiple range test ($P < 0.05$).



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wall associated with fruit ripening (Duan et al., 2011). The present results are similar to the ones obtained by Cefola et al. (2022), who studied the application of an antifungal active package to the raspberries based on green tea and rosemary ethanolic extracts, and found that essential oils tended to affect this parameter negatively (Cefola et al., 2022).

The phenolic content was also monitored since it indicates the fruit's degradation in the post-harvest period. The total phenolic content in raspberries did not show significant differences in test 1 for all the treatments. At the end of the experiment, the control and NAP_T had the highest values, with 114.67 and 107.72 mg of gallic acid equivalent/100g of fresh weight, respectively. In contrast, NAP_O decreased to 79.09 mg of gallic acid equivalent/100g of fresh weight (Table 3). In test 2 (Table 4), the trend was different. The control increased the total phenolic content from 60.25 to the maximum value of 160.50 mg of gallic acid equivalent/100g of fresh weight at 12 days. All the other treatments had similar trends, with an increase in 6 days, followed by a decrease at the end of the experiment; the differences, however were minimal and not statistically relevant. The fact that the concentration of phenols did not change significantly in the experiments could be associated with the absence of reactive oxygen species, leading to oxidative reactions (Gomes et al., 2017). The same trend was observed by Ishkeh et al. (2019) when applying lemon verbena essential oil to raspberries. They attributed it to high essential oil concentration, leading to faster breakdown of cellular structure. In fact, in test 2, with higher essential oil concentrations, this decrease was more evident than in test 1, where the essential oil quantities were distinctly lower.

The measurement of the antioxidant activity by the DPPH method showed a decrease after 6 days for NAP_T and NAP_O in test 1, with 2020 μM Trolox/g and 2088 μM Trolox/g, respectively. At the end of the test, no significant differences were noticed in the values of the various treatments, with the lowest value of 2397 μM Trolox/g from NAP_O, compared to the control, which had 3025 μM Trolox/g. Similarly, in test 2, no significant differences were found among the treatments during the storage of the fruits. In general, the values were higher than in test 1. The application of the essential oil helped maintain the levels of antioxidants but did not improve their effectiveness over time. This could mean that the antioxidant capacity assayed may be due to high amounts of antioxidants already in the fruits. For instance, Jin et al. (2012) found higher levels of antioxidants, such as anthocyanins, flavonoids, and phenolic acids, in raspberries.

The anthocyanins quantity measured in test 1 started to be significantly different only at 12 days, with NAP having the highest value of 86 mg/ml, compared to the control (70 mg/ml). For all treatments, the anthocyanins content increased over time, maybe due to the biosynthesis of phenolic compounds after harvest related to the ripening of the fruits (Gomes et al., 2017). Instead, in test 2, their values increased significantly in the first 6 days and then decreased again at 12 days (Table 4). In this case, the highest value was observed in the control (44 mg/ml) and the lowest in NAP_O (34 mg/ml), but without statistical difference. These results are in line with those by Guerreiro et al. (2015) who analyzed raspberries coated with pectin and alginate-based edible coating enriched with essential oil components citral and eugenol.

### 3.2.2. Microbial growth

The analysis of microbial growth in real setting application is fundamental to establishing the activated films' antimicrobial activity. In fact, they have to preserve the physical and chemical quality of the fruits and ensure effective protection against food pathogens. In this study, the prevention of post-harvest decay was carried out by the vapors from essential oils released by the nanocellulose films. Other studies have confirmed the effectiveness of this application against fungi growth (Mziouid et al., 2018).

Figure 8 shows the microbial analysis results for fungi (a, c) and mesophilic bacteria (b, d) for tests 1 and 2, respectively. The results from the psychrophilic bacteria have not been presented since they consistently showed no colonies in both tests and all the treatments.

In test 1, the control treatment showed fungi growth of about 2 log (CFU)/g of fresh weight in the first 6 days, ending in the highest value of 5.8 at 12 days. In contrast, in other treatments, it increased in the first 6 days and then decreased. NAP_O showed the lowest value of 2.6 log (CFU)/g of fresh weight, followed by NAP_T and NAP_C (3.4 and 4.0, respectively). Also, in test 2, all the treatments showed better results than the control at 12 days of storage, even if without statistical differences among them. They consistently decreased over time, with NAP_T and NAP_TO reaching a log (CFU)/g of 0 at the end of the experiment.

Regarding mesophilic bacteria, in the first six days, the log (CFU)/g was maintained at 0 for all the treatments in test 1. At 12 days, NAP_C had the highest growth, reaching 2.6 log (CFU)/g of fresh weight. On the other hand, NAP_O and NAP_T showed better results of 1.4 and 1.9, respectively, which were, in any case, higher than the control with 2.5 log (CFU)/g of fresh weight but higher than the initial value of 0.6 log (CFU)/g of fresh weight. In test 2, there was a slight decrease in the first 6 days, followed by an increase in the remaining duration of the test. Nevertheless, this increase reached a log (CFU)/g value, which was still lower than the one reached at the end of the first test. The best results were shown by NAP_O and NAP_TO, with 0.6 and 0.8 log (CFU)/g of fresh weight, respectively.

The different trends in the two experiments were probably due to the different quantities of oils present in the matrix. With more oils in test 2, it was possible to preserve the antimicrobial activity longer and with a stronger effect on the reduction of the colonies since the beginning of the application. In any case, the microbial evaluation never exceeded the accepted limit of 6 log (CFU)/g, according to Bierhals et al. (2011).

### 3.2.3. Sensory evaluation

Antimicrobial properties of essential oils help preserve the quality parameters and extend the shelf-life of packed food, but they could alter its sensory characteristics. For this reason, a sensory evaluation was performed to test the consumer perception and acceptance of the proposed active packaging technology.

Figure 9 shows the results of the sensory evaluation performed with the panelists. The test was performed after 12 days of storage for test 2 (Figure 9) to see the differences in consumer perception of treated fruits. Results showed that, after 12 days of storage, raspberries in active packaging had a better appearance than controls.

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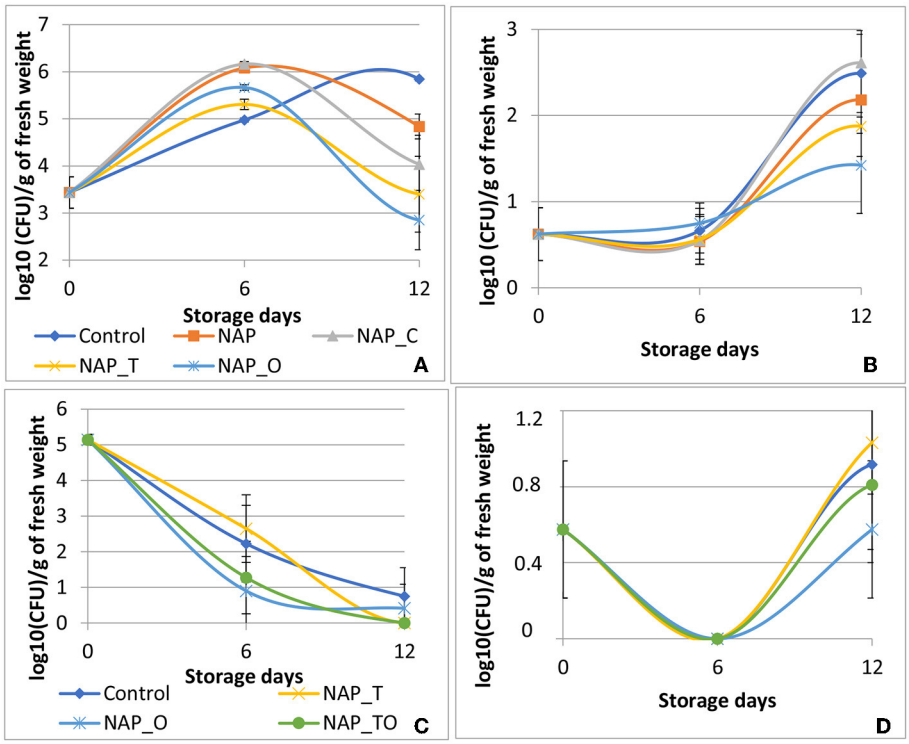


FIGURE 8
Microbial analysis of **(A, C)** fungi and **(B, D)** mesophilic bacteria on raspberries after 12 days of storage at 0.5°C, for test 1 and 2, respectively. The colors represent respectively: the control (blue), the pure nanocellulose without essential oils (orange), the nanocellulose with cinnamon essential oil (grey), the nanocellulose with thyme essential oil (yellow), the nanocellulose with oregano essential oil (light blue), and the nanocellulose with thyme+oregno essential oils (green).

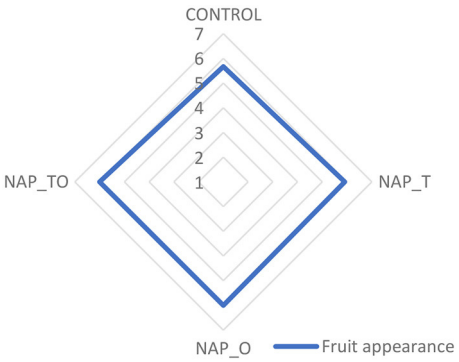


FIGURE 9
Sensory evaluation (fruit appearance) of fruit from test 2, after 12 days storage at 0.5°C, based on a 7-point hedonic scale: 1 – dislike very much; 2 – dislike; 3 – dislike slightly; 4 – either like or dislike; 5 – like slightly; 6 – like; 7 – like very much. Treatments are control, nanocellulose with oregano essential oil (NAP_O), nanocellulose with thyme essential oil (NAP_T), and nanocellulose with thyme+oregano essential oils (NAP_TO).

## 4. Conclusion

The present study focused on the antimicrobial activity of essential oils incorporated into nanocellulose matrix, given their use as an active packaging solution for the shelf-life extension of foods. Thyme, cinnamon, and oregano essential oils were studied *in vitro* to test their antimicrobial activity and in real food application to estimate their effect on the shelf-life elongation of raspberries. The first set of laboratory tests allowed us to calculate the MIC for each oil for *S. aureus* and *E. coli* bacterial strains. The thyme essential oil had a lower MIC with a value of 0.37 mg of oil/mg of matrix, reaching 100% antimicrobial effect against Gram-positive *S. aureus* and Gram-negative *E. coli*. In contrast, the oregano reached this antimicrobial effect at 0.62 mg/mg.

When using the MIC quantity directly on raspberry packaging, the oils successfully maintained the most general quality parameters of the fruits and reduced microbial growth, mainly NAP_T and NAP_O in test 1. Their effect on microbial growth was more evident after 12 days of storage, especially for fungi. When increasing the oil concentrations and applying the two best oils (thyme and oregano) and their combination, the positive effect was enhanced, with a positive impact on the sensorial appreciation.

From the results of this experiment, it is possible to conclude that active packaging, including nanocellulose films enriched with thyme and oregano essential oils with a concentration of 1.1 and 1.8 ml of oil/liter of air in the headspace of the package, respectively, is a promising technology to improve shelf-life of raspberries. Also, the synergistic effect of both essential oils combined shows an additional benefit in preserving main raspberry quality characteristics against fungi development and weight loss. Further study on a different fruit or different type of packaging could be helpful to confirm this hypothesis.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

MA, SC, MB, and MC: conceptualized the study and designed the experiments. SC and SN: performed the experiments. AG and CG: assisted in laboratory analysis. MA, SC, MC, SN, and MB: assisted results analysis and interpretation. MA, MB, and MC: assisted in the revision of the research. SC: wrote the article with the contribution of all authors. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1190979/full#supplementary-material

## References

Ahankari, S. S., Subhedar, A. R., Bhadauria, S. S., and Dufresne, A. (2021). Nanocellulose in food packaging: a review. *Carbohydrate Polymers* 255, 117479. doi: 10.1016/j.carbpol.2020.117479

Asgher, M., Qamar, S. A., Bilal, M., and Iqbal, H. M. N. (2020). Bio-based active food packaging materials: Sustainable alternative to conventional petrochemical-based packaging materials. *Food Res. Int.*, 137, 109625. doi: 10.1016/j.foodres.2020.109625

Atarés, L., and Chiralt, A. (2016). Essential oils as additives in biodegradable films and coatings for active food packaging. *Trends Food Sci. Technol.* 48, 51–62. doi: 10.1016/j.tifs.2015.12.001

Azeredo, H. M. C., Rosa, M. F., and Mattoso, L. H. C. (2017). Nanocellulose in bio-based food packaging applications. *Ind. Crops Prod.* 97, 664–671. doi: 10.1016/j.indcrop.2016.03.013

Bierhals, V. S., Chiumarelli, M., and Hubinger, M. D. (2011). Effect of cassava starch coating on quality and shelf life of fresh-cut pineapple (Ananas Comosus L. Merril cv "Pérola"). *J. Food Sci.* 76, E62–E72. doi: 10.1111/j.1750-3841.2010.01951.x

Bouhdid, S., Abrini, J., Zhiri, A., Espuny, M. J., and Manresa, A. (2009). Investigation of functional and morphological changes in Pseudomonas aeruginosa and Staphylococcus aureus cells induced by Origanum compactum essential oil. *J. Appl. Microbiol.* 106, 1558–1568. doi: 10.1111/j.1365-2672.2008.04124.x

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods - A review. *Int. J. Food Microbiol.*, 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Casalini, S., and Baschetti, M. G. (2022). The use of essential oils in chitosan or cellulose-based materials for the production of active food packaging solutions: a review. *J. Sci. Food Agriculture*. 5, 918. doi: 10.1002/jsfa.11918

Casalini, S., Baschetti, M. G., and Montanari, F. (2022). Diffusion of thyme, cinnamon and oregano essential oils in different nanocellulose matrices. *Carbohydrate Polymer Technol. Applications*. 5, 271. doi: 10.1016/j.carpta.2022.100271

Cefola, M., Pace, B., Vieira, T. M., Alves, V. D., and Martins, M. M. (2022). Application of an eco-friendly antifungal active package to extend the shelf life of fresh red raspberry (rubus idaeus l. cv. andlsquo;kweliandrsquo). *Foods*, 11, 1805. doi: 10.3390/foods11121805

di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., Mauriello, G., et al. (2007). Membrane toxicity of antimicrobial compounds from essential oils. *J. Agric. Food Chemistry*, 55, 4863–4870. doi: 10.1021/jf0636465

Duan, J., Wu, R., Strik, B. C., and Zhao, Y. (2011). Effect of edible coatings on the quality of fresh blueberries (Duke and Elliott) under commercial storage conditions. *Postharvest Biol. Technol.*, 59, 71–79. doi: 10.1016/j.postharvbio.2010.08.006

Duncan, D. B. (1955). Multiple range and multiple f tests. *Biometrics*, 11, 1. doi: 10.2307/3001478

Falleh, H. ben Jemaa, M., Saada, M., and Ksouri, R. (2020). Essential oils: a promising eco-friendly food preservative. *Food Chemistry* 330, 127268. doi: 10.1016/j.foodchem.2020.127268

Fan, Lt., and Singh, S. K. (1989). "Diffusion-controlled release," in *Controlled Release. Polymers*, Vol 13 (Berlin; Heidelberg: Springer). doi: 10.1007/978-3-642-74507-2_2

FAO (2021). *Food and Agriculture: Raspberry Production*. Available online at: https://www.fao.org/faostat/en/#data/QI (accessed May 6, 2023)

Gabrieli De Souza, A., Ferreira, R. R., Silva, E., Aguilar, F., Zanata, L., Dos, D., et al. (2021). Cinnamon essential oil nanocellulose-based pickering emulsions: processing parameters effect on their formation, stabilization, and antimicrobial activity. *Polysaccharides* 2, 608–25, 2.(3), 608–625. doi: 10.3390/polysaccharides2030037

Gomes, M. S., Cardos das G., Guimarães, A. C. G., Guerreiro, A. C., Gago, C. M. L., Vilas Boas, E. V. (2017). Effect of edible coatings with essential oils on the quality of red raspberries over shelf-life. *J. Sci. Food Agriculture*, 97, 929–938. doi: 10.1002/jsfa.7817

Guerreiro, A. C., Gago, C. M. L., Faleiro, M. L., Miguel, M. G. C., and Antunes, M. D. C. (2015). Raspberry fresh fruit quality as affected by pectin- and alginate-based edible coatings enriched with essential oils. *Scientia Hortic.* 194, 138–146. doi: 10.1016/j.scienta.2015.08.004

Guerreiro, A. C., Gago, C. M. L., Miguel, M. G. C., Faleiro, M. L., and Antunes, M. D. C. (2016). The influence of edible coatings enriched with citral and eugenol on the raspberry storage ability, nutritional and sensory quality. *Food Packaging Shelf Life*, 9, 20–28. doi: 10.1016/j.fpsl.2016.05.004

Han, Y., Yu, M., and Wang, L. (2018). Physical and antimicrobial properties of sodium alginate/carboxymethyl cellulose films incorporated with cinnamon essential oil. *Food Packaging Shelf Life*, 15, 35–42. doi: 10.1016/j.fpsl.2017.11.001

Hossain, F., Follett, P., Salmieri, S., Vu, K. D., Fraschini, C., Lacroix, M., et al. (2019). Antifungal activities of combined treatments of irradiation and essential oils (EOs) encapsulated chitosan nanocomposite films in *in vitro* and *in situ* conditions. *Int. J. Food Microbiol.* 295, 33–40. doi: 10.1016/j.ijfoodmicro.2019.02.009

Hu, J., Wang, X., Xiao, Z., and Bi, W. (2015). Effect of chitosan nanoparticles loaded with cinnamon essential oil on the quality of chilled pork. *LWT - Food Sci. Technol.* 63, 519–526. doi: 10.1016/j.lwt.2015.03.049

Inouye, S., Uchida, K., Maruyama, N., Yamaguchi, H., and Abe, S. (2006). A novel method to estimate the contribution of the vapor activity of essential oils in agar diffusion assay. *Nippon Ishinkin Gakkai Zasshi*, 47, 91–98. doi: 10.3314/jjmm.47.91

Ishkeh, S. R., Asghari, M., Shirzad, H., Alirezalu, A., and Ghasemi, G. (2019). Lemon verbena (Lippia citrodora) essential oil effects on antioxidant capacity and phytochemical content of raspberry (Rubus ulmifolius subsp. sanctus). *Scientia Hortic.* 248, 297–304. doi: 10.1016/j.scienta.2018.12.040

Jin, P., Wang, S. Y., Gao, H., Chen, H., Zheng, Y., Wang, C. Y., et al. (2012). Effect of cultural system and essential oil treatment on antioxidant capacity in raspberries. *Food Chem.* 132, 399–405. doi: 10.1016/j.foodchem.2011.11.011

Khan, A., Huq, T., Khan, R. A., Riedl, B., and Lacroix, M. (2014). Nanocellulose-based composites and bioactive agents for food packaging. *Critical Rev. Food Sci. Nutr.* 54, 163–174. doi: 10.1080/10408398.2011.578765

Lee, J., Durst, R. W., and Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *J. AOAC Int.* 88, 1269–1278. doi: 10.1093/jaoac/88.5.1269

Licciardello, F., Muratore, G., Mercea, P., Tosa, V., and Nerin, C. (2013). Diffusional behaviour of essential oil components in active packaging polypropylene films by multiple headspace solid phase microextraction–gas chromatography. *Packaging Technol. Sci.* 26, 173–185. doi: 10.1002/pts.1969

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A. I., Gutiérrez-Praena, D., et al. (2015). *In vitro* toxicological evaluation of essential oils and their main compounds used in active food packaging: a review. *Food Chem. Toxicol.* 81, 9–27. doi: 10.1016/j.fct.2015.03.030

López, P., Sánchez, C., Batlle, R., and Nerín, C. (2005). Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. *J. Agric. Food Chem.* 53, 6939–6946. doi: 10.1021/jf050709v

McGuire, R. G. (1992). Reporting of objective color measurements. *HortScience* 4, 1254-1255. doi: 10.21273/HORTSCI.27.12.1254

Mejía-Garibay, B., Palou, E., and López-Malo, A. (2015). Composition, diffusion, and antifungal activity of black mustard (brassica nigra) essential oil when applied by direct addition or vapor phase contact. *J. Food Protection* 78, 843–848. doi: 10.4315/0362-028X.JFP-14-485

Miguel, M. G., Gago, C., Antunes, M. D., Lagoas, S., Faleiro, M. L., Megías, C., et al. (2018). Antibacterial, antioxidant, and antiproliferative activities of Corymbia citriodora and the Essential oils of eight eucalyptus species. *Medicines.* 5, 1–12. doi: 10.3390/medicines5030061

Mohan, S., and Panneerselvam, K. (2022). Development of polylactic acid based functional films reinforced with ginger essential oil and curcumin for food packaging applications. *J. Food Measurement Character.* 16, 4703–4715. doi: 10.1007/s11694-022-01551-7

Montero, Y., Souza, A. G., Oliveira, É. R., and Rosa, D. (2021). Nanocellulose functionalized with cinnamon essential oil: a potential application in active biodegradable packaging for strawberry. *Sust. Mat. Technol.* 29, e00289. doi: 10.1016/j.susmat.2021.e00289

Moreno, M. A., Vallejo, A. M., Ballester, A. R., Zampini, C., Isla, M. I., López-Rubio, A., et al. (2020). Antifungal edible coatings containing Argentinian propolis extract and their application in raspberries. *Food Hydrocolloids* 107, 105973. doi: 10.1016/j.foodhyd.2020.105973

Mziouid, A., Senhaji, B., Heimeur, N., Chebli, H., Bounimi, S., Mayad, E., et al. (2018). Evaluation of the antifungal activity of five aromatic plants essential oils against Botrytis cinerea and their efficiency for keeping quality of fresh raspberries and strawberries. *Appl. J. Environ. Eng. Sci.* 4, 467–472. doi: 10.48422/IMIST.PRSM/ajees-v4i4.14278

Nunes, M. C. (2015). Correlations between subjective quality and physicochemical attributes of fresh fruits and vegetables. *Postharvest Biol. Technol.* 107, 43–54. doi: 10.1016/j.postharvbio.2015.05.001

Pandey, V. K., Islam, R. U., Shams, R., and Dar, A. H. (2022). A comprehensive review on the application of essential oils as bioactive compounds in Nano-emulsion based edible coatings of fruits and vegetables. *Appl. Food Res.* 2, 100042. doi: 10.1016/j.afres.2022.100042

Sharma, S., Barkauskaite, S., Jaiswal, A. K., and Jaiswal, S. (2021). Essential oils as additives in active food packaging. *Food Chemistry* 343, 128403. doi: 10.1016/j.foodchem.2020.128403

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144. doi: 10.5344/ajev.1965.16.3.144

Souza, A. G., Ferreira, R. R., Paula, L. C., Mitra, S. K., and Rosa, D. S. (2021). Starch-based films enriched with nanocellulose-stabilized Pickering emulsions containing different essential oils for possible applications in food packaging. *Food Packaging Shelf Life*, 27, 100615. doi: 10.1016/j.fpsl.2020.100615

Sthle, L., and Wold, S. (1989). Analysis of variance (ANOVA). *Chemometr. Int. Lab. Syst.* 6, 259–272. doi: 10.1016/0169-7439(89)80095-4

Subbuvel, M., and Kavan, P. (2022). Preparation and characterization of polylactic acid/fenugreek essential oil/curcumin composite films for food packaging applications. *Int. J. Biol. Macromol.* 194, 470–483. doi: 10.1016/j.ijbiomac.2021.11.090

Trache, D., Tarchoun, A. F., Derradji, M., Hamidon, T. S., Masruchin, N., Brosse, N., et al. (2020). Nanocellulose: from fundamentals to advanced applications. *Front. Chem.* 8, 392. doi: 10.3389/fchem.2020.00392

Wang, W., Zhang, Y., Yang, Z., and He, Q. (2021). Effects of incorporation with clove (Eugenia caryophyllata) essential oil (CEO) on overall performance of chitosan as active coating. *Int. J. Biol. Macromol.* 166, 578–586. doi: 10.1016/j.ijbiomac.2020.10.215

Wu, Y. M., Wang, Z. W., Hu, C. Y., and Nerín, C. (2017). Influence of factors on release of antimicrobials from antimicrobial packaging. *Materials* 58, 1108–1121. doi: 10.1080/10408398.2016.1241215

Wyrwa, J., and Barska, A. (2017). Innovations in the food packaging market: active packaging. *Eur. Food Res. Technol.* 243, 1681–1692. doi: 10.1007/s00217-017-2878-2

Yemiş, G. P., and Candogan, K. (2017). Antibacterial activity of soy edible coatings incorporated with thyme and oregano essential oils on beef against pathogenic bacteria. *Food Sci. Biotechnol.* 26, 1113–1121. doi: 10.1007/s10068-017-0136-9

Yousuf, B., Wu, S., and Siddiqui, M. W. (2021). Incorporating essential oils or compounds derived thereof into edible coatings: effect on quality and shelf life of fresh/fresh-cut produce. *Trends Food Sci. Technol.* 108, 245–257. doi: 10.1016/j.tifs.2021.01.016