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Impact of Oregano and Rosemary Oleoresins on Native Microflora and *E. coli* O157:H7 Growth on Sliced and Grated Carrots

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ABSTRACT

The microbiological safety of fresh produce has been a cause for concern among consumers and within the food industry. Considering the demand for current information, the biocontrol exerted on *E. coli* by the native microflora on carrots was studied. Oregano and rosemary oleoresins were applied and *E. coli* O157:H7 was inoculated at a medium level (4–5 $\log_{10}$ CFU/g) on sliced and grated carrots. Samples were stored at 20 and 10°C for 96 h to monitor changes in native microflora and pathogen growth. Biocontrol exerted on *E. coli* O157:H7 by the native microflora was not significant at storage temperatures of 20 and 10°C in both types of processed carrot. The use of oregano and rosemary at higher concentration exerted an inhibitory effect on the native microflora of sliced carrot and on *E. coli* O157:H7 growth in sliced and grated carrots. However, sensory analysis revealed that acceptance was inversely related to the concentration of the oleoresins used. Inhibition of the pathogen was more evident at 20 than at 10°C in both types of processed carrot. The bacteriostatic effect of oleoresins on the pathogen could be enhanced by synergistic effects together with other preservation technologies.

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INTRODUCTION

Minimally processed vegetables are experiencing increasing popularity, mainly due to their convenience, freshness and associated health benefits. Sliced and grated carrots have predominated in the fresh-cut vegetable market because of their pleasant flavor and nutritional benefits as well as versatility of use *(1)*.

Shredded or grated carrots usually have a short shelf life, about 3 to 4 days. The enhanced physiological/biochemical responses caused by the surface area increase created by shredding or slicing lead to increased microbial and non-microbial spoilage during storage, resulting in a negative impact on sensory quality *(1, 9)*.

In general, microbial counts on minimally processed vegetables after processing range from 4 to 6 $\log_{10}$ CFU/g representing predominantly Gram negative bacteria *(16, 17)*. Preservation treatments applied during minimal processing play a fundamental role in the stability, safety and overall quality of minimally processed vegetables. The use of bio-

preservatives *(4, 7)* for controlling or eliminating pathogenic bacteria while maintaining overall product quality *(7)* represents an interesting approach to improving the microbial safety of fresh produce. A wide range of natural products from plants, animals or microorganisms can be used to extend food shelf life. The antimicrobial properties of some types of bacteria are of interest to researchers involved in finding new biocontrol strategies to be used in foods. The native microorganisms present on the surface of fresh produce are assumed to play an important role in maintaining the safety of ready-to-eat vegetables *(12)* by out-competing pathogens for physical space and nutrients and/or by producing antagonistic compounds that negatively affect the viability of pathogens *(10, 13)*.

Another alternative for biopreservation is the use of plant extracts. Spice oleoresins constitute the true essence of spices in their most concentrated form, containing volatile as well as non-volatile components and differing from essential spice oils in that they have all the flavoring ingredients of a particular spice. In addition, they are the most convenient substitutes for raw spices in the food processing industry, since they are free of bacteria and may be standardized to a desired flavor strength *(15)*. It is well known that the antimicrobial potency of essential oils and oleoresins is generally lower in food systems than *in vitro*, depending on the food composition, processing steps and storage temperature, all of which could strongly influence the effectiveness of these antimicrobial agents *(4)*. Accordingly, larger amounts of oleoresins are required in food systems, which could seriously interfere with the food's final sensory properties. Among several oleoresins that may be useful as antimicrobial agents, oregano and rosemary may have the greatest potential for use in industrial applications *(5)*.

The incidence of foodborne infections caused by bacterial pathogens, especially *Escherichia coli* O157:H7, continues to be a problem in the industry. This pathogen attaches preferentially to cut edges of vegetables and penetrates into the plant tissue, where it remains unaffected by various sanitizing treatments *(19)*. These facts have led investigators to search for novel methods of controlling *E. coli* O157:H7 contamination in vegetables *(6, 18)*. Raw materials, such as carrots, have been subjected to processing operations that commonly increase the risk of contamination by this pathogen *(11)*. In this context, the aims of the present work were to evaluate the effectiveness of the endogenous microflora of sliced and grated carrots in controlling *E. coli* O157:H7 and to determine the effectiveness of oregano and rosemary oleoresins to control pathogen growth. In addition, the ability of the oleoresins to control growth of the native microflora were evaluated at two storage temperatures, 10 and 20°C.

MATERIALS AND METHODS

Sample preparation

Carrots (*Daucus carota L.*) were grown and harvested at optimal maturity in Sierra de los Padres, Mar del Plata, Argentina, and were immediately transported to the laboratory. Carrots of about 140–180 mm in length and 40 mm in diameter (upper end) were precleaned with tap water, knife-peeled, topped and then grated or sliced by use of a multi-purpose belt cutting machine manufactured by Braun (Kronberg, Germany). Finally, carrots were washed, centrifuged and aseptically transferred to packages of PET materials (10 × 10 cm).

Culture maintenance and inoculum preparation

E. coli O157:H7, ATCC 25158 provided by CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, La Plata, Argentina) was used. In previous work, Moreira et al. *(11)* reported no significant differences in sensitivity to essential oils among different *E. coli* strains (*E. coli* O157:H7, ATCC 25158, ATCC 32922 and CI and CII, isolated from foods). For this reason, only one strain of *E. coli* O157:H7 (ATCC 25158) was used in the present work.

A stock culture was maintained in tryptic soy broth (Britania, Buenos Aires, Argentina) at 4°C. Before use, *E. coli* O157:H7 was cultured in brain heart infusion broth (BHI, Britania, Buenos Aires, Argentina) for 24 h at 37°C. The culture was transferred to 9.0 ml of BHI at two consecutive 24-h intervals immediately before each experiment.

Effectiveness of endogenous microflora on *E. coli* O157:H7 control

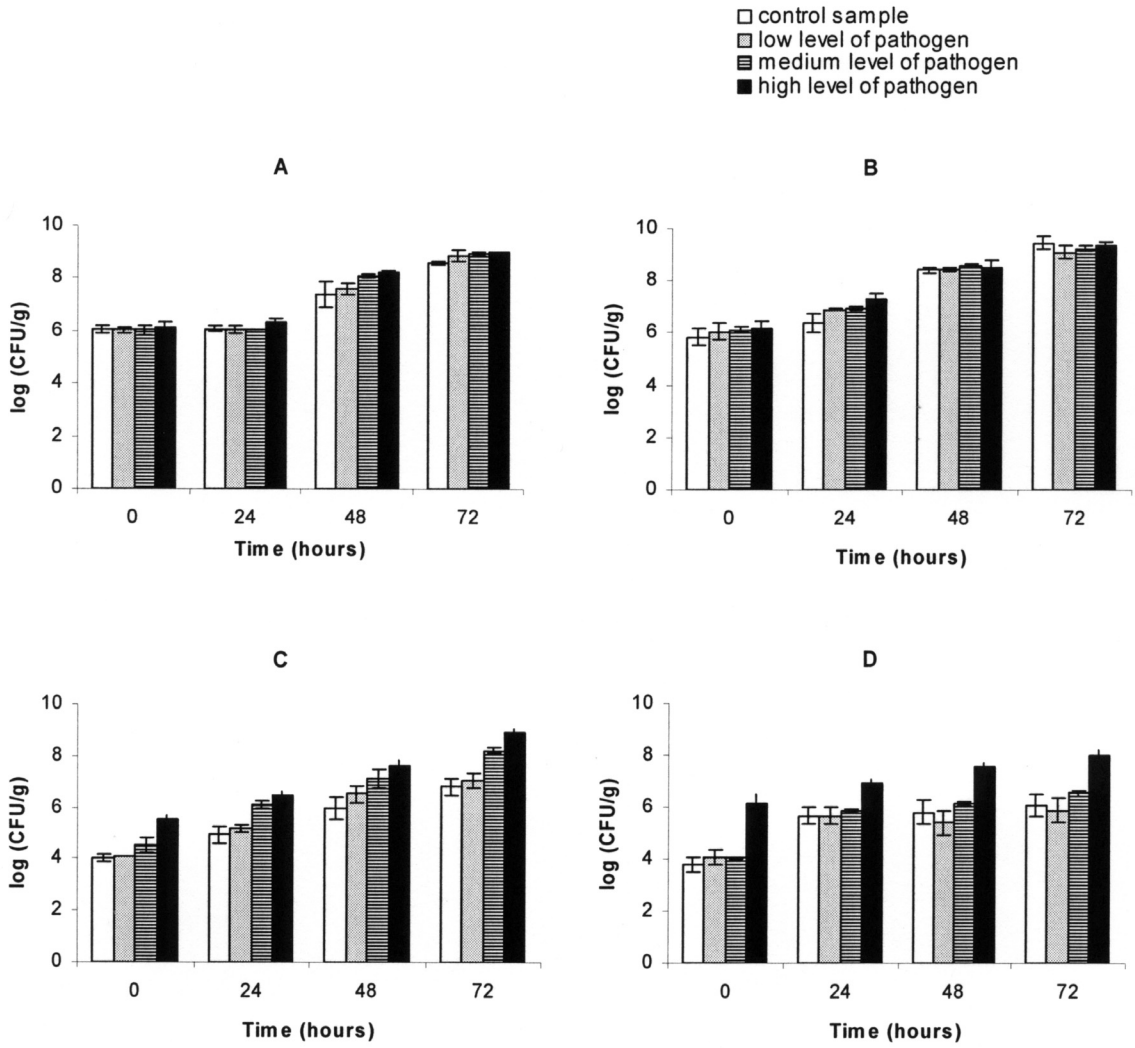
Grated or sliced carrots (100 g) were inoculated with 100 µl of *E. coli* O157:H7 active culture in the form of several drops. Carrot samples were vigorously shaken to distribute the inoculum and immediately covered with film (Resinite AF 50). The pathogen was inoculated at three levels (low: 2–3 $\log_{10}$, medium: 4–5 $\log_{10}$ and high: 6–7 $\log_{10}$, approximately). To account for possible growth of native *E. coli* strains, a control sample without added *E. coli* O157:H7 was used. Carrot samples were stored for 72 h at saturated relative humidity and two temperatures: 10°C (representing inadequate refrigeration storage) or 20°C (representing an abusive, room temperature). Each assay was performed in duplicate in four independent experimental runs.

Effect of oregano and rosemary on *E. coli* O157:H7 inoculated in carrot samples

Rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*) oleoresins used in this work were provided by Pionherb, Buenos Aires, Argentina. Food grade oleoresins were obtained from fresh herbs by alcohol steam distillation.

One hundred grams of grated or sliced carrots were thoroughly mixed with oleoresins at two concentrations: 0.5 and 1% w/w. Samples with oleoresins were immediately inoculated with *E. coli* O157:H7 by placing 100 µl bacterial suspension on grated or sliced carrots in the form of several drops, reaching a pathogen concentration of approximately 10^4–10^5 CFU/g, representing a medium contamination level. Inoculated carrot samples were shaken vigorously to evenly distribute the inoculum and immediately covered with film (Resinite AF 50). To compare the effectiveness of oleoresins on *E. coli* O157:H7 growth, a sample without oleoresin was used as control. Carrot samples were stored for 96 h at 10°C or 20°C. Each assay was performed in duplicate in four independent experimental runs.

FIGURE 1. Aerobic microflora in sliced (A) and grated (B) carrots and *E. coli* in sliced (A) and grated (B) carrots stored at 20°C



Enumeration of aerobic (mesophilic) microflora and *E. coli* O157:H7

Survival and growth of *E. coli* O157:H7 and aerobic background microflora on carrot samples were evaluated at regular intervals during the storage period. Samplings for viable cells were carried out at 0, 24, 48, 72 and 96 h. For both sliced and grated carrots, with or without oleoresins, extension of the storage period was established taking into account the organoleptic attributes. To allow an adequate contact between the oleoresins and the microorganisms, samples reported as corresponding to time 0 had 5 min of contact *(14).* After homogenization in a stomacher (Led Techno, Stomacher Lab-Blender 400), dilution series were made and the appropriate dilutions were pour-plated on eosin methylene blue agar (EMB; Britania, Buenos Aires, Argentina), a selective medium that allows the characterization of typical *E. coli* colonies (in this study, only those greenish and with a metallic sheen). Aerobic microflora were determined by surface plating on plate count agar (PCA, Britania, Buenos Aires, Argentina) and counting colonies after incubation at 37°C for 24–48 h. At each assay time, controls without oleoresin were also tested.

Sensory evaluation

During sensory evaluation, carried out by nine laboratory members, changes in taste, color, firmness, fresh-like aroma and general acceptance were assessed. A 5-point hedonic scale was used, where 5 = very good, 3 = limit of marketability and 1 = poor) *(14).*

Statistical analysis

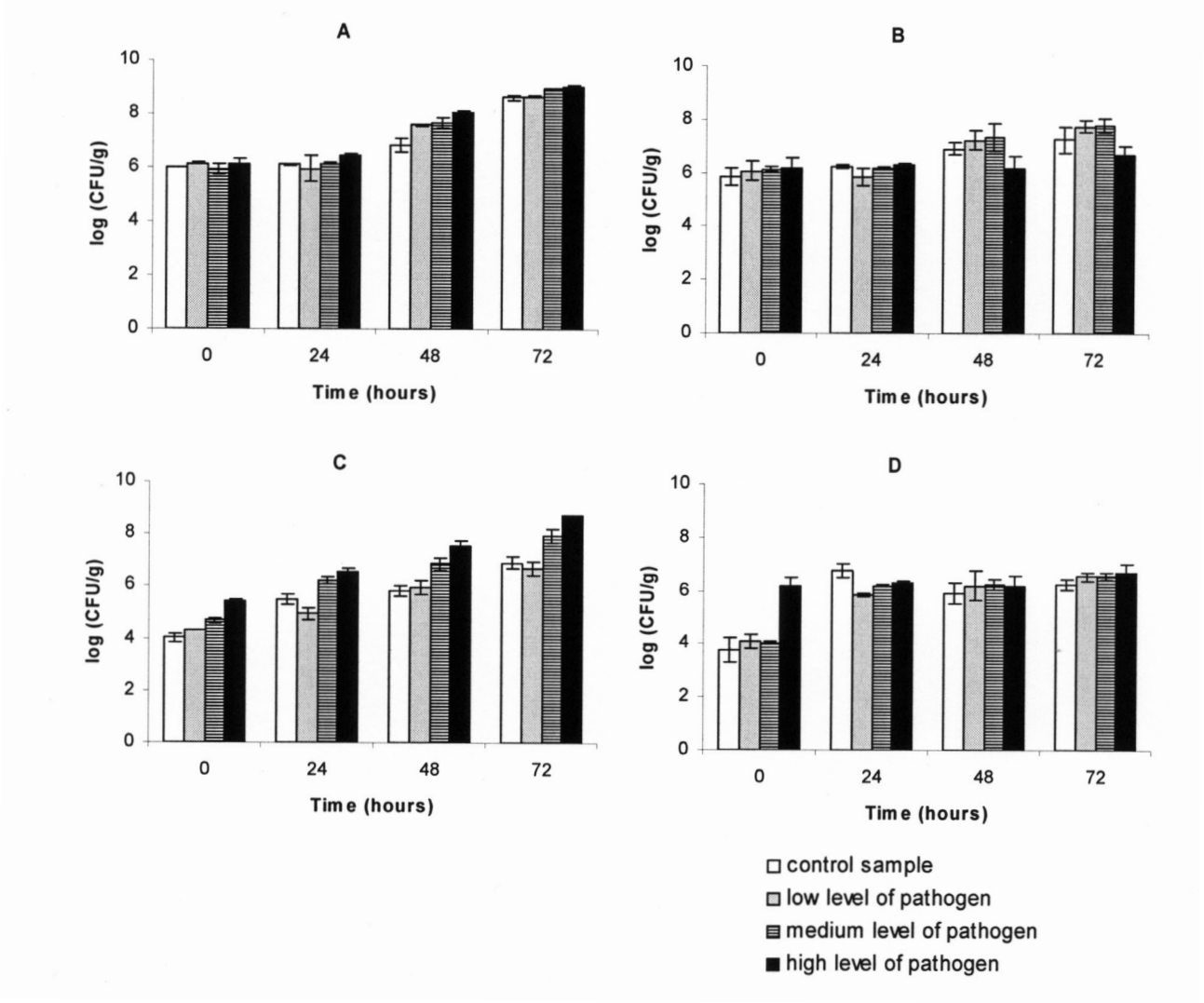
Each assay was performed in duplicate on three independent replicates. Differences among samples were tested by analysis of variance *(3).* When differences are reported as significant, a 99.9% confidence level was used.

RESULTS

Effectiveness of carrot native microflora in *E. coli* control

Figure 1 shows results obtained from grated and sliced carrots inoculated with three inoculum levels of *E. coli* O157:H7 and stored at 20°C. Initial counts of native microflora in the control and in the three inoculated sliced carrot samples were approximately 6.00 $\log_{10}$ CFU/g (Fig. 1A). Similar values were obtained for grated carrot (Fig.

FIGURE 2. Aerobic microflora in sliced (A) and grated (B) carrots and *E. coli* in sliced (A) and grated (B) carrots stored at 10°C



1B). At 72 h of storage, native microflora counts in sliced carrot samples were in the range of 8.50–9.00 $\log_{10}$ CFU/g. At the same time, bacteria counts in the control grated carrot (Fig. 1B) were approximately 1 log higher compared to control sliced carrot (Fig. 1A). In contrast, there were no significant differences in aerobic bacterial counts between sliced and grated carrot samples inoculated with three pathogen levels at 72 h of storage (Fig. 1A-B).

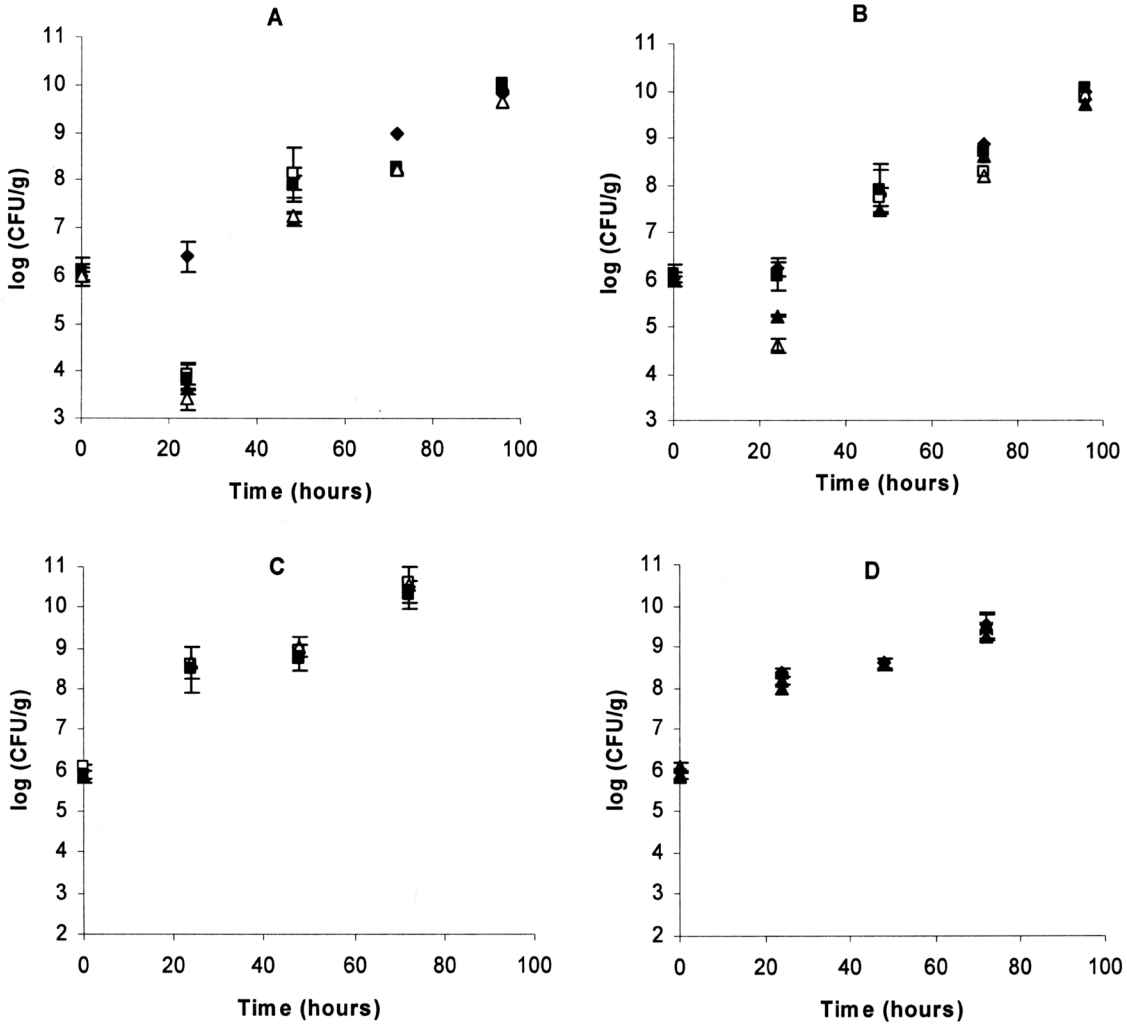
Initial *E. coli* counts in control and samples inoculated with low and medium levels of pathogens were similar in both types of processed carrot (Fig. 1C and D). At the end of storage, *E. coli* counts in all sliced carrot samples were in the range of 6.80–8.90 $\log_{10}$ CFU/g (Fig. 1C), whereas lower *E. coli* counts (6.00–8.00 $\log_{10}$ CFU/g) were observed in grated carrot samples (Fig. 1D).

Figure 2 shows the results obtained from sliced and grated carrots inoculated with three levels of *E. coli* O157:H7 and stored at 10°C. In both types of processed carrot samples, initial aerobic microflora counts were in the range of 5.80–6.20 $\log_{10}$ CFU/g. At 72 h of storage, aerobic counts in sliced carrot were 1.80–2.20 log higher than the counts in grated carrot (Fig. 2A and B). In agreement with what was observed at 20°C, no significant differences were found between initial *E. coli* counts corresponding to control samples and low and medium inoculated samples (Fig. 2C and D). At 72 h of storage, *E. coli* counts in sliced and grated carrot samples were in the range of 6.70–7.80 and 6.20–6.70 $\log_{10}$ CFU/g, respectively. The biocontrol exerted by native microflora on *E. coli* growth, in both types of processed carrot, was not significant. Storage temperature was not a factor, as similar results were observed in carrot samples stored at 20 and 10°C.

Inhibitory effects of oregano and rosemary oleoresins on native microflora and *E. coli* O157:H7 inoculated in carrot samples stored at 10 and 20°C

The effect of oleoresin application on *E. coli* in carrots was determined immediately after treatment and during storage. The microbial load of aerobic mesophilic microflora and E. coli present in sliced and grated carrots was determined. For the inoculated sample with the addition of oleoresins, we selected a medium level of pathogen inoculation, simulating an accidental contamination.

FIGURE 3. Aerobic microflora counts in sliced carrot at 20 (A) and 10°C (B) and in grated carrots at 20 (C) and 10°C (D). Samples were treated with oregano and rosemary at different concentrations: Control sample (♦); carrot plus oregano at 0.5% (□) and 1% (□); rosemary 0.5% (□) and 1% (Δ)



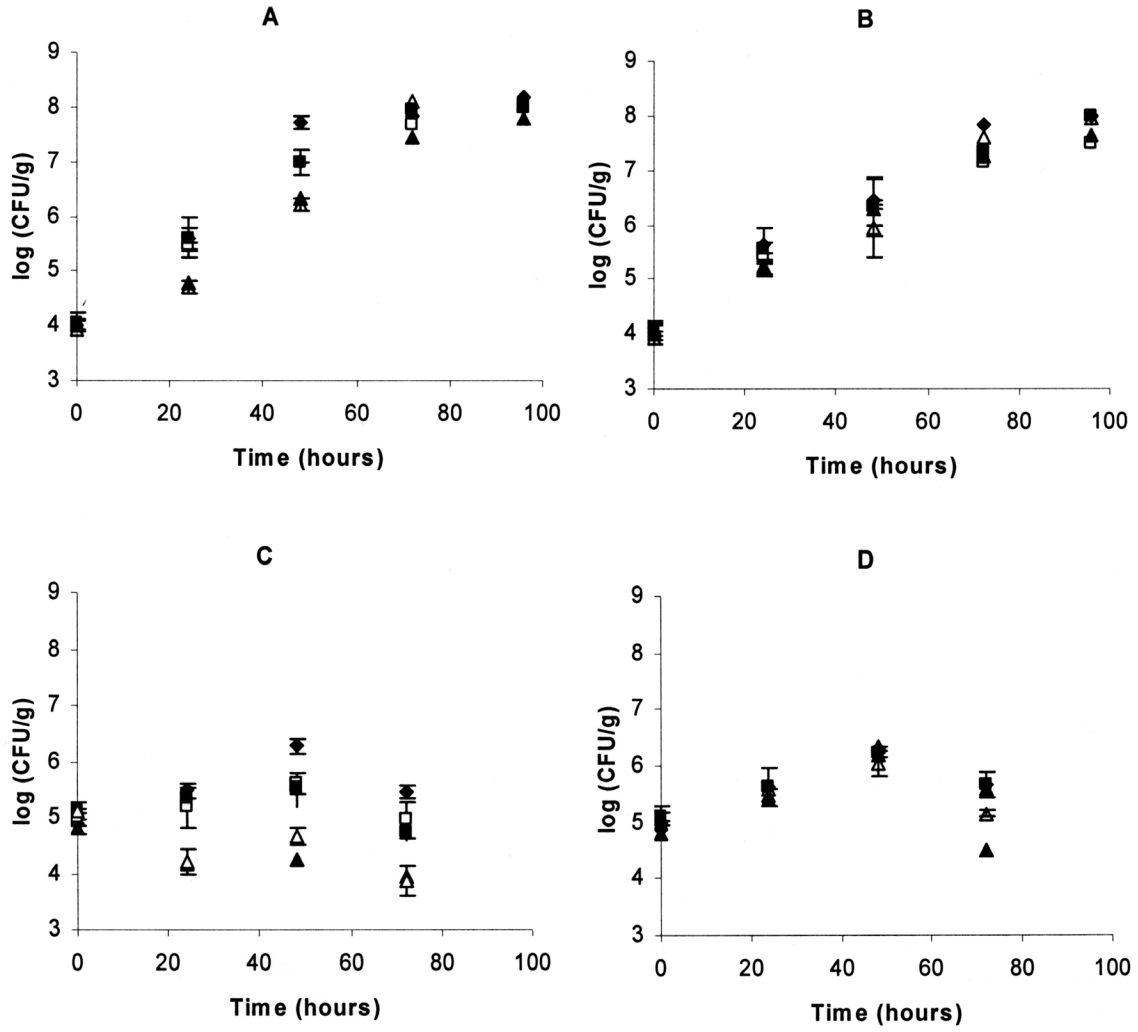
Effectiveness of oleoresins on native microflora of carrot samples

Effects of rosemary and oregano at 0.5 and 1% on aerobic microflora growth during storage of sliced and grated carrots are shown in Fig. 3. Figure 3A and B presents the changes of the native microflora in sliced carrot samples, during storage at 20 and 10°C, respectively. Initial aerobic bacterial counts were not significantly different in control and treated samples stored at either temperature. When the sliced carrot samples were stored at 20°C (Fig. 3A) oleoresins at 0.5 and 1% exerted a significant inhibitory effect on native microflora growth at 24 h of storage (2.5 to 3.0 log reductions). At 10°C, oleoresins exerted an inhibitory effect on native microflora of sliced carrot samples only at the higher concentration (1%) (1.0 to 1.6 log reductions) at 24 h of storage (Fig 3B). At the end of storage, these inhibitory effects were no longer observed (Fig 3A and B).

In grated carrot samples stored at both temperatures (Fig. 3C and D), no significant differences were recorded in the initial microbial counts (5.88–6.00 $\log_{10}$ CFU/g) between control samples and samples treated with different oleoresins. During storage, no significant differences were observed in total microflora counts between all samples, indicating that changes were similar for treated and non-treated samples. A linear increment was observed with total bacteria counts between 3.5 and 4.5 $\log_{10}$ CFU/g higher at the end of storage. In contrast to the results obtained with sliced carrots (Fig. 3A and B), oleoresins caused no observed inhibitory effect on native microflora of grated carrots.

Figure 4 presents *E. coli* counts in sliced and grated carrots treated with oregano and rosemary oleoresins during storage at 20 and 10°C. Figure 4A-B shows that initial *E. coli* counts were not significantly ($P < 0.05$) different between sliced control samples and those treated with oleoresins at either of the two storage temperatures. During storage at 20°C, *E. coli* populations in untreated samples and in samples treated with oleoresins at 0.5% presented a similar increase, reaching values approximately 4.0 $\log_{10}$ CFU/g higher at the end of storage. Oleoresins applied at 1% exerted a bactericidal effect at 24 and 48 h (0.8 to 1.4 log reductions) on treated sliced carrot samples (Fig. 4A).

FIGURE 4. *E. coli* counts in sliced carrot at 20 (A) and 10°C (B) and in grated carrots at 20 (C) and 10°C (D). Samples were treated with oregano and rosemary at different concentrations: Control sample (♦); carrot plus oregano at 0.5% (□) and 1% (□); rosemary 0.5% (□) and 1% (Δ)



As shown in Fig. 4C and D, initial *E. coli* counts were not significantly ($P < 0.05$) different in grated control carrot and samples treated with oleoresins at both storage temperatures. When grated carrot was stored at 20°C, *E. coli* populations in control samples increased (1.4 $\log_{10}$ CFU/g) up to 48 h, and then slightly decreased. A similar change in *E. coli* counts was observed in grated carrot treated with oleoresins at 0.5%. Samples treated with 1% oregano and rosemary had a significant ($P < 0.05$) decrease (0.85–1.2 log) in *E. coli* counts, from 24 h until the end of storage (Fig. 4C). During storage at 10°C, *E. coli* populations in control and treated grated samples showed an increase (1–1.3 log) up to 48 h of storage and then a slow decrease, which became more evident in samples treated with rosemary and oregano at 1%, with reductions of 1 and 1.5 log CFU/g, respectively, compared with control samples and samples treated with oleoresins at 0.5% (Fig. 4C and D).

Sensory evaluation

Grated and sliced carrot samples as well as samples treated with the two oleoresins (0.5 and 1%) during storage at 10 and 20°C were evaluated organoleptically. Initially, oleoresin application produced no perceptible changes in overall acceptability, color, texture and aroma of fresh sliced and grated carrots stored at 10 and 20°C. During storage (24 h) at both temperatures, severe browning was observed in both sliced and grated samples treated with oleoresins. However, flavors in treated samples were imperceptible up to 24 h of storage. At 72 h and 20°C, all grated samples were unacceptable and sliced samples were within acceptability limits (score 3). However, for samples stored at 10°C, higher scores for overall quality and texture indices (3.75) were obtained in both types of processed carrots.

DISCUSSION

Technologies that substantially reduce or inhibit Gram negative bacteria by food-grade compounds are of considerable interest to the food industry, since there are both public health and economic concerns. In food protection, Gram negative spoilage organisms and pathogens are especially problematic because of their inherent resistance to some antimicrobials

that are applicable or present in food *(2)*. One alternative for natural preservation technologies is the use of plant extracts. Consequently, the objectives of the present study were to examine the effectiveness of the endogenous microflora of sliced and grated carrots in controlling *E. coli* O157:H7 and to determine the effectiveness of oregano and rosemary oleoresins on growth of this pathogen and on native microflora.

Even though several studies have been conducted on the *in vitro* antibacterial properties of plant essential oils and extracts, only a few studies on the activity of essential oils in food systems have been published *(4, 8, 11, 14)*. It is well known that the antimicrobial potency of essential oils and oleoresins in food systems is generally reduced when compared to *in vitro* work, as the presence of fats, carbohydrates, proteins, salts and pH strongly influence the effectiveness of these agents. Accordingly, larger amounts of oleoresins and essential oils are required in food systems, and such amounts can seriously interfere with organoleptic properties. Among several essential oils and oleoresins that may be useful as antimicrobial agents, oregano and rosemary are the most active against strains of *E. coli,* and thus they may have the greatest potential for use in industrial application *(4, 5)*.

Results obtained in this work indicate that the cutting method (slicing, grating) did not introduce any difference in the final aerobic counts on carrot samples stored at 20°C (Fig. 1A and B). However, when carrot samples were stored at 10°C, native microflora counts in sliced carrots were approximately 2.0 logs higher than in grated carrot at the end of storage (Fig. 2A and B). This fact could be attributed to better temperature transference in grated carrot samples, enhancing the effect of refrigerated storage.

The competitive power of native microflora is important in controlling pathogen growth. Wei et al. *(21)* indicated that the native microflora/pathogen ratio and competition for binding sites could play an important role. However, in this work the biocontrol exerted by the native microflora on *E. coli* growth, in both types of processed carrot, stored at 10 and 20°C, was not significant (Fig. 1 and 2). This fact could be attributed to the surface roughness and exposed surface area of grated and sliced carrots that allowed native microflora and the pathogen to proliferate. The large exposed surface and the physical conditions of processed carrot have a major impact on the adhesion of *E. coli* O157:H7 and therefore on the pathogen control exerted by the native microflora. In agreement with our results, Wang et al. *(20)* working with orange, apple, and cantaloupes, reported a positive correlation of roughness and adhesion strength of *E. coli* on the ineffectiveness of the sanitizer.

On the other hand, it was evident that the initial endogenous *E. coli* counts in control samples were so high that they could mask any potential bacteriostatic effect of native microflora. Therefore, at 10 and 20°C the native microflora did not exert significant control on pathogen growth inoculated in both sliced and grated carrot samples (Figs. 1 and 2).

Oregano and rosemary oleoresins, applied on sliced carrot, exerted a significant inhibitory effect on the native microflora at 24 h of storage at 10 and 20°C. Similarly, Burt *(4)* reported antimicrobial activity of oregano and rosemary against native microflora of vegetables.

The antimicrobial action of oleoresins on *E. coli* growth depended on the concentration of oleoresins and the storage temperature (Fig. 4). Inhibition of the pathogen was more evident at high than at low temperature in both types of processed carrot, a fact that could be explained by the higher diffusion coefficients of volatile components. Similar results were found by Moreira et al. *(11)* working with blanched spinach treated with essential oils and stored at 10 and 20°C. Similiarly, Singh et al. *(18)* analyzed the effect of oregano essential oils on survival of *E. coli* on lettuce and carrot and found a significant reduction in pathogen counts.

In this work, we report an inhibitory effect obtained by applying oleoresins at high concentration. However, the effect was limited from the point of view of guaranteeing vegetable safety. The scant inhibitory effects of oleoresins on *E. coli* growth could be explained by the fact that the resistance of Gram-negative bacteria is mainly due to the outer membrane, which acts as an efficient barrier against biopreservatives *(4)*.

Consequently, higher oleoresin concentrations may be required to produce a particular level of pathogen inhibition in vegetables. However, sensory analysis revealed that acceptance is inversely related to the concentration of the oleoresins used. As a result, synergistic effects need to be exploited to maximize the antibacterial activity of oleoresins and to minimize the concentrations required to achieve a particular antibacterial effect without adversely affecting the sensory acceptability.

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