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Comparative efficacy of *Zataria multiflora* Boiss., *Origanum compactum* and *Eugenia caryophyllus* essential oils against *E. coli* O157:H7, feline calicivirus and endogenous microbiota in commercial baby-leaf salads

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ABSTRACT

Ready-to-eat salads using baby-leaf and multi-leaf mixes are one of the most promising developments in the fresh-cut food industry. There is great interest in developing novel decontamination treatments, which are both safe for consumers and more efficient against foodborne pathogens. In this study, emulsions of essential oils (EOs) from *Origanum compactum* (oregano), *Eugenia caryophyllus* (clove), and *Zataria multiflora* Boiss (zataria) were applied by spray (0.8 ml) after the sanitizing washing step. The aim was to investigate their ability to control the growth of potentially cross-contaminating pathogens and endogenous microbiota in commercial baby leaves, processed in a fresh-cut produce company. Zataria EO emulsions of 3%, 5% and 10% reduced *Escherichia coli* O157:H7 by 1.7, 2.2 and 3.5 log cfu/g in baby-leaf salads after 5 days of storage at 7 °C. By contrast, reductions in *E. coli* O157:H7 counts remained the same when clove was applied at concentrations of 5% and 10% (2.5 log cfu/g reduction). Oregano (10%) reduced inoculated *E. coli* O157:H7 counts in baby-leaf salads by a maximum of 0.5 log cfu/g after 5 days of storage. Zataria showed strong antimicrobial efficacy against *E. coli* O157:H7 and also against the endogenous microbiota of baby-leaf salads stored for 9 days. Feline calicivirus (FCV), a norovirus surrogate, survived on inoculated baby-leaf salads during refrigerated storage (9 days at 7 °C) regardless of treatment. Refrigeration temperatures completely annulled the effectiveness of the EOs against FCV inoculated in baby-leaf salads as occurred in FCV cultures. This study shows that EOs, and zataria in particular, have great potential use as an additional barrier to reduce contamination-related risks in baby-leaf salads. However, further research should be done into foodborne viruses in order to improve food safety.

1. Introduction

Lettuce is one of the most important ready-to-use products and ranks highly among vegetables both in production and economic value. The world production of salad and especially the production of lettuce and chicory (the two plants being combined by the FAO for reporting purposes) was 23,622,366 metric tons (FAO, 2012) for calendar year 2010. Although prepared salads consist mainly of iceberg lettuce, other types of lettuce, with attractive colors and shapes, are used in salad mixes called “mesclun” in France or “spring mix” in the U.S. (Martinez-Sanchez et al., 2012). As consumers are looking for softer textures, baby-sized leaves using baby-leaf at immature stage and multi-leaf at mature stage have proven one of the most promising fresh-cut developments (Martinez-Sanchez et al., 2012). These new salads represent a new challenge to the food industry, because differences in the morphology and maturity stages of the numerous types of leafy vegetables that compose them affect sanitizing efficacy. The increase in fresh produce consumption has led to a higher incidence of foodborne illnesses since these vegetables are eaten raw (Warriner et al., 2009). Among the foodborne pathogens related to fresh produce such as spinach, lettuce, alfalfa sprouts and mixed salads, *Escherichia coli* O157 has been identified as the source of around the 21% of outbreaks (Olaimat and Holley, 2012; Rangel et al., 2005). Enteric viruses, and particularly norovirus, form another group of pathogens that are closely related to fresh produce (Sivapalasingam et al., 2004). Moreover, noroviruses are the most common cause of foodborne illness and have been listed in the top 5 pathogens in a cost-related ranking of foodborne illness in the United States (Scharff, 2012). Foods may be contaminated by contact with human fecal samples in the field or by unhygienic manipulation by a food handler infected by virus.

Most of the literature available on the decontamination of fresh vegetables has concluded that sanitizing washing processes reduce the

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endogenous microbial populations on the surface of the produce by only 2 to 3 log cfu/g (Gil et al., 2009). Furthermore, despite initial differences in bacterial load, after storage total counts are similar whether the produce is washed with tap water or a sanitizing solution. Therefore, sanitizing agents are useful to maintain water quality and prevent cross-contamination during washing; however, they have scarce or no efficacy in preventing microbial growth during product storage as they do not have a residual effect (Gil et al., 2009). This fact prompts an interest in the use of natural and safe compounds with an antimicrobial effect to be added to processed fruits and vegetables during storage. Essential oils (EOs) have long been applied as flavoring agents to foods such as meat and have shown a wide spectrum of antimicrobial activity on several foodborne pathogens and spoilage bacteria, both *in vitro* and in food matrices (Burt, 2004; Ponce et al., 2011). Composition and activity of some EOs such as oregano, clove, cinnamon, garlic, coriander, rosemary, mint, basil, and parsley have been reported in studies *in vitro* (Angioni et al., 2004; Elizaquivel et al., 2012; Karagözlü et al., 2011; Oussalah et al., 2007; Piskernik et al., 2011; Tsigarida et al., 2000) and demonstrate they exert antimicrobial effects against different foodborne pathogens. The antibacterial effect of some EOs on lettuce at mature stages, mainly iceberg and romaine varieties, has also been investigated (Karagözlü et al., 2011; Ponce et al., 2011; Yossa et al., 2013). All these studies examined endogenous or pathogenic bacteria reduction in fresh produce when EOs were used as sanitizing agents during the washing step, but only one of them studied other technological applications of the EOs (Ponce et al., 2011). Ponce et al. (2011) assayed spray, immersion and lactose capsule applications of tea tree, clove and rosemary EOs on processed romaine lettuce. Only EO spray application maintained an acceptable quality of the product throughout the entire storage period. In addition, there are other EOs which are traditionally used in some parts of the world, whose antimicrobial activity in foods has not been studied in detail. *Zataria multiflora* Boiss (*Z. multiflora*), belonging to the family *Laminacae*, is native to Iran, Pakistan and Afghanistan. This plant is traditionally used in foods, especially in yoghurt flavoring, as a stimulant, condiment, carminative and for treatment of pre-mature labor pains and rupture (Ali et al., 2000; Hosseinzadeh et al., 2000). This EO exhibits beneficial properties against respiratory tract infections and irritable bowel syndrome (Ali et al., 2000). Moreover, *Z. multiflora* antimicrobial activity has been demonstrated by *in vitro* experiments against fungi (Khosravi et al., 2012) and bacterial pathogens such as *Staphylococcus aureus, Salmonella enterica* or *Listeria monocytogenes* (Basti et al., 2007; Moradi et al., 2011) as well as norovirus surrogates such as feline calicivirus (FCV) (Elizaquivel et al., 2013a). However, the application and antimicrobial effect of *Z. multiflora* on fresh vegetables remains unexplored to date.

In the present study, we have investigated the potential of the EOs from *Origanum compactum* (oregano), *Eugenia caryophyllus* (clove) and *Z. multiflora* Boiss. as antimicrobial biopreservatives when applied by spraying on commercial baby-leaf salads processed in a fresh-cut produce company. Their antimicrobial effect, 15 min after application and during the shelf-life, has been tested on the endogenous microbiota of baby-leaf salads, as well as on *E. coli* O157:H7 and FCV, a norovirus surrogate, artificially inoculated in the same product.

2. Materials and methods

2.1. Essential oils

Commercially available EOs from oregano and clove, supplied by Pronarôm International (Ghislenghien, Belgium), and zataria EO (produced in-house) were used in this study. The main antimicrobial compounds present in each of these EOs are shown in Table 1. Oregano and clove EOs were diluted in 70% ethanol according to the manufacturer's instructions and stored at 4 °C before use. Zataria EO was obtained from *Z. multiflora* Boiss. collected in the Fars province (Iran). Air-dried aerial parts of the plant were subjected to steam distillation for 2 hours, using Clevenger-type apparatus as previously described (Basti et al., 2007). The obtained EO composition was determined by GC and GC-MS at the Institute of Medicinal Plants, Medical University of Tehran, Iran (Table 1) (Azizkhani et al., 2013). The main antimicrobial compound it contains is carvacrol (71.1%). Zataria EO was stored in airtight glass vials covered with aluminum foil at 4 °C, then prior to its application, zataria EO was resupended 1:10 *v*/*v* in 50% ethanol.

Table 1
Main compounds of the selected EOs.

Plant species	Common name	Origin	Distilled part	Main compounds (%)
Origanum compactum	Oregano	Morocco	Flowering plant	Carvacrol (46.88) Thymol (15.26) p-cymene (13.10) γ-terpinene (11.61)
Eugenia caryophyllus	Clove	Madagascar	Flower bud	Eugenol (83.96) Eugenile acetate (10.75) β-caryophyllene (3.25)
Zataria multiflora Boiss	Zataria	Iran	Aerial parts	Carvacrol (71.12) γ-terpinene (7.34) α-pinene (4.26) Eucaliptol (3.37) Globulol (2.32) Others compounds (<1)

2.2. Bacterial strain, culture conditions and inoculum preparation

Escherichia coli O157:H7 CECT 5947 (non-toxigenic) supplied by the Spanish Type Culture Collection (CECT) was used in this study. This strain is recommended for food safety control assays since gene *stx*2 (virulence factor) has been replaced with gene *cat*. A nalidixic acid-resistant (Nal^R) *E. coli* O157:H7 strain was obtained by consecutive 24-h transfers of brain heart infusion (BHI, Merck, Darmstadt, Germany) cultures to BHI containing increasing concentrations of nalidixic acid (Nal) up to 100 mg/ml. Then Nal^R *E. coli* O157:H7 colonies were consecutively subcultured twice in 5 ml of BHI supplemented with nalidixic acid (Nal^R, 100 mg/ml) at 37 °C for 20 h. This Nal^R *E. coli* O157:H7 strain was routinely grown on tryptic soy broth (TSB, Merck) at 37 °C for 18 h, and enumerated by plate count on tryptic soy agar (TSA, Merck) under the same incubation conditions.

Cultures for inoculation experiments were prepared by transferring 100 μl of the overnight culture to 10 ml of TSB with 100 μg/ml nalidixic acid, and incubated at 37 °C for 4 hours (ca. 10^8 cfu/ml). Thereafter, cultures were serially diluted in phosphate buffered saline (PBS) to obtain a final cell density of 10^6 cfu/ml.

2.3. Virus strain and cell line

The cytopathogenic F9 strain of FCV (ATCC VR-782) was propagated and assayed in CRFK cells. Semi-purified stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at 660 ×*g* for 30 min.

2.4. Baby-leaf salad preparation

Mixed salad containing baby red and green Batavia, Lollo Rosso lettuce, spinach and arugula at a ratio of 1:1:1:1:0.3 were processed in a fresh-cut vegetable processing company (Verdifresh, Valencia, Spain). Leaves with defects, such as bruising or discoloration, were hand removed before washing. They were well mixed until homogeneous and then washed by immersion for 30–60 s in 100 mg/ml chlorine solution (NaOCl) adjusted to pH 6.5 with phosphoric acid, and using a redox potential of 650–750 mV. Leaves were drained for 30 s and then rinsed with a tap water shower for 30–60 s. Excess water was removed by centrifugation. Samples of 100 g were mechanically non-vacuum packed