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Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils

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ABSTRACT

The antimicrobial activity of the vapour generated by a combination of cinnamon and clove essential oils against the growth of four Gram-negative (*Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis*) and four Gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* and *Enterococcus faecalis*) was assessed by means of the fractional inhibitory concentration index (*FIC*) of the mixture. The presence of synergism or antagonism effects depended on the reference parameter used to estimate such an index. If the minimal inhibitory concentrations were applied, the vapours of the combination of essential oils exerted an antagonistic effect on the growth of *E. coli*, while they wielded a synergistic effect for the inhibition of *L. monocytogenes*, *B. cereus* and *Y. enterocolitica* when the concentrations of maximal inhibition were used. This fact revealed a clear concentration-dependent interaction.

The headspace of the cinnamon and clove essential oils and their combination was sampled by solid-phase microextraction (SPME) and the constituents identified and quantified by gas chromatography–ion trap mass spectrometry (GC/ITMS). Eugenol was the most abundant compound for the three antibacterial atmospheres. The differences in behaviour could be attributed to minor compounds. The combined headspace contained slightly larger amounts of 1,8-cineole and camphor, which are believed to enhance the eugenol activity. The mechanisms responsible for the antagonism are, however, less known and much further investigation is required.

To the best of our knowledge this is the first time a combination of essential oils in the vapour phase has been tested as a preservative method to prevent microorganism proliferation.

1. Introduction

Numerous food products require protection against microbial spoilage during their shelf life. The growing demand of consumers for safe and natural products, without chemical preservatives, has resulted in thorough investigations from food authorities and researchers to assess the feasibility of mild preservation techniques and to improve the microbial quality and safety of products, while maintaining their good nutritional and organoleptic properties.

Essential oils (EOs) are volatile oily liquids obtained from different plant parts and widely used as food flavours (Burt, 2004). In spite of having been long recognised for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Kordali et al., 2005; Pezo, Salafranca, & Nerin, 2006), the recent interest in alternative natural substances has lead to a new scientific awareness of these substances. Some authors have even suggested the use of EOs for prevention of the transmission of resistant and harmful pathogens strains, such as methicillin-resistant *S. aureus* (MRSA) (Penalver et al., 2005).

Despite the high efficiency of the EOs and their constituents against food-borne pathogens and spoilage microorganisms when *in vitro* tests are conducted (Chorianopoulos et al., 2004; Fisher & Phillips, 2006), the same effect in food is only achieved with higher concentration of EOs (Burt, 2004; Hulin, Mathot, Mafart, & Dufosse, 1998). This fact may imply an organoleptic impact, caused by altering the natural taste of the food by exceeding the acceptable flavour thresholds (Hsieh, Mau, & Huang, 2001; Nazer, Kobilinsky, Tholozan, & Dubois-Brissonnet, 2005). Few approaches have been proposed to minimise EO concentrations and reduce the sensory effect.

One solution would consist of combining plant extracts. Although EOs were concluded to have greater activity than mixtures of their major components (Gill, Delaquis, Russo, & Holley, 2002; Mourey & Canillac, 2002), the combination of these major components with other constituents with a weaker activity might

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result in a synergistic, additive or antagonist effect (Ultee, Kets, Alberda, Hoekstra, & Smid, 2000). The combination of clove and rosemary exerted all three effects, depending on the corresponding microorganism (Fu et al., 2007). Oregano EO combined with thyme EO at low doses has been reported as a potential means of controlling the growth of pathogens and spoilage microorganisms, whereas the combinations of oregano with marjoram EO or thyme with sage EO might be useful for targeted control of key Gram-negative or Gram-positive bacteria, respectively (Gutierrez, Barry-Ryan, & Bourke, 2008). Since EOs are generally recognised as safe (GRAS) (Kabara, 1991), the possibility of reinforcing their natural antimicrobial effects by the addition of small amounts of other natural preservatives may be a way of attaining a balance between sensory acceptability and antimicrobial efficiency.

Lopez et al. have assessed the antimicrobial activity in the vapour phase of a wide number of EOs and their main constituents (Lopez, Sanchez, Batlle, & Nerin, 2005, 2007b) with promising results, which concluded with the development of an antimicrobial packaging (Lopez, Sanchez, Batlle, & Nerin, 2007a; Rodriguez, Batlle, & Nerin, 2007), which achieved similar inhibition for the *in vitro* tests and the assays conducted with food (Rodriguez, Nerin, & Batlle, 2008). The innovative film creates a protective atmosphere with a negligible organoleptic alteration. Nevertheless, and to the best of our knowledge, there is no published report regarding the antimicrobial effectiveness of combinations of EOs in the vapour phase.

The aim of the present study was the assessment of the susceptibility of various strains of microorganisms to cinnamon, clove and a mixture of cinnamon and clove EOs, all in the vapour phase, to detect synergistic, additive or antagonistic effects. The atmosphere generated by the different antimicrobial natural agents has been sampled by solid-phase microextraction (SPME) and analysed by gas chromatography–ion trap mass spectrometry (GC–ITMS). Finally, a correlation between the chemical composition and the antimicrobial activity has been proposed.

2. Experimental section

2.1. Bacterial strains

The following food-borne bacterial strains were selected, due to their relevance in the food industry: the Gram-negative bacteria *Escherichia coli* (American Culture Collection, ATCC 29252), *Yersinia enterocolitica* (Colección Española de Cultivos Tipo, CECT 4315), *Salmonella choleraesuis* (CECT 4000), and *Pseudomonas aeruginosa* (ATCC 27853); the Gram-positive bacteria *Bacillus cereus* (CECT 495), *Listeria monocytogenes* (ATCC 7644), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213). The strains were cultured on Mueller-Hinton agar (MHA, Bio-Rad, La Coquette, France) at 30 °C for 48 and 24 h for Gram-positive and Gram-negative microorganisms, respectively, and stored at −80 °C in sterile skimmed milk.

2.2. Essential oils

The essential oils (EOs) were supplied by ARTIBAL (Sabiñanigo, Spain). Oils from the following plant species were tested in this work: *Cinnamon zeylanicum* [cinnamon, Chemical Abstract Service (CAS), registry number 8015-91-6], and *Syzgium aromaticum* (clove, CAS No. 8000-34-6).

2.3. Chemicals

The following chemicals were used as standards to identify the composition of the atmospheres generated by the EOs and their mixtures: *trans*-cinnamaldehyde (99%, CAS 14371-10-9), β-caryophyllene (99.5%, CAS 87-44-5), bornyl acetate (95%, CAS 5655-61-8), estragol (98%, CAS 140-67-0), borneol (98%, CAS 464-43-7), *R*-(β)-pinene (98%, CAS 80-56-8), thymol (99.5%, CAS 89-83-8), 1,8-cineole (99%, CAS 470-82-6), D-limonene (97%, CAS 5989-27-5), camphor (96%, CAS 76-22-2), benzyl benzoate (99%, CAS 126-51-4), linalool (97%, CAS 78-70-6), eugenol (99%, CAS 97-53-0), α-pinene (99%, CAS 8172-673), camphene (95%, CAS 79-92-5), α-humulene (99.5%, CAS 6753-98-6) supplied by Sigma–Aldrich (St. Louis, MO); α-cubenene (97%, CAS 17699-14-8), α-copaene (90%, CAS 3856-25-5), (−)-verbenone (97%, CAS 1196-01-6), γ-terpinene (97%, CAS 99-85-4), α-terpinolene (97%, 582-67-9), α-phellandrene (95%, CAS 4221-98-1), α-terpinene (95%, CAS 99-86-5) supplied by Fluka (Bellefonte, PA), and α-terpineol (98%, CAS 562-74-3) supplied by Chem Service (West Chester, PA).

2.4. Antimicrobial activity test

Solid diffusion tests: The susceptibility of the bacteria to the EOs was determined by an agar diffusion disc method (Lopez et al., 2005). The appropriate solidified medium was inoculated with 100 μl of bacterial suspension containing 10^5 cfu/ml of the microorganism under study. Afterwards, 10 μl of each dilution of either the pure essential oil or their combinations (1:1) were added to 10 mm sterile blank filter discs and placed in direct contact with the agar medium. Previously, it was estimated that 10 μl of pure essential oil corresponded to 10 mg weight on the disc.

Vapour diffusion tests: Solidified medium was inoculated with 100 μl of bacterial suspension containing 10^5 cfu/ml of the microorganism under study. Each pure essential oils or their combinations (1:1) were diluted in ethyl ether (GC quality, Merck, Darmstadt, Germany) to obtain serial dilutions. Then, 10 μl of each dilution were added to 10-mm diameter sterile blank filter discs and placed in the centre of the lid of the Petri dish (Lopez et al., 2005). The Petri dishes were then sealed using sterile adhesive tape (Deltalab, Rubi, Spain). No hermetic sealing was needed because experiments were designed to simulate a worst-case situation, when leaking of the active components to the atmosphere can occur, thus increasing the probability for microorganism contamination. Blanks were prepared by adding 10 μl of ethyl ether to the filter discs, which was demonstrated to have no effect on the viability of any of the tested bacteria. Analyses were carried out in triplicate. The concentration of essential oil was expressed as weight per unit volume (mg/l air). The tested concentrations varied from 180 to 1.8 mg/l in the headspace of the Petri dish.

The effectiveness of the essential oil was calculated by measuring the diameter (in mm) of the zone of microorganism growth inhibition above the disc. The size of the zone with visible growth reduction around the inhibition zone was also measured.

The minimum inhibitory concentration (*MIC*) was defined as the lowest essential oil concentration resulting in the lack of visible microorganism growth. The reduction concentration (*RC*) was defined as the lowest essential oil concentration resulting in a visible microorganism growth reduction. The essential oil concentration which yielded the biggest inhibition zone was named as C_{max}.

A fractional inhibition concentration index (*FIC*) was estimated for all tested microorganisms to determine the antimicrobial effect of the mixture of cinnamon and clove EOs, within the limits of the method used (White, Burgess, Manduru, & Bosso, 1996). The *FIC* was calculated by the following equation:

$$FIC = [\text{concentration of essential oil combination which inhibits bacteria}]/[\text{concentration of pure essential oil which inhibits bacteria}].$$

Table 1
Inhibition and growth reduction zones, in millimetres, provided by cinnamon EO (CI), clove EO (CL) and their mixture (CI–CL). Results are expressed as mean ± standard deviation.

	Direct contact			Vapour phase		
	CI	CL	CI–CL	CI	CL	CI–CL
Gram-negative						
E. coli	18 ± 1	18 ± 1	18 ± 1	22 ± 3 (27)[a]	**30 ± 1**[b]	25 ± 3 (29)
Y. enterocolitica	21 ± 2	23 ± 3	22 ± 2	32 ± 3 (35)	35 ± 2 (38)	**50 ± 2 (54)**
S. choleraesuis	21 ± 1	23 ± 2	22 ± 1	13 ± 3	13 ± 4 (18)	16 ± 4 (28)
P. aeruginosa	12 ± 1	0 ± 1	12 ± 1	0	0	0
Gram-positive						
B. cereus	30 ± 2	32 ± 1	30 ± 1	26 ± 4 (32)	21 ± 3 (24)	**32 ± 2 (42)**
L. monocytogenes	19 ± 1	18 ± 2	20 ± 1	26 ± 2 (32)	**13 ± 1 (22)**	25 ± 3 (29)
E. faecalis	14 ± 1	15 ± 0	17 ± 1	15 ± 5 (18)	12 ± 3 (17)	12 ± 3 (14)
S. aureus	19 ± 2	19 ± 1	20 ± 1	24 ± 3 (28)	23 ± 3 (24)	25 ± 1 (29)

[a] The growth reduction zone in brackets. 90 mm means total inhibition.
[b] Significant different results in bold.

Table 2
Minimum inhibitory concentration (*MIC*), reduction concentration (*RC*) and concentration of maximal inhibition (C_{max}), expressed as mg EO/l headspace in vapour phase of cinnamon EO (CI), clove EO (CL) and their combination (CI–CL).

	CI			CL			CI–CL (1:1)		
	MIC	*RC*	C_{max}	*MIC*	*RC*	C_{max}	*MIC*	*RC*	C_{max}
Gram-negative									
E. coli	18 ± 0	18 ± 0	54 ± 0	27 ± 0	27 ± 0	180 ± 0	90 ± 0	90 ± 0	90 ± 0
Y. enterocolitica	18 ± 0	13 ± 2	90 ± 0	9 ± 5	9 ± 2	180 ± 25	18 ± 0	18 ± 0	90 ± 10
S. choleraesuis	136 ± 25	54 ± 10	181 ± 0	54 ± 20	35 ± 10	180 ± 25	135 ± 0	36 ± 0	180 ± 0
P. aeruginosa	0	0	0	0	0	0	0	0	0
Gram-positive									
B. cereus	18 ± 5	13 ± 2	181 ± 25	18 ± 5	18 ± 5	135 ± 25	36 ± 0	36 ± 0	135 ± 0
L. monocytogenes	54 ± 0	54 ± 2	181 ± 25	18 ± 5	18 ± 0	135 ± 25	90 ± 0	90 ± 0	180 ± 0
E. faecalis	54 ± 0	54 ± 0	181 ± 0	90 ± 0	35 ± 0	180 ± 25	90 ± 5	90 ± 0	135 ± 25
S. aureus	36 ± 5	27 ± 5	136 ± 25	27 ± 0	27 ± 0	90 ± 0	54 ± 0	36 ± 0	135 ± 25

2.5. Solid-phase microextraction (SPME)

Fully-retracted SPME fibres (Supelco, Bellefonte, PA), coated with an 85-μm layer of polyacrylate (PA) for clove EO or a 100-μm layer of polydimethylsiloxane (PMDS) for cinnamon EO and the clove–cinnamon mixture were used. The optimum conditions as well as the type of fibre used were based on the results of previous studies (Lopez, Huerga, Batlle, & Nerin, 2006). The atmosphere generated in the vapour diffusion tests was measured by placing a fully-retracted SPME fibre into the headspace of the Petri dish (Lopez et al., 2005). Sampling was performed over 24 h. The volume of the atmosphere sampled on every occasion was 57.3 cm^3. The atmosphere composition inside the Petri dishes was estimated for the pure EOs and/or mixture under the same conditions of temperature and culture medium as the microbiological tests, but without inoculating any strain.

2.6. Gas chromatography–ion trap mass spectrometric (GC–ITMS) analysis

GC–ITMS analyses were carried out using a Varian CP 3800 gas chromatograph (Varian Inc., Palo Alto, CA) equipped with a VF-5 MS (Varian) column (60 m × 0.25 mm, 0.25 μm film thickness) coupled to a Saturn 2000 ITMS detector; a split–splitless injector operated in splitless mode, (splitless time 2 min) with a 0.8 mm id SPME-specific liner (Varian), and an MS version 6.03 Chemstation. The carrier gas was helium (C-50, Carburos Metálicos, Zaragoza, Spain) at a constant flow rate of 1.0 ml/min.

Two different sets of chromatographic conditions were used, according to a previous study (Lopez et al., 2006). For clove analysis, the injector temperature was 265 °C, and the oven programme was as follows: initial temperature, 45 °C, held for 1 min; 15 °C/min to 90 °C; 5 °C/min to 170 °C; then 5 °C/min to 200 °C, and held for 15 min. For cinnamon, and the mixture of clove–cinnamon EOs, the injector temperature was held at 250 °C, whereas the oven temperature was initially held at 45 °C for 1 min, raised at 2 °C/min to 85 °C, by 5 °C/min to 170 °C, then finally at 15 °C/min to 200 °C, and held for 2 min.

The MS was operated in electron impact (EI) ionisation mode, and complete scans from 40 to 350 amu were recorded. Compounds were identified by matching their mass spectra with those

Table 3
Fractional inhibitory concentration (*FIC*) index for each individual EO and its combination (CI, cinnamon EO; CL, clove EO; A, antagonism; S, synergism; I, indifferent effect; ad, additive effect).

	FIC_{CI}	FIC_{CL}	$FIC_{CI} + FIC_{CL}$
(a) Considering *MIC* values as reference			
E. coli	2.5 (A)	1.7 (I)	**4.2 (A)**[a]
Y. enterocolitica	0.5 (S)	1.0 (I)	1.5 (ad)
S. choleraesuis	0.5 (S)	1.3 (I)	1.8 (ad)
B. cereus	1.0 (I)	1.0 (I)	2.0 (ad)
L. monocytogenes	0.8 (I)	2.5 (A)	3.3 (ad)
E. faecalis	0.8 (I)	0.5 (S)	1.3 (ad)
S. aureus	0.7 (I)	1.0 (I)	1.8 (ad)
(b) Considering concentrations of maximal inhibition (C_{max}) as reference			
E. coli	0.8 (I)	0.3 (S)	1.1 (ad)
Y. enterocolitica	0.1 (S)	0.1 (S)	**0.2 (S)**[b]
S. choleraesuis	0.5 (S)	0.5 (S)	1.0 (ad)
B. cereus	0.2 (S)	0.2 (S)	**0.4 (S)**
L. monocytogenes	0.2 (S)	0.3 (S)	**0.5 (S)**
E. faecalis	0.5 (S)	0.5 (S)	1.0 (ad)
S. aureus	0.4 (S)	0.8 (I)	1.1 (ad)

[a] Antagonism effect in bold.
[b] Synergism effect in bold.

in the NIST commercial library (purity criterion, >85%). When available, the retention times and fragmentation spectra of pure standards (>95%) were obtained for confirmation. All analyses were carried out in triplicate.

2.7. Statistical analysis

The triplicate data obtained are presented in Tables 1 and 2 as means ± standard deviation (SD). Significant differences were determined by ANOVA at the 95% significance level using STATGRAPHICS Plus 5.1 (Statistical Graphic Corporation, Warrenton, VA).

3. Results

3.1. Antimicrobial activity

3.1.1. Direct contact versus vapour phase

The antimicrobial activity of cinnamon and clove EOs and their combination (1:1), both by direct contact or through vapour phase, against the eight microbial species described in Section 2.1, was qualitatively and quantitatively assessed by the presence or absence of inhibition zone. The diameter of the inhibition zone is given in Table 1.

The mass of tested EO was 10.4 mg, which corresponded to a concentration of 181 mg/l in the vapour phase. No significant differences were observed among the size of the inhibition zone for the mixture and the individual EOs when direct contact, while the combination provided a significant increase of the activity for *Y. enterocolitica*, *B. cereus* and *L. monocytogenes*.

Essential oils showed higher activity in the vapour phase. *P. aeruginosa* was the only microorganism that was better inhibited when in direct contact. The size of the inhibition zone in the vapour phase generally increased in the following order:

Cinnamon EO: *S. choleraesuis* < *E. faecalis* < *L. monocytogenes* ≈ *E. coli* ≈ *S. aureus* ≈ *B. cereus* < *Y. enterocolitica;* clove EO: *S. choleraesuis* ≈ *L. monocytogenes* ≈ *E. faecalis* < *S. aureus* ≈ *B. cereus* < *E. coli* < *Y. enterocolitica;* and cinnamon–clove combination: *E. faecalis* < *S. choleraesuis* < *S. aureus* ≈ *L. monocytogenes* ≈ *E. coli* ≪ *B. cereus* < *Y. enterocolitica.*

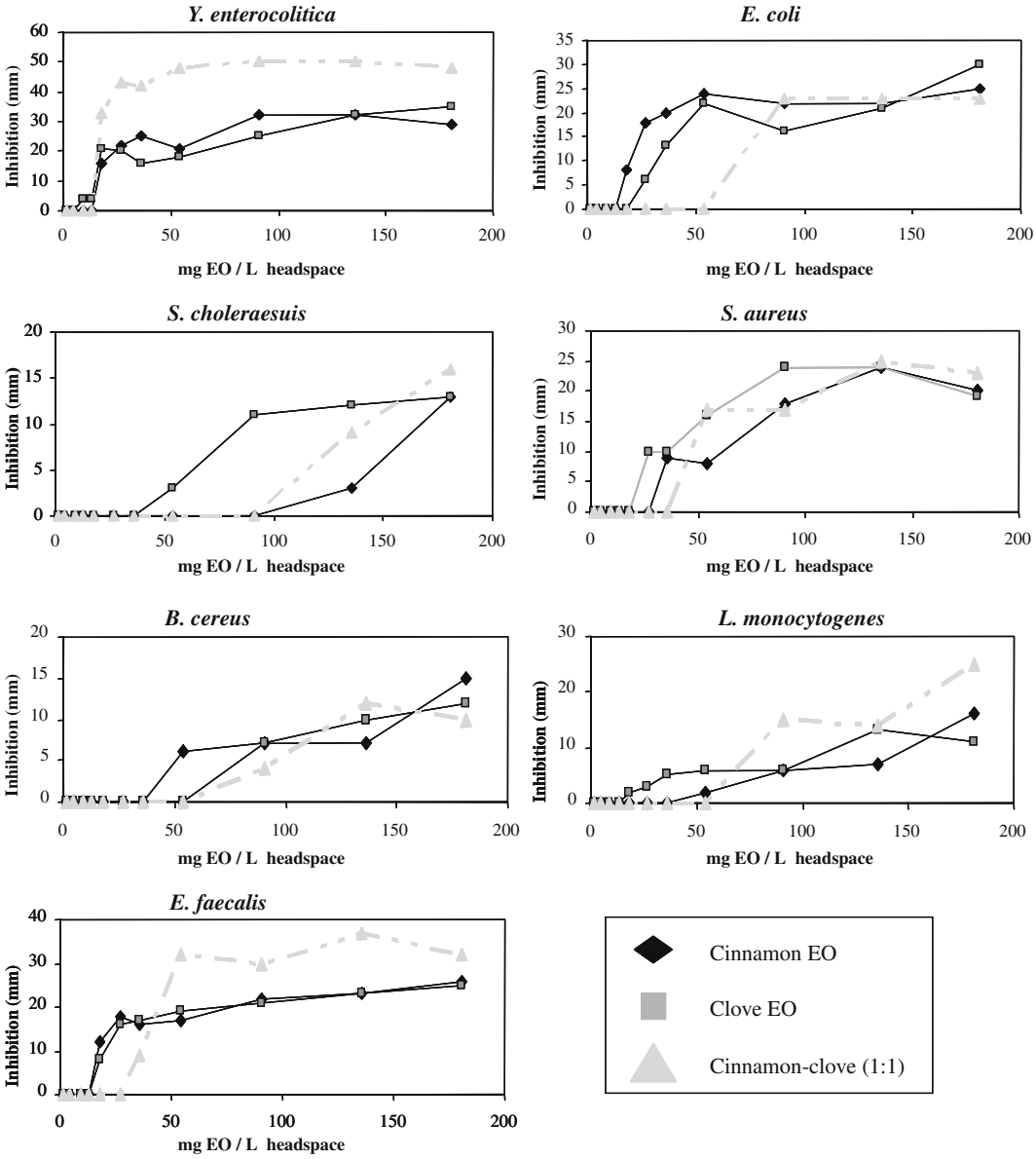

Fig. 1. Antimicrobial activity of the single EOs and their combination in the vapour phase.

However, no relationship between the Gram-positive or Gram-negative structure and the activity of the essential oils was observed, with the exception of *P. aeruginosa*, which provided a slight activity in direct contact with cinnamon.

3.1.2. Minimum inhibitory (MIC), reduction concentration (RC) and concentration of maximal inhibition (C_{max}) in vapour phase

Table 2 shows the minimum inhibitory concentration (*MIC*), the reduction concentration (*RC*) and the concentration of maximal inhibition for the atmosphere generated by cinnamon and clove EO and their mixture (1:1).

Regarding the *MIC* values, *Y. enterocolitica* was the most sensitive microorganism, providing the lowest microorganism growth, followed by *B. cereus* and *S. aureus* whatever the tested EO. Although cinnamon EO and its combination with clove EO provided the same *MIC* values against *Y. enterocolitica*, an increase in the inhibition zone, without bacterial growth, was observed for the combination of EOs. As expected, *P. aeruginosa* was the least susceptible strain.

As far as the C_{max}-values were concerned, the EO mixture was more effective than the single EOs against *E. faecalis*, *Y. enterocolitica* or *B. cereus* growth. In the cases of *Y. enterocolita* and *B. cereus*, the C_{max}-value of the combination was equal to that of one of the single oils; however, the inhibition zone was larger.

3.1.3. Synergistic, antagonistic or additive effect

An additive effect is observed when the combined effect is equal to the sum of the individual effects. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied (Davidson & Parish, 1989).

In order to numerically define the influence of the concentration in the antagonistic/synergistic effects, the fraction inhibitory concentration index (*FIC* index) was calculated for each EO in the tested combination (White et al., 1996) using two approaches. The *MIC* values were considered as the reference in the first approach, while the concentrations of maximal inhibition (C_{max}) were the key parameters in the second approach. For instance, the *FIC* of cinnamon EO (FIC_{CI}) was equal to the *MIC* of cinnamon EO in combination divided by the *MIC* of cinnamon EO alone, following the first approach (Table 3a), and equal to C_{max} of cinnamon EO in combination divided by C_{max} of cinnamon EO alone, following the second approach (Table 3b). Synergistic effect was defined as an *FIC* index less than or equal to 0.5; additive effect when *FIC* = 0.5–0.75, indifferent effect when *FIC* = 0.76–2.0; and antagonistic effect when *FIC* was greater than or equal to two. The *FIC* mixture index was estimated as the sum of the individual *FIC* values for each EO. An *FIC* mixture index between 0.5 and 4 provided additive (non-antagonistic) interactions. Antagonistic interactions were defined as those with *FIC* index greater than four and synergistic effect as *FIC* < 0.5.

Following the *MIC* approach, no synergistic effect was obtained for the combination of EOs. On the other hand, such a mixture provided an antagonism on the inhibition of *E. coli*. Nevertheless, at higher concentrations (see Fig. 1) the individual EOs and the mixture possessed the same activity against the growth of *E. coli*.

Fig. 1 also shows a clear concentration-dependent interaction between the individual EOs. Like *E. coli*, *L. monocytogenes* and *B. cereus* required higher concentrations of the mixture to be inhibited; however, they were more sensitive to the mixture than to the single EO, even achieving a synergism effect at C_{max}. Considering the C_{max} approach, a synergistic effect was also observed for the inhibition of *Y. enterocolitica*.

As for the rest of the tested bacteria, the combination of the single EOs did not contribute to improving the inhibition.

Table 4
Composition of the atmosphere generated by cinnamon EO, clove EO and their combination (1:1), expressed as percentages of total ion counts identified ($n = 3$).

Peak number	Compound	CI[a]	CL[a]	CI–CL
1	α-Pinene	1.1 ± 0.4	–	0.3 ± 0.0
2	Camphene	0.4 ± 0.2	–	0.1 ± 0.0
3	β-Pinene	0.8 ± 0.2	–	0.2 ± 0.0
4	α-Phellandrene	1.2 ± 0.6	–	0.3 ± 0.1
5	*p*-Cymene	1.2 ± 0.1	–	0.6 ± 0.2
6	Limonene	0.9 ± 0.5	–	0.3 ± 0.1
7	1,8-Cineole	1.2 ± 0.1	–	6.2 ± 1.7
8	Linalool	3.0 ± 0.4	–	2.5 ± 0.3
9	Camphor	0.4 ± 0.3	–	1.3 ± 0.5
10	Citronellal	0.2 ± 0.1	–	0.4 ± 0.1
11	Borneol	0.2 ± 0.1	–	0.2 ± 0.0
12	1-Terpinen-4-ol	0.3 ± 0.2	–	–
13	α-Terpineol	0.5 ± 0.1	–	0.2 ± 0.1
14	Estragol	1.7 ± 0.5	0.2 ± 0.1	2.6 ± 0.5
15	(*E*)-Cinnamaldehyde	0.4 ± 0.0	–	0.2 ± 0.0
16	Safrol	2.8 ± 0.9	–	1.8 ± 0.1
17	Thymol	0.1 ± 0.0	0.1 ± 0.0	–
18	(*E*)-Cinnamyl alcohol	0.1 ± 0.0	–	–
19	α-Cubebene	0.1 ± 0.0	–	–
20	Eugenol	67 ± 12	82 ± 2	61 ± 4
21	Benzenepropy lacetate	0.1 ± 0.0	–	0.1 ± 0.0
22	α-Copaene	2.1 ± 0.7	–	1.2 ± 0.2
23	β-Caryophyllene	8.6 ± 1.7	10 ± 2	14 ± 2
25	α-Aromadendrene	0.1 ± 0.0	–	–
26	(*E*)-Cinnamyl acetate	0.7 ± 0.1	–	0.6 ± 0.1
27	α-Humelene	1.7 ± 0.6	2.9 ± 0.5	2.6 ± 1.2
28	γ-Muurolene	0.2 ± 0.0	–	–
29	Ledene	0.2 ± 0.0	–	–
30	α-Muurolene	0.1 ± 0.0	–	–
31	Eugenol acetate	0.6 ± 0.5	0.5 ± 0.3	0.5 ± 0.2
32	δ-Cadinene	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2
33	Calamenene	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
34	Benzyl benzoate	1.1 ± 0.4	–	1.0 ± 0.1
	Not identified	0.5 ± 0.2	0.3 ± 0.0	0.9 ± 0.4
Total		100 ± 1	100 ± 3	100 ± 0

[a] CI, cinnamon EO; CL, clove EO.

3.2. Chemical composition of the antibacterial atmosphere

The identified components of the atmosphere generated by cinnamon EO, clove EO and their combination are listed in Table 4. As can be seen in Fig. 2, the antibacterial headspaces were characterised with a prominent (>60%) concentration of eugenol. In addition to eugenol, the headspace created by clove EO also contained β-caryophyllene (10%) and α-humulene (3%) as main constituents and traces of estragol, eugenol acetate, δ-cadinene and calamenene. Cinnamon EO headspace was characterised by the presence of a larger number of volatile compounds, most of them terpenes at trace level. The main constituents were eugenol (67%), β-caryophyllene (8.6%), linalool (3%) and safrol (2.8%). Finally, the combined mixture provided an antibacterial atmosphere more similar to the one generated by single cinnamon EO.

4. Discussion

The present study has demonstrated the potential of the combination of cinnamon EO and clove EO (1:1, v/v) as an antibacterial agent in the vapour phase. The effectiveness of these two EOs against microbial growth when in direct contact with the inoculated culture had already been widely reported (Ouattara, Simard, Holley, Piette, & Begin, 1997; Smith-Palmer, Stewart, & Fyfe, 1998; Valero & Salmeron, 2003), although their application in the vapour phase entails a relative innovative approach (Lopez et al., 2005). In accordance with other authors, when bacteria were exposed to the vapours of essential oils, the inhibitory effects were noticeably different from those found by direct contact (Edwards-Jones, Buck,

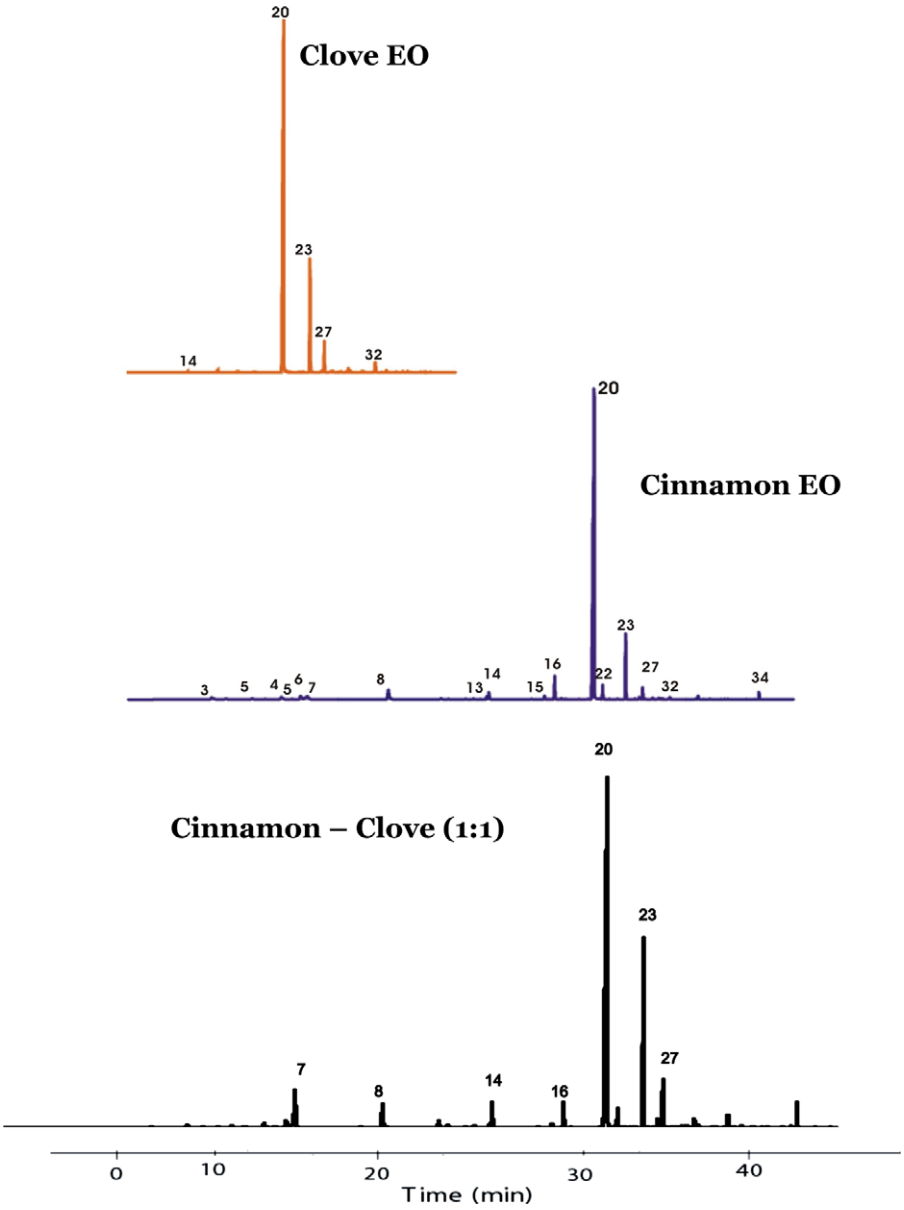

Fig. 2. GC–ITMS chromatograms of the two essential oils (cinnamon (CI) and clove (CL)) and their combination (CI–CL) evaluated in this study as antimicrobial agents in the vapour phase. For peak identification, see Table 4.

Shawcross, Dawson, & Dunn, 2004). Whereas *Y. enterocolitica* and *S. choleraesuis* were the most sensitive strains and showed similar behaviour when in direct contact, *S. choleraesuis* was more resistant to the vapours. These differences may be explained by considering the physicochemical properties of the antimicrobial agents and the culture media and how the contact between the microorganism and the agent occurs. The antimicrobial effect in direct-contact experiments is mostly due to the activity of the more hydrophilic (water-soluble) and less volatile substances, whereas an equilibrium is attained during the vapour-phase experiments among the volatile compounds released in the headspace (both hydrophilic and hydrophobic) and part of the more hydrophilic ones, absorbed in the agar surface. The agar diffusion method is considered unsuitable in estimating the antimicrobial activity of EOs since the active volatile components are likely to be evaporated, together with the dispersing solvent, and their apolar nature prevents them from diffusion through the agar media (Kalemba & Kunicka, 2003; Kubo, Muroi, & Kubo, 1995). Therefore, the vapour phase experiments are more reliable in determining the antibacterial properties of EOs.

The mode of action of antimicrobial agents depends on the type of microorganism and evidence indicates that in the case of EOs it is mainly associated with cell membrane damage. Their chemical constituents are characteristically hydrophobic and will accumulate in the lipid-rich environments of cell membrane structures and cause structural and functional damage (Cox et al., 2000; Lambert, Skandamis, Coote, & Nychas, 2001; Sikkema, Debont, & Poolman, 1995). However, hydrophobicity and ability to damage cell membrane structures are not the only factors involved (Becerril, Gomez-Lus, Goni, Lopez, & Nerin, 2007) and it is clear that toxicity is linked to an optimum range of hydrophobicity. It has been suggested that aqueous solubility is a factor that limits the extent to which hydrophobic compounds can accumulate to lethal levels in cell membranes (Cox, Mann, & Markham, 2001). Antimicrobial monoterpenes typically have aqueous solubilities ranging from around 800–2000 ppm (Griffin, Wyllie, Markham, & Leach, 1999).

However, it is also clear that other factors should be considered for inhibiting the growth of *P. aeruginosa*, which displays an intrinsic resistance to a wide variety of EOs and their constituents (Cox & Markham, 2007).

The antimicrobial activity of the EOs and their combination in vapour phase is closely associated with the composition of the headspace. The phenolic compounds are widely reported to possess high levels of antimicrobial activity (Baydar, Sagdic, Ozkan, & Karadogan, 2004; Dorman & Deans, 2000; Lambert et al., 2001). Eugenol, which is the main component of the three characterised atmospheres, exhibited both antimicrobial and antifungal activity in the vapour phase (Lopez et al., 2007b; Matan et al., 2006; Valverde et al., 2005). Bactericidal properties of clove oil are comparable to those of disinfectants applied in hospitals and eugenol has been proved to kill even *L. monocytogenes*, *E. coli* and some antibiotic-resistant bacteria (Gill & Holley, 2006; Nostro et al., 2004). The atmosphere of cinnamon EO and hence the atmosphere of the cinnamon–clove combination, comprised other compounds in trace levels, such as cinnamaldehyde, a known powerful antimicrobial agent (Lopez et al., 2007b; Valero & Giner, 2006), and linalool, 1,8-cineole, *p*-cymene and α-pinene (Bagamboula, Uyttendaele, & Debevere, 2004; Belaiche, TantaouiElaraki, & Ibrahimy, 1995; Santoyo et al., 2005), which are less effective in inhibiting the growth of microorganisms.

The minimum inhibitory concentration (*MIC*) is cited by most researchers as a measure of the antimicrobial performance of EOs. Among all the different definitions found in literature (Burt, 2004), the concepts applied in this work were the lowest concentration inhibiting visible growth of the test organism (here defined as *MIC*) and the lowest concentration required for complete inhibition (here defined as C_{max}). Given that the effect of the mixture of cinnamon and clove EO (synergistic, antagonistic or additive) depended on the concentrations of the single EOs (see Fig. 1), it is important to specify which value (either *MIC* or C_{max}) was used to calculate the *FIC* index. Using *MIC* values, the mixture of cinnamon and clove EO (1:1, v/v) exhibited a clear antagonistic effect against *E. coli*, whereas it revealed a synergistic effect against *Y. enterocolitica*, *L. monocytogenes* and *B. cereus* when C_{max} was used. Burt (2004) also suggested that the minor components of EOs are more critical to the activity than mixtures of the main EO components, and may have either an additive or synergistic effect. For instance, terpinen-4-ol is thought to diffuse into and damage cell membrane structures, causing increased fluidity or disordering membrane structure and inhibition of membrane-bound enzymes (Sikkema et al., 1995). Camphor and 1,8-cineole, whose concentration in the mixture headspace was five times greater than for the single cinnamon EO, could contribute to the enhancement of eugenol activity, since the oxygenated terpenes are believed to present a higher activity than the terpene hydrocarbons (Caccioni & Guizzardi, 1994; Knobloch, Pauli, & Iberl, 1989).

There are some generally accepted mechanisms of antimicrobial interaction that produce synergism: sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, combinations of cell wall active agents, and use of cell wall active agents to enhance the uptake of other antimicrobials (Santiesteban-Lopez, Palou, & López-Malo, 2007). Mechanisms of antimicrobial interaction that produce antagonism are less known, although they include combinations of bactericidal and bacteriostatic agents, use of compounds that act on the same target of the microorganism and chemical (direct or indirect) interactions among compounds. For instance, non-oxygenated monoterpene hydrocarbons such as γ-terpinene and *p*-cymene appear to produce antagonistic effects, since they reduce the aqueous terpene solubility and, therefore, the microbial availability of the active components (Cox et al., 2001). Therefore, specific modes of action of plant constituents with antimicrobial properties on the metabolic activities of microorganisms still need to be clearly defined, even when the antimicrobials are used individually (Alzamora, López-Malo, Guerrero, & Palou, 2003).

All the tested combinations were prepared at 50% in volume, but the study of the effect with different proportions is now in progress. It is remarkable the low probability of appearance of resistances, as was demonstrated by the fact that some bacteria, like *Y. enterocolitica* or *S. Choleraesuis*, were not able to develop resistance after more than 20 passes at subinhibitory concentrations of cinnamon, either by direct contact or by vapour phase (data not shown).

5. Conclusion

This work enables us to evaluate and compare the antimicrobial activity of the combination of cinnamon and clove essential oils at 50% in volume against a wide range of bacteria in vapour phase. The results showed that a synergistic effect could be achieved for some of the tested microorganisms, this effect being concentration-dependent.

The experimental results also provide the first approach to developing an antimicrobial packaging with less active concentrations of the active essential oils. This fact is of paramount importance from the point of view of food safety and food organoleptic properties.

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