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Antimicrobial Activity of Plant Essential Oils Using Food Model Media: Efficacy, Synergistic Potential and Interaction with Food Components

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Title: **"Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components"**

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Running Title: **Antibacterial efficacy of plant EO's in food model media**

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Abstract

The aim of this study was to optimize the antimicrobial efficacy of plant essential oils (EO's) for control of *Listeria* spp. and spoilage bacteria using food model media based on lettuce, meat and milk. The EO's evaluated were lemon balm, marjoram, oregano and thyme and their minimum inhibitory concentrations (MIC) were determined against *Enterobacter* spp., *Listeria* spp., *Lactobacillus* spp., and *Pseudomonas* spp. using the agar dilution method and/or the absorbance based microplate assay. MICs were significantly lower in lettuce and beef media than in TSB. *Listeria* strains were more sensitive than spoilage bacteria, and oregano and thyme were the most active EO's. EO combinations were investigated using the checkerboard method and Oregano combined with thyme had additive effects against spoilage organisms. Combining lemon balm with thyme yielded additive activity against *Listeria* strains. The effect of simple sugars and pH on antimicrobial efficacy of oregano and thyme was assessed in a beef extract and tomato serum model media. EO's retained greater efficacy at pH5 and 2.32% sugar, but sugar concentrations above 5% did not negatively impact EO efficacy. In addition to proven antimicrobial efficacy, careful selection and investigation of EO's appropriate to the sensory profile of foods and composition of the food system is required. This work shows that EO's might be more effective against food-borne pathogens and spoilage bacteria when applied to foods containing a high protein level at acidic pH, as well as moderate levels of simple sugars.

1. Introduction

Illnesses caused due to the consumption of foods contaminated with pathogens such as *Listeria monocytogenes* has a wide economic and public health impact worldwide (Gandhi and Chikindas 2007). *L. monocytogenes* can adapt to survive and grow in a wide range of environmental conditions as well as in a large variety of raw and processed foods, including milk and dairy products, various meats and meat products or fresh produce. Food spoilage includes physical damage, chemical changes, such as oxidation, color changes, or appearance of off-flavors and off-odors resulting from microbial growth and metabolism in the product (Gram et al. 2002). The spoilage of refrigerated meat is caused in part by *Pseudomonas* species which are responsible for the off-odors, off-flavors, discoloration, gas production and slime production (Oussalah et al. 2006a). In some cases, a change in atmosphere by vacuum-packing inhibits the aerobic pseudomonads in meats causing a shift in the microflora to lactic acid bacteria (LAB) and Enterobacteriaceae (Gram et al. 2002). The pseudomonads are also found in pasteurized milk and are generally from post-process contamination (Eneroth et al. 2000). The spoilage microflora associated with fresh vegetables includes *Pseudomonas* spp. as well as other Gram-negative bacteria, such as Enterobacteria (Ragaert et al. 2007). Current technologies for preservation and shelf life extension of food include chemical preservatives, heat processing, modified atmosphere packaging (MAP), vacuum packaging (VP) or refrigeration. Unfortunately, these steps do not eliminate undesirable pathogens such as *L. monocytogenes* from these products or delay microbial spoilage entirely. Alternative preservation techniques such as novel non-thermal technologies and

naturally derived antimicrobial ingredients are under investigation for their application to food products.

Greater consumer awareness and concern regarding synthetic chemical additives has led researchers and food processors to look for natural food additives with a broad spectrum of antimicrobial activity (Marino et al. 2001). In this context, plant essential oils are gaining interest for their potential as preservative ingredients or decontaminating treatments, as they have GRAS status and a wide acceptance from consumers (Burt et al. 2004). The antimicrobial components are commonly found in the essential oil fractions and it is well established that many have a wide spectrum of antimicrobial activity, with potential for control of *L. monocytogenes* and spoilage bacteria within food systems (Smith-Palmer et al. 1998, Hammer et al. 1999, Elgayyar et al. 2001, Dorman and Deans 2002, Moreira et al. 2005, Oussalah et al. 2006b, Gutierrez et al., 2008a). Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) are amongst the most active EO's, while lemon balm (*Melissa officinalis*) and marjoram (*Origanum majorana*) display a good antimicrobial activity against Gram-positive and Gram-negative bacteria, respectively. Recently, some researchers have reported the efficacy of plant EO´s as antimicrobial agents against food borne pathogens and spoilage microflora in meat (Busatta et al., 2008; Carramiñana et al., 2008). Although some studies have shown that plant extracts are useful for reduction of pathogens associated with meat (Mytle et al. 2006, Ahn et al. 2007), others reported very low antimicrobial activity or no effect against *L. monocytogenes* or *Salmonella* when EO's were applied to beef or chicken (Uhart et al. 2006, Firouzi et al. 2007). Thus, the application of plant EO's for control of food-borne pathogens and food spoilage bacteria requires the evaluation of efficacy

within food products or in model systems that closely simulate food composition. In general, the efficacy of many added and naturally occurring antimicrobials may be reduced by certain food components (Glass and Johnson 2004). Therefore, to successfully apply EO's in food systems, primary studies in representative food model media should be employed to determine potential interactions between EO's and food components that could impact on their antimicrobial efficacy.

Another aspect for the optimized application of EO's in foods is the impact on sensory acceptability. If high concentrations are required to achieve useful EO antimicrobial activity, unacceptable levels of inappropriate flavours and odours may result. We previously reported that lettuce samples treated with thyme and lemon balm at concentrations of 500 and 1,000 ppm, respectively, were rejected by panelists as they perceived strong chemical odors from these samples (Gutierrez et al. 2008a). Therefore, research in this area should be focused on optimizing EO combinations and applications to obtain effective antimicrobial activity at sufficiently low concentrations so as not to adversely affect the organoleptic acceptability of foods. Furthermore, the use of antimicrobials can reduce or eliminate target microorganisms but it may also produce favorable conditions for other microorganisms (Davidson and Branen 2005). It is recognized that this situation is less likely to develop towards substances that have more than one mode of action (Ippolito and Nigro 2003). It is suggested that the antimicrobial activity of EO's is attributed to more than one mechanism (Burt 2004, Moreira 2005). Thus, combining EO's could lead to useful efficacy against both spoilage and pathogenic target organisms. Whole plant extracts have a higher antimicrobial activity than when

major components are mixed, and minor components of plant EO's may be critical to activity with potentiating influence or synergistic effects (Burt 2004).

Thus, the main objectives of this work were: (i) to evaluate the antimicrobial activity of plant essential oils (EO's) against *Listeria* spp. and spoilage bacteria in food model media, in order to optimize product application, (ii) to assess the efficacy of EO's in combination against selected bacteria to determine potential for their synergistic application at low doses; and (iii) to monitor and quantify the effect of food components on the EO efficacy. The sensitivity of different antimicrobial assays was also assessed and compared in order to select those that were the most suitable to calculate MICs.

2. Material and methods

2.1. Essential oils

The essential oils (EO's) used in this study were lemon balm (*Melissa officinalis*), marjoram (*Origanum majorana*), oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*). They were selected based on previously reported efficacy (Gutierrez et al. 2008a), and were obtained from Guinness Chemical Ltd. (Portlaoise, Ireland) as CO_2 soluble supercritical fluid extracts.

2.2. Bacteria

The bacteria used in this study are listed in Table 1. All cultures were maintained at -70ºC in 20% glycerol and grown in Tryptic Soy Broth (TSB, pH 7.2, Scharlau Chemie) for 24 hours at 30ºC, except for the *Listeria* strains, which were incubated at 37ºC, in order to

obtain sub-cultures. Working cultures were prepared in selected model media from sub-cultures and grown under optimal conditions for each bacterium for 24 hours. Working cultures were adjusted to the required concentration of 10^6 CFU/ml using the McFarland standard (Biomerieux Inc.).

2.3. Food model media

Lettuce leaf model media (L) was prepared as described by Francis et al. (1998) but with some modifications. 50g of iceberg lettuce (*Lactuca sativa* sp.) were added to 100ml of sterile deionized water and shaken for 1 min. The suspension was filtered using 18.5 cm Whatman filters and pH was adjusted from 5.6 to 7.2 by mixing two parts of lettuce media with one part 0.3M potassium phosphate buffer, giving a final concentration of 0.1M phosphate buffer, pH 7.2. The buffered medium was then autoclaved at 121ºC for 15 min. To investigate the EO efficacy in meat-based model media, experiments were performed with autoclaved beef extract (BE, 12% protein, Scharlau Chemie). Milk model media (M) was made mixing skimmed milk powder (Scharlau Chemie) with agar solution (Scharlau Chemie), both autoclaved separately, in order to obtain a final solid media solution with 1.5% agar. Beef extract and milk model media were adjusted to pH 7.2 to separate pH effects.

2.4. Kinetic analyses

Bacteria which were grown in TSB, lettuce leaf model media or beef extract (Table 1), were monitored in a microplate spectrophotometer (PowerWave, Biotek) at 600 nm over 24 h at 30 min intervals. Growth curves were analyzed using Gen5 software (Biotek) and

the increase in lag phase (λ) and the maximum specific growth rate (μmax) were calculated. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, U.S.A). Data represent the means of experiments performed in duplicate and replicated at least twice. Differences between bacteria were analyzed by ANOVA followed by LSD ($p < 0.05$). Differences between control media (TSB) and model media were examined using paired sample t-tests ($p < 0.05$).

2.5. Antimicrobial assays

The Agar-well Diffusion Test (ADT), Agar Dilution Method (ADM) and absorbance based Microtitre Plate Assay (MPA) were used to determine the MICs of selected EO's. MICs were considered as the lowest concentration of the EO resulting in a complete inhibition of growth and were obtained from at least 3 different experiments and expressed in ppm. Differences between antimicrobial assays were analyzed by ANOVA followed by LSD ($p < 0.05$).

2.5.1. Agar-Well Diffusion Test (ADT)

The ADT was performed as previously described (Bagamboula et al. 2004, Schelz et al. 2006) but with some modifications. 20 ml of Tryptic Soy Agar (TSA, pH 7.2, Scharlau Chemie) were inoculated with 10^6 CFU/ml of the indicator strain and then poured onto a Petri dish and allowed to solidify. Wells of 6.5-mm diameter were aseptically bored into the agar, and 50 μl of serially-diluted EO solutions in ethanol, were added to the wells. The plates were kept at 4^oC for 2 h to allow dispersal and subsequently incubated under optimal conditions for growth of the target strains. The antimicrobial activity was visually

appraised as inhibition zones surrounding the wells. Ethanol was used as negative control and the indicator strains were *L. innocua* NCTC11288 and *P. fluorescens*.

2.5.2. Agar Dilution Method (ADM)

The ADM was performed as described by Hammer et al. (1999) and Oussalah et al. (2006b), but with some modifications. TSA or Milk Model Media were inoculated with the appropriate EO and serially diluted using the same model media to the appropriate concentrations, poured onto a Petri dish and allowed to solidify. Plates were then seeded with 10^2 CFU of the target microorganism, and incubated at the appropriate temperature. The positive control consisted of TSA or Milk Model Media inoculated with the same amount of cells but without any EO, while uninoculated plates containing the EO served as negative control. Target microorganisms were previously grown in TSB or liquid model media to allow the cells to adapt to the food environment. *L. innocua* NCTC11288 and *L. monocytogenes* NCTC11994 were the target *Listeria* strains seeded into TSA and the milk model media, respectively. *P. fluorescens* was selected as target in both media. Plates were evaluated for the presence or the absence of colonies after 24 hours of incubation at conditions optimal for each bacterium.

2.5.3 Absorbance based Microtitre Plate Assay (MPA)

Ninety-six well microtitre plates were used (Sarstedt Ltd) to perform the MPA. This assay was based on previous work (Schelz et al. 2006) but with the following modifications, where aliquots of EO solutions in growth media (200 µl) were added into the first row of a microtitre plate. The remainder of the wells were filled with 100 µl of the appropriate

medium. The EO's were then diluted two fold along each column. Finally, 100 µl of media containing $2x10^6$ CFU/ml of the indicator strain was added to all wells. Positive controls contained growth media inoculated with the organism under investigation. Negative controls contained EO's and sterile growth media only. The plates were then placed in the Biotek microplate spectrophotometer set at the appropriate temperature for each test organism. The absorbance was recorded at 600 nm every 30 minutes over a 24 hour incubation period.

2.6. Synergy studies: checkerboard method

The checkerboard method was performed using 96-well microtitre plates (Schelz et al., 2006) to obtain the fractional inhibitory concentration (FIC) index of EO combinations EO's in the lettuce leaf model media. Plates consisted of columns containing 50 µl of EO_A diluted twofold in lettuce model media along the x axis as well as rows with the same amount of EO_B diluted twofold in the same media along the y axis. Subsequently, 100 µl of the lettuce media containing $2x10^6$ CFU/ml of the indicator strain were added to all wells. Plates were then incubated at 37°C for 24 h. The FIC indices were calculated as $FIC_A + FIC_B$, where $FIC_A = (MIC_A \text{ combination} / MIC_A \text{ alone})$ and $FIC_B = (MIC_B \text{ combination} / MIC_B \text{ alone})$. The results were interpreted as synergy ($FIC < 0.5$), addition ($0.5 \leq FIC \leq 1$), indifference ($1 < FIC \leq 4$) or antagonism ($FIC > 4$). Experiments were performed in triplicate.

Combinations of oregano, thyme, basil and marjoram were tested against spoilage bacteria, whereas mixtures of oregano, thyme, lemon balm and sage were tested against the *Listeria* strains. Concentrations used for the combinations were based on MIC values

obtained in lettuce leaf model media and assays were performed in duplicate and then replicated.

2.7. Interactive effects of food ingredients and pH in beef extract and tomato serum media (BE-TS)

The effect of food ingredients and pH on the antimicrobial efficacy of EO's was performed using a range of model media consisting of beef extract mixed with tomato serum (Scharlau Chemie) at different ratios (Table 2). The concentrations of protein, fat and salt were suitable for optimal EO efficacy (Gutierrez et al., 2008b), while percentage of carbohydrates, mainly composed of glucose and fructose, increased from 0 to 11.6% and the pH range was from 7.06 to 4.43. *L. monocytogenes* NCTC1194, *L. sakei* ATCC15521 and *P. putida* were chosen as target microorganisms. The growth of selected bacteria in each model medium with EO was monitored using the 96 well-microplates, which were performed and assessed in the Biotek microplate spectrophotometer. A second batch of experiments was performed in the same model media but adjusted to pH 7.2. The effect of food components on EO efficacy was evaluated considering the MIC and the growth parameters of target bacteria, as described in sections 2.4 and 2.5.3, respectively. Positive controls contained model media inoculated with the organism under investigation. Negative controls contained EO's and sterile model media only.

3. Results

3.1. Kinetic analysis in food model media

1 The lag phase and μmax of bacteria grown in TSB, lettuce media or BE are shown in
2 Table 3. Bacterial growth was a function of the media used. The lag phase and μmax of
3 all bacteria grown in lettuce media was longer and lower respectively, than in TSB or BE
4 ($p < 0.05$). In general, no significant differences were observed between lag phase and
5 growth rates values of bacteria grown in TSB and BE ($p < 0.05$). Growth rate of the
6 reference strain *L. monocytogenes* NCTC1194 was significantly lower ($p < 0.05$) in BE
7 than in TSB. In lettuce media, the lag phase of spoilage bacteria was considerably shorter
8 than that of *Listeria* spp. ($p < 0.05$). Growth rates of all bacteria cultured in lettuce media
9 were similar, whereas in TSB growth rates of spoilage organisms were lower than those
10 for *Listeria* strains ($p < 0.05$).
11
12 *3.2. Sensitivity of antimicrobial assays*
13 When MICs of selected EO's were compared using 3 different antimicrobial assays
14 (MPA, ADM and ADT), no significant differences were observed between MICs of
15 oregano, thyme or lemon balm tested by MPA and ADM (Table 4). Furthermore, the
16 MICs of oregano and thyme against both target microorganisms as well as those of lemon
17 balm against the *Listeria* strain, were significantly lower ($p< 0.05$) using MPA or ADM
18 than those recorded by ADT. When indicator strains were exposed to marjoram only, the
19 MICs calculated by ADT were the same as those observed by MPA or ADM. Therefore,
20 ADM and MPA protocols were selected as most appropriate for calculating MICs in solid
21 and liquid food model media, respectively.
22
23 *3.3. Antimicrobial efficacy in food model media (MPA method)*

The MIC values obtained for each EO in TSB, lettuce leaf model media and beef extract are presented in Table 5. The average efficacy of EO's against *Listeria* spp. was in the following order: oregano ≥ thyme > lemon balm, while the efficacy order of EO's against the spoilage bacteria was: oregano ≥ thyme > marjoram. When *P. fluorescens* and the *Listeria* spp. were exposed to the EO's in lettuce media, the MIC values were approximately 10 fold lower than in TSB for all EO's. However, when *E. cloacae* was exposed to EO's within TSB or vegetable model media, the MIC values were comparable. *E. cloacae* was more susceptible to the EO's than *P. fluorescens* in TSB. In BE, MICs of EO's against *Listeria* spp. were significantly lower ($P < 0.05$) than in TSB. MICs of lemon balm against the food-borne pathogen in BE were comparable to those observed in the vegetable media. MICs of oregano and thyme against *Pseudomonas* spp. in BE were similar to those found in TSB, whilst the MIC of marjoram against the same spoilage bacteria was significantly lower ($p<0.05$) in BE than in TSB. *Listeria s*trains were always more sensitive than the spoilage bacteria.

Furthermore, when *L. monocytogenes* NCTC11994 and *P. fluorescens* were exposed to oregano or thyme on milk model media (M), it was observed that the MICs of these EO's were approximately 10 fold higher than those obtained on the control media TSA. MICs of oregano and thyme against the *Listeria* strain on the milk model media were 1,000 and 3,000 ppm respectively. *P. fluorescens* was more resistant to both oregano and thyme on same food model media, with corresponding MICs of 10,000 and 20,000 ppm, respectively.

3.4. Synergy studies

The FIC indices for the EO combinations in lettuce leaf model media are shown in Table 6. With reference to the FIC scale, no synergistic effect (< 0.5) was found, but addition occurred with a number of combinations. More incidences of additive effects were found with EO combinations against *Listeria* strains. Combinations of oregano with thyme or lemon balm were more effective against *L. monocytogenes*. The combination of thyme with lemon balm had greater efficacy against *L. innocua*. Only one combination (oregano with thyme) had additive effects against both spoilage microorganisms. No antagonism was observed for any of the combinations evaluated.

3.5. Influence of BE-TS model media composition on bacterial growth

As shown in figure 1, the μmax of *L. monocytogenes* and *P. putida* increased significantly ($p < 0.05$) when grown in medium B (pH 6.09, 1.16% sugars, see Table 2) than in medium A (pH 7.06; 0% sugars). A similar trend was observed when *L. sakei* grew in medium C (pH 5.92; 2.32% sugars), by comparison with medium B. On the contrary, the μmax values of *L. monocytogenes* and *L. sakei* grown in medium D (pH 5.32; 5.80% sugars) were significantly lower ($p < 0.05$) than those obtained in medium C. Considering the lag phase of selected bacteria, no significant differences were observed between media A and B. However, the lag phase values of *L. monocytogenes* and *L. sakei* grown in media D and C, respectively, were significantly longer ($p < 0.05$) than those obtained in medium C, for *L. monocytogenes*, or medium B, for *L. sakei*. The opposite was observed for *P. putida* since its lag phase was significantly reduced ($p < 0.05$) in medium C, compared to media B or A. None of the target micro organisms were capable of growing in model medium E (pH 4.43; 11.6% sugars). *P. putida* was also unable to grow in medium D.

3.6. Influence of BE-TS model media composition on EO efficacy

The EO efficacy increased significantly ($p < 0.05$) in BE-TS model media containing a major percentage of sugars as well as more acidic pH values (Table 7). However, the MICs of oregano or thyme against *P. putida* were the same ($p < 0.05$) when tested in the different food model media. Growth experiments with the selected bacteria were also performed in the same BE-TS model media but adjusted to pH 7.2, in order to investigate the effect of sugars on the EO antimicrobial activity. In general, the μmax of the cultures exposed to oregano or thyme decreased when the percentage of sugars increased (Fig. 2). Moreover, when *L. monocytogenes* was grown in medium B (1.16% sugars) containing the EO's, the growth rate values increased, by comparison with those recorded in medium A (0.00% sugars). Similar trends were observed with controls. However, the μmax of *Listeria* cultures in medium C (2.32% sugars) with oregano or thyme was lower ($p < 0.05$) than that observed in medium B with the same EO's. The growth rate values of *Listeria* control cultures in media B and C were not significantly different ($p < 0.05$). When *L. sakei* and *P. putida* were exposed to thyme in media B and C, respectively, the μmax decreased ($p < 0.05$) compared to those obtained in medium A, for *L. sakei*, and medium B, for *P. putida*. With respect to the control cultures, there was no significant difference in the growth rate of *L. sakei* in media A and B as well as that of *P. putida* in media B and C ($p < 0.05$). In general, the lag phase of cultures grown in neutralized model media regardless of presence or absence of EO's increased significantly ($p < 0.05$) in medium E (Fig. 3). Furthermore, inclusion of oregano or thyme led to a significantly

longer lag phase with 0 to 2.32% of sugars, by comparison with control ($p < 0.05$). The lag phase of *P. putida* grown in model medium C containing oregano was longer than in medium B. When the same bacterium was exposed to thyme, the lag phase increased significantly in medium B, by comparison with medium A. In the control cultures, no significant differences were observed between lag phase values in media A, B and C.

4. Discussion

Most researchers currently use agar or broth dilution series to assess antimicrobial activity of spices, herbs and their EO's, and in some cases both assays for comparative purposes because antimicrobial performance in the two systems can vary (Holley and Patel 2005). In this work, no significant differences were observed between MIC values using the Microplate Assay (MPA) or the Agar Dilution Method (ADM). Furthermore, these methods proved to be more sensitive than the Agar well-Diffusion Test (ADT). Although tube macrodilution and diffusion from inhibitor-impregnated paper discs on agar surfaces are still used, there is heavy reliance on microwell plate systems containing inhibitors and target microorganisms in broth. Some authors have suggested that the agar well/disk diffusion tests might only be used as a selection method when large numbers of EO's and or bacterial isolates have to be screened, since the comparison of published data are not feasible (Dorman and Deans 2000, Burt 2004). The hydrophobicity of EO components is known to limit the value of these diffusion tests for estimating antimicrobial potency accurately (Holley and Patel 2005). Although several substances have been used to dissolve the EO or to stabilize it in water-based culture media, such as ethanol, methanol, Tween-20, Tween-80, acetone, polyethylene glycol, propylene glycol, n-hexane, dimethyl sulfoxide or

1 agar (Burt 2004), we did not find any improvement on the EO efficacy by using some of
2 these substances, in agreement with other researchers, such as Smith-Palmer et al. (1998),
3 Dorman and Deans (2000) or Elgayyar et al. (2001).

4 Over the last decade many tests have been carried out in synthetic growth media in
5 order to evaluate the EO antimicrobial activity against spoilage and food-borne pathogens
6 associated with meat, milk and vegetables. However, results obtained in model media
7 may be more useful prior to further studies on real food, rather than those observed using
8 standard laboratory media, since these product liquid models may assist in the optimised
9 final application of EO's and would also reflect the nutrient availability and composition
10 of food produce. In this respect, some authors have already used fruit and vegetable
11 model media to investigate EO efficacy (Cerrutti and Alzamora 1996, Del Campo et al.
12 2000, Hsieh et al. 2001, Ultee and Smid 2001, Valero and Salmeron, 2003). In most of
13 these cases the plant extracts efficacy' decreased in the food model media, by comparison
14 with the in vitro control media In this study, the antimicrobial efficacy of plant EO's was
15 evaluated in different food model media and compared to that observed in lab control
16 media (TSB) using their MIC values against spoilage bacteria and *Listeria* spp.

17 Since food system composition is known to impact on the antimicrobial efficacy of
18 EO's, Burt (2004) suggested that the low fat content of vegetables may contribute to the
19 success of EO's in fresh produce. In most cases the efficacy of EO's in lettuce model
20 media was 10 fold times higher than that in TSB (Table 5). The fact that the lag phase
21 and the growth rate of all bacteria in lettuce media was longer and lower respectively, by
22 comparison to those observed within TSB (Table 3), may have contributed to the higher

efficacy of EO's in the vegetable media. The rich nutrients in TSB compared to lettuce media may enable bacteria to repair damaged cells faster, as suggested by Gill et al. (2002). However, the EO's were more effective in BE than in TSB and the MIC of lemon balm in the meat based model media was comparable to that obtained in lettuce media. Gutierrez et al. (2008b) observed that the presence of high concentrations of protein in BE promoted the growth of *L. monocytogenes*, but the efficacy of oregano and thyme was also greater at these higher concentrations of protein. These authors explained that peptones with hydrophobic properties might display interactions with EO's to facilitate their dissolution in BE. Baranauskien et al. (2006) reported that proteins usually possess a high binding capacity for flavor volatile compounds.

Recently, some studies have recorded the EO antimicrobial efficacy, alone or in combination with other preservation methods, against spoilage and food-borne pathogens when applied to meat (Mytle et al. 2006, Ahn et al. 2007, Ghalfi et al. 2007, Solomakos et al. 2008) or milk (Cava et al. 2007). Particularly, Careaga et al. (2003) observed that chilli extracts (*Capsicum annuum*) had a bacteriostatic effect against *P. aeruginosa* at concentrations of 3,000 ppm. In this study the MICs of oregano and thyme against the *Pseudomonas* strains were 1,500 and 2,500 ppm, respectively, in BE. When Cava et al. (2007) assessed the antimicrobial activity of EO's of cinnamon bark, cinnamon leaf, and clove against *L. monocytogenes* in semi skimmed milk incubated at 7°C for 14 days, they observed that the MIC was 500 ppm for cinnamon bark EO and 3,000 ppm for the cinnamon leaf and clove EO's. Concentrations increased to 1,000 ppm for cinnamon bark EO, 3,500 ppm for clove EO, and 4,000 ppm for cinnamon leaf EO when the semi skimmed milk was incubated at 35°C for 24 h. The antimicrobial efficacy of oregano and

thyme against *L. monocytogenes* in the milk model media used in this work was very similar, with corresponding MICs of 1,000 ppm and 3,000 ppm, respectively. The EO's possessing the strongest antibacterial properties are usually composed of phenolic compounds, such as eugenol (clove, cinnamon leaf), cinnamic acid (chilli, cinnamon bark), carvacrol (oregano) or thymol (thyme) (Burt 2004, Holley and Patel 2005), thus it seems reasonable that their mechanism of action and antimicrobial efficacy would be similar.

Oregano and thyme were the most effective EO's for inhibition of *Listeria* and spoilage organisms in all the food model media (Table 5). Marjoram also displayed a high antimicrobial activity against the Gram-negative bacteria, while lemon balm had good efficacy against the Gram-positive *Listeria* spp. (Table 5). The high antimicrobial activity of marjoram against Gram-negative bacteria might be due to the presence of hydroxyl groups in EO compounds, as described previously (Elgayyar et al. 2001, Burt 2004, Oussalah et al. 2006b). Longaray Delamare et al. (2005) attributed the strong activity of sage against Gram-positive bacteria to the presence of β-caryophyllene, a compound that is found in the composition of the lemon balm EO's used in this study.

Plant EO's are generally more active against gram-positive bacteria than gram-negative bacteria (Burt 2004). Some authors suggest that the outer membrane surrounding the cell wall of gram-negative bacteria may restrict diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara 1992, Davidson and Branen 2005). In the current work, gram-negative strains, *P. fluorescens*, *P. putida* and *E. cloacae* were more resistant to the action of the EO's than the Gram-positive *Listeria* spp.

(Table 5). As the lag phase values of both *Listeria* strains in lettuce media were much longer than those obtained for spoilage organisms (Table 1), this may have also promoted the efficacy of the EO's. However, the MIC values for oregano and thyme against *Listeria* spp. were similar to those observed with the same EO's against *E. cloacae* (Table 5) in TSB. The growth rate of *E. cloacae* in TSB was approximately 2 fold lower than those attained by the *Listeria* strains. Although the growth rate of *P. fluorescens* was not significantly different to that for *E. cloacae*, the *Pseudomonas* strains were the most resistant to oregano and thyme in TSB (Table 1). *Pseudomonas* spp. are known to show consistently high resistance to plant antimicrobials (Hammer et al. 1999, Holley and Patel 2005). However, both *E. cloacae* and *P. fluorescens* had the same sensitivity to the EO's in the lettuce model media (Table 5). Both of these spoilage organisms were isolated from lettuce and the lag phase for *E. cloacae* within the vegetable media was shorter than that of *P. fluorescens* (Table 3).

Combinations of EO's were assessed for synergistic activity at lower concentrations in order to reduce undesirable impacts on organoleptic properties of food (Table 6). No synergy as described by FIC indices was observed in lettuce model media but an important number of combinations displayed additive effects at very low concentrations, such as oregano combined with thyme against spoilage bacteria and thyme in combination with lemon balm against *L. innocua*. Some studies have concluded that whole EO's have a greater antibacterial activity than the major components mixed (Gill et al. 2002, Mourey and Canillac 2002). Burt (2004) suggested that the minor components present in the EO's extracts are more critical to the activity than EO main components mixed, and may have synergistic effects or a potentiating influence. As many plant EO's

possess compounds with similar structures, their combinations may exhibit additive rather than synergistic effects. Furthermore, as the EO efficacy also depends on lipophilic properties, potency of functional groups or their aqueous solubility (Dorman and Deans 2000), the mixture of compounds within whole EO's may contribute to that "additive" effect.

Furthermore, since another important aspect for the optimised application of EO's in food is the evaluation of interaction with food ingredients, five different model media were prepared using beef extract and tomato serum in order to assess and quantify the effect of pH and sugars on the antimicrobial efficacy of oregano and thyme. In general, the antimicrobial activity of these EO's increased when the pH decreased. Previously, it was also observed that the inhibitory effect of plant extracts was greater at acidic pH values (Del Campo et al. 2000, Hsieh et al. 2001). The susceptibility of bacteria to EO's appears to increase with lower pH values since the hydrophobicity of EO's increases at low pH, consequently enabling easier dissolution in the lipids of the cell membrane of target bacteria (Juven et al. 1994). The major efficacy of EO's at pH 5.32 or 5.92 was confirmed with the lag phase and growth rate results at these pHs, which were longer and lower, respectively, than at higher pH levels. As the pH was reduced, the lag phase increased and the growth rate declined for *Listeria* and *L. sakei*, and consequently, the addition of either oregano or thyme enhanced the EO efficacy. However, no significant differences were observed between lag phase and growth rate values of *L. sakei* at pH 7.06 or 6.09 but the MICs of selected EO's decreased at more reduced pH. The same trend was observed with *P. putida* although maximum specific growth rate and lag phase

values increased and decreased, respectively, at more acidic pH. Thus, EO efficacy may also have been promoted by the presence of sugars.

The increase of sugars percentage up to 2.32% seemed to improve the antimicrobial efficacy of oregano and thyme. Moreover, the presence of high concentrations of carbohydrates (5.80 or 11.6%) did not have any negative impact on the EO efficacy, in agreement with the general observation that carbohydrates in foods do not protect bacteria from the action of EO's as much as fat and protein do (Shelef et al. 1984). However, Gutierrez et al. (2008b) reported a protective effect of carbohydrate for bacteria where starch at 5 or 10% had a negative impact on the antimicrobial activity of oregano and thyme. Therefore, EO application should be orientated to food products containing more simple sugars than complex carbohydrates.

This work shows a method for the evaluation of the antimicrobial activity of EO's in food model media prior to optimised further application in real food, as well as a link between organoleptic impact, food composition and EO efficacy. Both agar and broth dilution antimicrobial assays were suitable to calculate MICs of selected EO's against *Listeria* and spoilage bacteria in vegetable, meat or milk based model media, which might be the first step in order to approach optimising EO efficacy when applied to food. On the other hand, oregano and thyme and their combination could have potential for controlling spoilage bacteria in fresh product challenge studies. Combinations of lemon balm with thyme might be useful to reduce the presence of or control *Listeria* spp. in final products. Our results show that EO combinations acted against pathogens and natural spoilage microflora and therefore have potential for use at combined low concentrations to assist in reduction of the sensory impact associated with high concentrations of EO's in food.

Thus, potential combinations that may address spoilage, shelf life as well as safety concerns associated with ready to use foods should be evaluated using product challenge studies. These should incorporate standard processing steps to ensure their efficacy in real systems as well as concurrent sensory analysis.

Furthermore, the antimicrobial efficacy of the EO's in this study was found to be a function of ingredient manipulation. The antimicrobial activity of oregano and thyme was increased at high concentrations of protein and acidic pH conditions. Concentrations above 5% of sugars did not reduced EO efficacy. Therefore, the application of EO's should be further investigated for control of microbial safety and spoilage concerns in proteinaceous foods and/or foods containing simple sugars with low pH values, which may promote the antibacterial efficacy of EO's. The retention of anti-microbial efficacy of EO's within suitable food systems should be evaluated alone as well as taking hurdle effects of other preservation methods into account.

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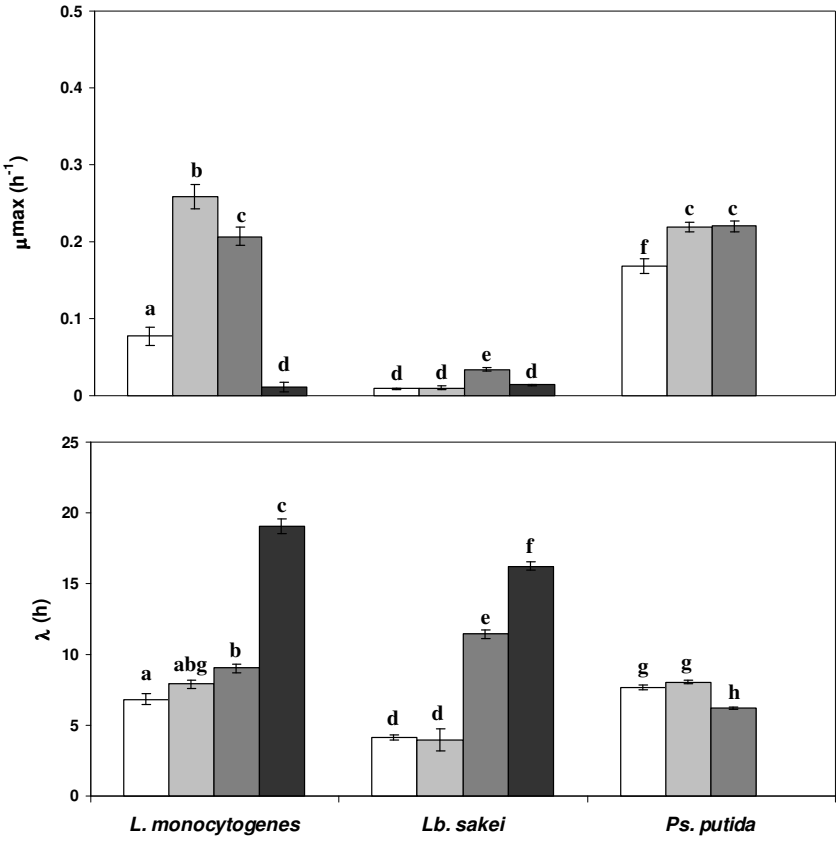
Figures legend

Fig.1. Maximum specific growth (μmax) rate and lag phase (λ) of *L. monocytogenes* NCTC1194, *Lb. sakei* ATCC15521 and *Ps. putida* grown in beef extract and tomato serum model media A (□, pH 7.06), B (▫, pH 6.09), C (▪, pH 5.92), D (■, pH 5.32), and E (■ , pH 4.43). Different letters signify statistical differences between values ($p<0.05$).

Fig. 2. Maximum specific growth rate (μmax) of *L. monocytogenes* (i), *Lb. sakei* (ii) and *Ps. putida* (iii) in neutralized beef extract and tomato serum model media A (□, 0.00% sugars), B (▫, 1.16% sugars), C (▪, 2.32% sugars), D (■, 5.80% sugars), and E (■, 11.60% sugars) containing oregano (31.25 ppm) or thyme (62.5 ppm). Different letters signify statistical differences between values ($p<0.05$).

Fig. 3. Lag phase (λ) of *L. monocytogenes* (i), *Lb. sakei* (ii) and *Ps. putida* (iii) in neutralized beef extract and tomato serum model media A (□, 0.00% sugars), B (▫, 1.16% sugars), C (▪, 2.32% sugars), D (■, 5.80% sugars), and E (■, 11.60% sugars) containing oregano (31.25 ppm) or thyme (62.5 ppm). Different letters signify statistical differences between values ($p<0.05$).



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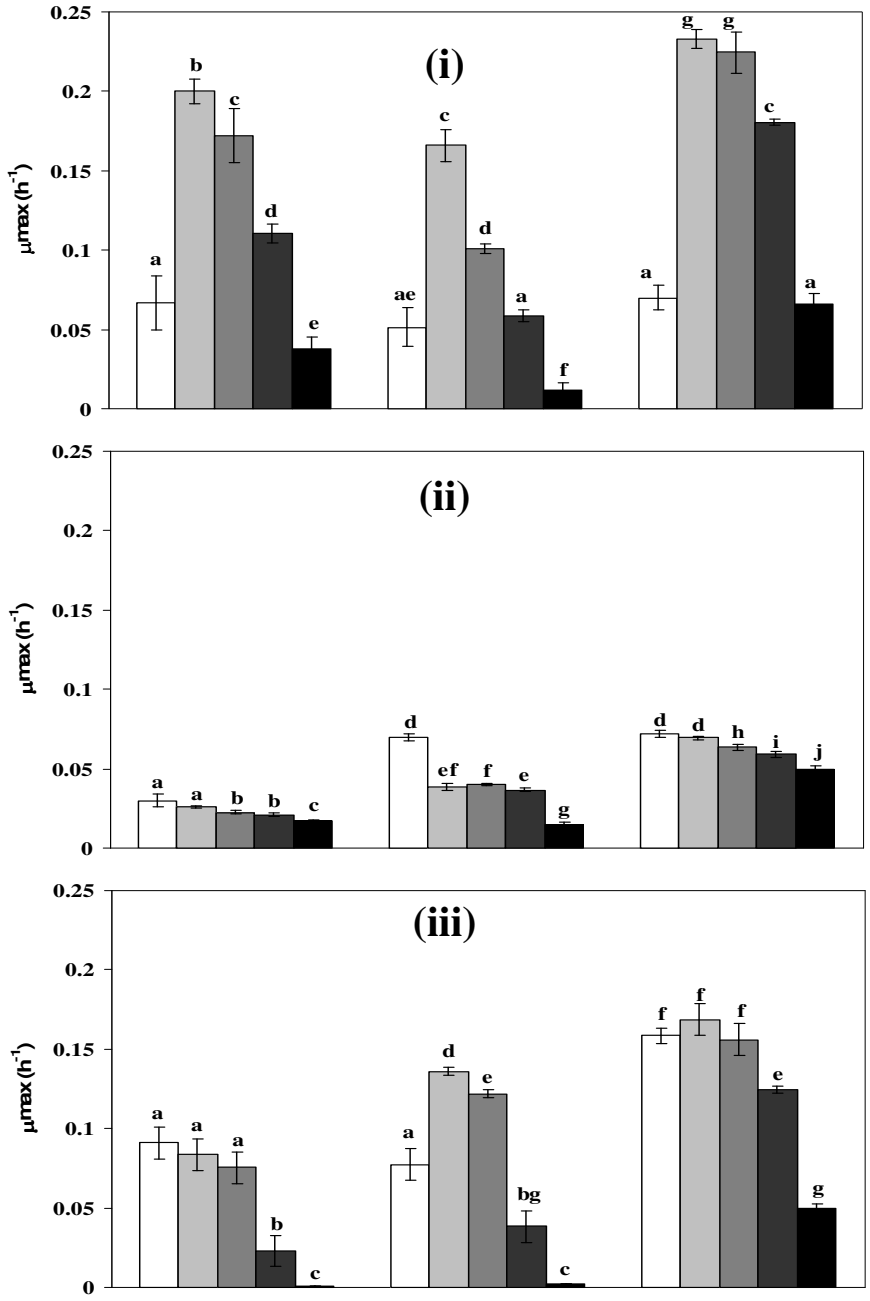

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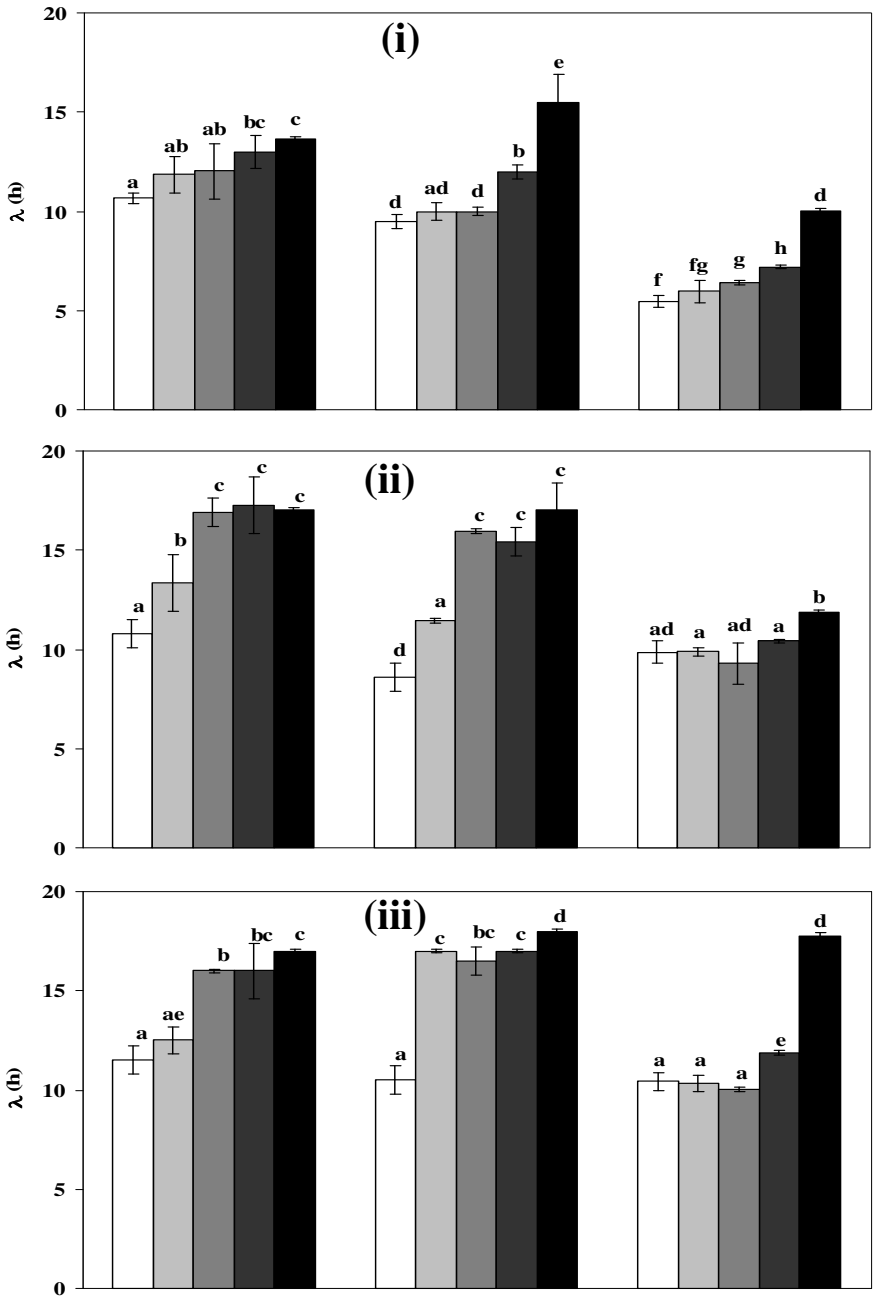

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Table 1

Microorganisms used in this study

Strain	**Reference**[a]	**Origin**	**Food model media**[b]
Enterobacter cloacae	*	Iceberg lettuce	TSB, L
Pseudomonas fluorescens	*	Iceberg lettuce	TSB, L, M, BE
Pseudomonas putida	*	Iceberg lettuce	TSB, BE
Lactobacillus sakei	ATCC 15521	Fermented drink	TSB, BE
Listeria innocua	NCTC 11288	Cow brain	TSB, L, BE
Listeria monocytogenes	NCTC 11994	Cheese	TSB, M, BE
Listeria monocytogenes	IL 323*	Iceberg lettuce	TSB, L

[a] Strains indicated with an asterisk were provided by the Department of Life Sciences, University of Limerick, Ireland

[b] Bacteria were grown in control media (TSB), lettuce leaf model media (L), milk (M) or beef extract (BE).

Table 2

Composition of the food model media containing beef extract and tomato serum at different ratios

Food model media	**Ingredients (%)**				**pH**
	Protein	Fat	Carbohydrates	Salt	
(A) BE-TS[a] 100:0[c]	12.00	0.00	0.00	1.000	7.06
(B) BE-TS[b] 95:5	12.21	0.02	1.16	0.951	6.09
(C) BE-TS 90:10	12.42	0.03	2.32	0.902	5.92
(D) BE-TS 75:25	10.00	0.40	5.80	0.756	5.32
(E) BE-TS 50:50	8.00	0.80	11.60	0.512	4.43

[a]BE: Beef extract

[b]TS: Tomato serum

[c]Ratios are expressed in percentage

Table 3

Lag phase and maximum specific growth rate of selected bacteria in TSB, lettuce leaf model media and beef extract

Microorganism	TSB				Lettuce media				Beef extract			
	λ [a]		μmax [b]		λ		μmax		λ		μmax	
E.. aerogenes	5.57	± 1.01[c]	0.136	± 0.017	7.65	± 1.72	0.025	± 0.002	ND		ND	
L. innocua NCTC11288	6.10	± 0.29	0.222	± 0.015	17.44	± 1.15	0.026	± 0.007	6.68	± 0.12	0.210	± 0.018
L. monocytogenes IL323	6.72	± 0.32	0.325	± 0.008	17.46	± 1.34	0.032	± 0.008	ND		ND	
L. monocytogenes NCTC1194	5.78	± 0.08	0.352	± 0.029	ND[d]		ND		6.38	± 0.83	0.077	± 0.012
P. fluorescens	5.86	± 2.28	0.170	± 0.027	9.58	± 1.85	0.024	± 0.002	6.18	± 0.10	0.168	± 0.009
P. putida	7.01	± 0.17	0.196	± 0.027	ND		ND		7.70	± 0.18	0.172	± 0.008

[a] Lag phase is expressed in hours.

[b] Maximum specific growth rate is expressed in $hours^{-1}$

[c] Standard deviation

[d] ND, not determined

Data represent the means of experiments performed in duplicate and replicated at least twice

Table 4

MICs of selected EO's comparing the Microplate Assay (MPA), the Agar Dilution Method (ADM) and the Agar well-Diffusion Test (ADT)

Microorganism	Oregano			Thyme			Lemon balm			Marjoram		
***L. innocua* NCTC11288**												
MPA	**100**	± 0	a	**125**	± 30	a	**1,250**	± 290	a	**5,000**	± 0	a
ADM	**75**	± 30	a	**375**	± 145	a	**1,750**	± 870	a	**3,000**	± 2,310	a
ADT	**375**	± 145	b	**1,750**	± 875	b	**5,000**	± 0	b	**5,000**	± 0	a
P. fluorescens												
MPA	**1,250**	± 500	a	**1,500**	± 575	a	**75,000**	± 28,900	a	**37,500**	± 14,425	a
ADM	**875**	± 250	a	**1,750**	± 875	a	**50,000**	± 0	ab	**10,000**	± 0	b
ADT	**2,500**	± 0	b	**3,750**	± 1,445	b	**25,000**	± 0	b	**17,500**	± 8,660	b

MICs are expressed in ppm. For each microorganism, means in the same column followed by different letters are significantly different ($p<0.05$).

All experiments were performed in duplicate and replicated at least three times.

Table 5

MIC of EO's used in this study against the selected bacteria in TSB (A), lettuce leaf model media (B) or beef extract (C).

Microorganism	Oregano	Thyme	Marjoram	Lemon Balm
(A)				
E. cloacae	400	600	6,000	ND
L. innocua NCTC11288	200	200	ND[a]	2,500
L. monocytogenes IL323	200	200	ND	2,500
L. monocytogenes NCTC1194	200	200	ND	2,500
P. fluorescens	2,000	2,000	50,000	ND
P. putida	2,000	2,000	50,000	ND
(B)				
E. cloacae	250	250	2,000	ND
L. innocua NCTC11288	20	30	ND	250
L. monocytogenes IL323	20	30	ND	250
P. fluorescens	250	250	2,000	ND
(C)				
L. innocua NCTC11288	60	125	ND	500
L. monocytogenes NCTC1194	60	125	ND	500
P. fluorescens	1,500	2,500	12,500	ND
P. putida	1,500	2,500	12,500	ND

[a] ND, not determined

All experiments were performed in duplicate and replicated at least three times.

Table 6

FIC values of EO combinations in lettuce leaf model media

EO combinations	***E. cloacae***		***P. fluorescens***		***L. innocua* NCTC11288**		***L. monocytogenes* IL323**	
	FIC	Std Dev.*	FIC	Std Dev.*	FIC	Std Dev.*	FIC	Std Dev.*
Oregano + Marjoram	1.75 (I)	± 0.35	2.00 (I)	± 0.00	ND		ND	
Oregano + Lemon balm	ND[a]		ND		1.50 (I)	± 0.71	1.25 (I)	± 0.43
Oregano + Thyme	0.75 (A)	± 0.00	0.88 (A)	± 0.18	1.00 (A)	± 0.00	1.18 (I)	± 0.30
Thyme + Marjoram	1.00 (A)	± 0.00	1.38 (I)	± 0.90	ND		ND	
Thyme + Lemon balm	ND		ND		0.75 (A)	± 0.00	1.25 (I)	± 0.35

Results are interpreted as synergy (**S**, FIC < 0.5), addition (**A**, 0.5 ≤ FIC ≤ 1), indifference (**I**, 1 < FIC ≤ 4) or antagonism (**AN**, FIC > 4)

[a] ND, not determined

Table 7

MICs of selected EO's in the beef extract and tomato serum model media at different ratios

Strain	Beef extract and Tomato Serum Model Media (BE-TS)				
	Media A (100:0, pH 7.06)	**Media B** (95:5, pH 6.09)	**Media C** (90:10, pH 5.92)	**Media D** (75:25, pH 5.32)	**Media E** (50:50, pH 4.43)
***L. monocytogenes* NCTC1194**					
Oregano	**62.50** ± 0.00 a	**31.25** ± 0.00 b	**15.63** ± 0.00 c	**7.81** ± 0.00 d	**NG**
Thyme	**125.00** ± 0.00 a	**93.75** ± 36.08 ab	**70.31** ± 39.32 b	**15.63** ± 0.00 c	**NG**
Lemon balm	**500.00** ± 0.00 a	**375.00** ± 144.34 b	**250.00** ± 0.00 c	**54.69** ± 15.63 d	**NG**
Marjoram	**3,125.00** ± 0.00 a	**2,343.75** ± 902.11 b	**1,562.50** ± 0.00 c	**781.25** ± 0.00 d	**NG**
***L. sakei* ATCC15521**					
Oregano	**312.50** ± 125.00 a	**375.00** ± 144.34 a	**125.00** ± 0.00 b	**62.50** ± 0.00 c	**NG**
Thyme	**500.00** ± 0.00 a	**500.00** ± 0.00 a	**250.00** ± 0.00 b	**125.00** ± 0.00 c	**NG**
Lemon balm	**10,000.00** ± 0.00 a	**10,000.00** ± 0.00 a	**5,000.00** ± 0.00 b	**1,562.50** ± 625.00 c	**NG**
Marjoram	**4,687.50** ± 1,804.22 a	**3,125.00** ± 0.00 ab	**2,343.75** ± 902.11 bc	**1,171.88** ± 451.06 cd	**NG**
P. putida					
Oregano	**1,562.50** ± 625.00 a	**1,250.00** ± 0.00 a	**1,250.00** ± 0.00 a	**NG**	**NG**
Thyme	**2,500.00** ± 0.00 a	**2,500.00** ± 0.00 a	**2,500.00** ± 0.00 a	**NG**	**NG**
Lemon balm	**62,500.00** ± 25,000.00 a	**50,000.00** ± 0.00 ab	**31,250.00** ± 12,500.00 b	**NG**	**NG**
Marjoram	**12,500.00** ± 0.00 a	**7,812.50** ± 3,125.00 b	**6,250.00** ± 0.00 c	**NG**	**NG**

NG, No growth was observed in control media without any EO

MICs are expressed in ppm. Means in the same row followed by different letters are significantly different for each bacterial population ($p<0.05$). All experiments were performed in duplicate and replicated at least three times.