

2009-01-01

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

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Recommended Citation

Gutierrez, J., Barry-Ryan, C. & Bourke, P. (2009) Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interaction with food components. *Food Microbiology*, Vol. 26, issue 2, April 2009, pp.142-50. doi:10.1016/j.fm.2008.10.008

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1 Title: **“Antimicrobial activity of plant essential oils using food model**
2 **media: efficacy, synergistic potential and interactions with food**
3 **components”**

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

22 Key words: **essential oils, antimicrobial, synergy, food model, food composition**

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24

1 **Abstract**

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

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1 **1. Introduction**

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

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

3 Greater consumer awareness and concern regarding synthetic chemical additives has
4 led researchers and food processors to look for natural food additives with a broad
5 spectrum of antimicrobial activity (Marino et al. 2001). In this context, plant essential oils
6 are gaining interest for their potential as preservative ingredients or decontaminating
7 treatments, as they have GRAS status and a wide acceptance from consumers (Burt et al.
8 2004). The antimicrobial components are commonly found in the essential oil fractions
9 and it is well established that many have a wide spectrum of antimicrobial activity, with
10 potential for control of *L. monocytogenes* and spoilage bacteria within food systems
11 (Smith-Palmer et al. 1998, Hammer et al. 1999, Elgayyar et al. 2001, Dorman and Deans
12 2002, Moreira et al. 2005, Oussalah et al. 2006b, Gutierrez et al., 2008a). Oregano
13 (*Origanum vulgare*) and thyme (*Thymus vulgaris*) are amongst the most active EO's,
14 while lemon balm (*Melissa officinalis*) and marjoram (*Origanum majorana*) display a
15 good antimicrobial activity against Gram-positive and Gram-negative bacteria,
16 respectively. Recently, some researchers have reported the efficacy of plant EO's as
17 antimicrobial agents against food borne pathogens and spoilage microflora in meat
18 (Busatta et al., 2008; Carramiñana et al., 2008). Although some studies have shown that
19 plant extracts are useful for reduction of pathogens associated with meat (Mytle et al.
20 2006, Ahn et al. 2007), others reported very low antimicrobial activity or no effect
21 against *L. monocytogenes* or *Salmonella* when EO's were applied to beef or chicken
22 (Uhart et al. 2006, Firouzi et al. 2007). Thus, the application of plant EO's for control of
23 food-borne pathogens and food spoilage bacteria requires the evaluation of efficacy

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

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

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

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

10

11 **2. Material and methods**

12

13 *2.1. Essential oils*

14 The essential oils (EO's) used in this study were lemon balm (*Melissa officinalis*),
15 marjoram (*Origanum majorana*), oregano (*Origanum vulgare*) and thyme (*Thymus*
16 *vulgaris*). They were selected based on previously reported efficacy (Gutierrez et al.
17 2008a), and were obtained from Guinness Chemical Ltd. (Portlaoise, Ireland) as CO₂
18 soluble supercritical fluid extracts.

19

20 *2.2. Bacteria*

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

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

5

6 2.3. *Food model media*

7 Lettuce leaf model media (L) was prepared as described by Francis et al. (1998) but with
8 some modifications. 50g of iceberg lettuce (*Lactuca sativa* sp.) were added to 100ml of
9 sterile deionized water and shaken for 1 min. The suspension was filtered using 18.5 cm
10 Whatman filters and pH was adjusted from 5.6 to 7.2 by mixing two parts of lettuce
11 media with one part 0.3M potassium phosphate buffer, giving a final concentration of
12 0.1M phosphate buffer, pH 7.2. The buffered medium was then autoclaved at 121°C for
13 15 min. To investigate the EO efficacy in meat-based model media, experiments were
14 performed with autoclaved beef extract (BE, 12% protein, Scharlau Chemie). Milk model
15 media (M) was made mixing skimmed milk powder (Scharlau Chemie) with agar solution
16 (Scharlau Chemie), both autoclaved separately, in order to obtain a final solid media
17 solution with 1.5% agar. Beef extract and milk model media were adjusted to pH 7.2 to
18 separate pH effects.

19

20 2.4. *Kinetic analyses*

21 Bacteria which were grown in TSB, lettuce leaf model media or beef extract (Table 1),
22 were monitored in a microplate spectrophotometer (PowerWave, Biotek) at 600 nm over
23 24 h at 30 min intervals. Growth curves were analyzed using Gen5 software (Biotek) and

1 the increase in lag phase (λ) and the maximum specific growth rate (μ_{max}) were
2 calculated. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago,
3 U.S.A). Data represent the means of experiments performed in duplicate and replicated at
4 least twice. Differences between bacteria were analyzed by ANOVA followed by LSD (p
5 < 0.05). Differences between control media (TSB) and model media were examined
6 using paired sample t-tests ($p < 0.05$).

7

8 *2.5. Antimicrobial assays*

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

15

16 *2.5.1. Agar-Well Diffusion Test (ADT)*

17 The ADT was performed as previously described (Bagamboula et al. 2004, Schelz et al.
18 2006) but with some modifications. 20 ml of Tryptic Soy Agar (TSA, pH 7.2, Scharlau
19 Chemie) were inoculated with 10^6 CFU/ml of the indicator strain and then poured onto a
20 Petri dish and allowed to solidify. Wells of 6.5-mm diameter were aseptically bored into
21 the agar, and 50 μ l of serially-diluted EO solutions in ethanol, were added to the wells.
22 The plates were kept at 4°C for 2 h to allow dispersal and subsequently incubated under
23 optimal conditions for growth of the target strains. The antimicrobial activity was visually

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

3

4 2.5.2. Agar Dilution Method (ADM)

5 The ADM was performed as described by Hammer et al. (1999) and Oussalah et al.
6 (2006b), but with some modifications. TSA or Milk Model Media were inoculated with
7 the appropriate EO and serially diluted using the same model media to the appropriate
8 concentrations, poured onto a Petri dish and allowed to solidify. Plates were then seeded
9 with 10^2 CFU of the target microorganism, and incubated at the appropriate temperature.
10 The positive control consisted of TSA or Milk Model Media inoculated with the same
11 amount of cells but without any EO, while uninoculated plates containing the EO served
12 as negative control. Target microorganisms were previously grown in TSB or liquid
13 model media to allow the cells to adapt to the food environment. *L. innocua* NCTC11288
14 and *L. monocytogenes* NCTC11994 were the target *Listeria* strains seeded into TSA and
15 the milk model media, respectively. *P. fluorescens* was selected as target in both media.
16 Plates were evaluated for the presence or the absence of colonies after 24 hours of
17 incubation at conditions optimal for each bacterium.

18

19 2.5.3 Absorbance based Microtitre Plate Assay (MPA)

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

1 medium. The EO's were then diluted two fold along each column. Finally, 100 μ l of
2 media containing 2×10^6 CFU/ml of the indicator strain was added to all wells. Positive
3 controls contained growth media inoculated with the organism under investigation.
4 Negative controls contained EO's and sterile growth media only. The plates were then
5 placed in the Biotek microplate spectrophotometer set at the appropriate temperature for
6 each test organism. The absorbance was recorded at 600 nm every 30 minutes over a 24
7 hour incubation period.

8

9 *2.6. Synergy studies: checkerboard method*

10 The checkerboard method was performed using 96-well microtitre plates (Schelz et al.,
11 2006) to obtain the fractional inhibitory concentration (FIC) index of EO combinations
12 EO's in the lettuce leaf model media. Plates consisted of columns containing 50 μ l of
13 EO_A diluted twofold in lettuce model media along the x axis as well as rows with the
14 same amount of EO_B diluted twofold in the same media along the y axis. Subsequently,
15 100 μ l of the lettuce media containing 2×10^6 CFU/ml of the indicator strain were added to
16 all wells. Plates were then incubated at 37°C for 24 h. The FIC indices were calculated as
17 $FIC_A + FIC_B$, where $FIC_A = (MIC_A \text{ combination} / MIC_A \text{ alone})$ and $FIC_B = (MIC_B$
18 $\text{combination} / MIC_B \text{ alone})$. The results were interpreted as synergy ($FIC < 0.5$), addition
19 ($0.5 \leq FIC \leq 1$), indifference ($1 < FIC \leq 4$) or antagonism ($FIC > 4$). Experiments were
20 performed in triplicate.

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

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

3

4 *2.7. Interactive effects of food ingredients and pH in beef extract and tomato serum media* 5 *(BE-TS)*

6 The effect of food ingredients and pH on the antimicrobial efficacy of EO's was
7 performed using a range of model media consisting of beef extract mixed with tomato
8 serum (Scharlau Chemie) at different ratios (Table 2). The concentrations of protein, fat
9 and salt were suitable for optimal EO efficacy (Gutierrez et al., 2008b), while percentage
10 of carbohydrates, mainly composed of glucose and fructose, increased from 0 to 11.6%
11 and the pH range was from 7.06 to 4.43. *L. monocytogenes* NCTC1194, *L. sakei*
12 ATCC15521 and *P. putida* were chosen as target microorganisms. The growth of selected
13 bacteria in each model medium with EO was monitored using the 96 well-microplates,
14 which were performed and assessed in the Biotek microplate spectrophotometer. A
15 second batch of experiments was performed in the same model media but adjusted to pH
16 7.2. The effect of food components on EO efficacy was evaluated considering the MIC
17 and the growth parameters of target bacteria, as described in sections 2.4 and 2.5.3,
18 respectively. Positive controls contained model media inoculated with the organism under
19 investigation. Negative controls contained EO's and sterile model media only.

20

21 **3. Results**

22

23 *3.1. Kinetic analysis in food model media*

1 The lag phase and μ_{max} 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 μ_{max} 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)

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

15 Furthermore, when *L. monocytogenes* NCTC11994 and *P. fluorescens* were exposed
16 to oregano or thyme on milk model media (M), it was observed that the MICs of these
17 EO's were approximately 10 fold higher than those obtained on the control media TSA.
18 MICs of oregano and thyme against the *Listeria* strain on the milk model media were
19 1,000 and 3,000 ppm respectively. *P. fluorescens* was more resistant to both oregano and
20 thyme on same food model media, with corresponding MICs of 10,000 and 20,000 ppm,
21 respectively.

22

23 *3.4. Synergy studies*

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

9 3.5. Influence of BE-TS model media composition on bacterial growth

10 As shown in figure 1, the μ_{max} of *L. monocytogenes* and *P. putida* increased
11 significantly ($p < 0.05$) when grown in medium B (pH 6.09, 1.16% sugars, see Table 2)
12 than in medium A (pH 7.06; 0% sugars). A similar trend was observed when *L. sakei*
13 grew in medium C (pH 5.92; 2.32% sugars), by comparison with medium B. On the
14 contrary, the μ_{max} values of *L. monocytogenes* and *L. sakei* grown in medium D (pH
15 5.32; 5.80% sugars) were significantly lower ($p < 0.05$) than those obtained in medium C.
16 Considering the lag phase of selected bacteria, no significant differences were observed
17 between media A and B. However, the lag phase values of *L. monocytogenes* and *L. sakei*
18 grown in media D and C, respectively, were significantly longer ($p < 0.05$) than those
19 obtained in medium C, for *L. monocytogenes*, or medium B, for *L. sakei*. The opposite
20 was observed for *P. putida* since its lag phase was significantly reduced ($p < 0.05$) in
21 medium C, compared to media B or A. None of the target micro organisms were capable
22 of growing in model medium E (pH 4.43; 11.6% sugars). *P. putida* was also unable to
23 grow in medium D.

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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 μ_{max} 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 μ_{max} 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 μ_{max} 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

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

6

7 **4. Discussion**

8 Most researchers currently use agar or broth dilution series to assess antimicrobial
9 activity of spices, herbs and their EO's, and in some cases both assays for comparative
10 purposes because antimicrobial performance in the two systems can vary (Holley and Patel
11 2005). In this work, no significant differences were observed between MIC values using
12 the Microplate Assay (MPA) or the Agar Dilution Method (ADM). Furthermore, these
13 methods proved to be more sensitive than the Agar well-Diffusion Test (ADT). Although
14 tube macrodilution and diffusion from inhibitor-impregnated paper discs on agar surfaces
15 are still used, there is heavy reliance on microwell plate systems containing inhibitors and
16 target microorganisms in broth. Some authors have suggested that the agar well/disk
17 diffusion tests might only be used as a selection method when large numbers of EO's and
18 or bacterial isolates have to be screened, since the comparison of published data are not
19 feasible (Dorman and Deans 2000, Burt 2004). The hydrophobicity of EO components is
20 known to limit the value of these diffusion tests for estimating antimicrobial potency
21 accurately (Holley and Patel 2005). Although several substances have been used to dissolve
22 the EO or to stabilize it in water-based culture media, such as ethanol, methanol, Tween-20,
23 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

1 efficacy of EO's in the vegetable media. The rich nutrients in TSB compared to lettuce
2 media may enable bacteria to repair damaged cells faster, as suggested by Gill et al.
3 (2002). However, the EO's were more effective in BE than in TSB and the MIC of lemon
4 balm in the meat based model media was comparable to that obtained in lettuce media.
5 Gutierrez et al. (2008b) observed that the presence of high concentrations of protein in
6 BE promoted the growth of *L. monocytogenes*, but the efficacy of oregano and thyme was
7 also greater at these higher concentrations of protein. These authors explained that
8 peptones with hydrophobic properties might display interactions with EO's to facilitate
9 their dissolution in BE. Baranauskien et al. (2006) reported that proteins usually possess a
10 high binding capacity for flavor volatile compounds.

11 Recently, some studies have recorded the EO antimicrobial efficacy, alone or in
12 combination with other preservation methods, against spoilage and food-borne pathogens
13 when applied to meat (Mytle et al. 2006, Ahn et al. 2007, Ghalfi et al. 2007, Solomakos
14 et al. 2008) or milk (Cava et al. 2007). Particularly, Careaga et al. (2003) observed that
15 chilli extracts (*Capsicum annuum*) had a bacteriostatic effect against *P. aeruginosa* at
16 concentrations of 3,000 ppm. In this study the MICs of oregano and thyme against the
17 *Pseudomonas* strains were 1,500 and 2,500 ppm, respectively, in BE. When Cava et al.
18 (2007) assessed the antimicrobial activity of EO's of cinnamon bark, cinnamon leaf, and
19 clove against *L. monocytogenes* in semi skimmed milk incubated at 7°C for 14 days, they
20 observed that the MIC was 500 ppm for cinnamon bark EO and 3,000 ppm for the
21 cinnamon leaf and clove EO's. Concentrations increased to 1,000 ppm for cinnamon bark
22 EO, 3,500 ppm for clove EO, and 4,000 ppm for cinnamon leaf EO when the semi
23 skimmed milk was incubated at 35°C for 24 h. The antimicrobial efficacy of oregano and

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

8 Oregano and thyme were the most effective EO's for inhibition of *Listeria* and
9 spoilage organisms in all the food model media (Table 5). Marjoram also displayed a
10 high antimicrobial activity against the Gram-negative bacteria, while lemon balm had
11 good efficacy against the Gram-positive *Listeria* spp. (Table 5). The high antimicrobial
12 activity of marjoram against Gram-negative bacteria might be due to the presence of
13 hydroxyl groups in EO compounds, as described previously (Elgayyar et al. 2001, Burt
14 2004, Oussalah et al. 2006b). Longaray Delamare et al. (2005) attributed the strong
15 activity of sage against Gram-positive bacteria to the presence of β -caryophyllene, a
16 compound that is found in the composition of the lemon balm EO's used in this study.

17 Plant EO's are generally more active against gram-positive bacteria than gram-
18 negative bacteria (Burt 2004). Some authors suggest that the outer membrane
19 surrounding the cell wall of gram-negative bacteria may restrict diffusion of hydrophobic
20 compounds through its lipopolysaccharide covering (Vaara 1992, Davidson and Branen
21 2005). In the current work, gram-negative strains, *P. fluorescens*, *P. putida* and *E.*
22 *cloacae* were more resistant to the action of the EO's than the Gram-positive *Listeria* spp.

1 (Table 5). As the lag phase values of both *Listeria* strains in lettuce media were much
2 longer than those obtained for spoilage organisms (Table 1), this may have also promoted
3 the efficacy of the EO's. However, the MIC values for oregano and thyme against
4 *Listeria* spp. were similar to those observed with the same EO's against *E. cloacae* (Table
5 5) in TSB. The growth rate of *E. cloacae* in TSB was approximately 2 fold lower than
6 those attained by the *Listeria* strains. Although the growth rate of *P. fluorescens* was not
7 significantly different to that for *E. cloacae*, the *Pseudomonas* strains were the most
8 resistant to oregano and thyme in TSB (Table 1). *Pseudomonas* spp. are known to show
9 consistently high resistance to plant antimicrobials (Hammer et al. 1999, Holley and Patel
10 2005). However, both *E. cloacae* and *P. fluorescens* had the same sensitivity to the EO's
11 in the lettuce model media (Table 5). Both of these spoilage organisms were isolated
12 from lettuce and the lag phase for *E. cloacae* within the vegetable media was shorter than
13 that of *P. fluorescens* (Table 3).

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

1 possess compounds with similar structures, their combinations may exhibit additive
2 rather than synergistic effects. Furthermore, as the EO efficacy also depends on lipophilic
3 properties, potency of functional groups or their aqueous solubility (Dorman and Deans
4 2000), the mixture of compounds within whole EO's may contribute to that "additive"
5 effect.

6 Furthermore, since another important aspect for the optimised application of EO's in
7 food is the evaluation of interaction with food ingredients, five different model media
8 were prepared using beef extract and tomato serum in order to assess and quantify the
9 effect of pH and sugars on the antimicrobial efficacy of oregano and thyme. In general,
10 the antimicrobial activity of these EO's increased when the pH decreased. Previously, it
11 was also observed that the inhibitory effect of plant extracts was greater at acidic pH
12 values (Del Campo et al. 2000, Hsieh et al. 2001). The susceptibility of bacteria to EO's
13 appears to increase with lower pH values since the hydrophobicity of EO's increases at
14 low pH, consequently enabling easier dissolution in the lipids of the cell membrane of
15 target bacteria (Juven et al. 1994). The major efficacy of EO's at pH 5.32 or 5.92 was
16 confirmed with the lag phase and growth rate results at these pHs, which were longer and
17 lower, respectively, than at higher pH levels. As the pH was reduced, the lag phase
18 increased and the growth rate declined for *Listeria* and *L. sakei*, and consequently, the
19 addition of either oregano or thyme enhanced the EO efficacy. However, no significant
20 differences were observed between lag phase and growth rate values of *L. sakei* at pH
21 7.06 or 6.09 but the MICs of selected EO's decreased at more reduced pH. The same
22 trend was observed with *P. putida* although maximum specific growth rate and lag phase

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

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

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

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

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

14

15 **Acknowledgments**

16 This work was supported by funding from Irish Department of Agriculture and Food as
17 part of the National Development Plan 2000-2006.

18

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1 **Figures legend**

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3 Fig.1. Maximum specific growth (μ_{max}) rate and lag phase (λ) of *L. monocytogenes*
4 NCTC1194, *Lb. sakei* ATCC15521 and *Ps. putida* grown in beef extract and tomato
5 serum model media A (□, pH 7.06), B (▣, pH 6.09), C (▤, pH 5.92), D (▥, pH 5.32),
6 and E (▦, pH 4.43). Different letters signify statistical differences between values
7 ($p < 0.05$).

8

9 Fig. 2. Maximum specific growth rate (μ_{max}) of *L. monocytogenes* (i), *Lb. sakei* (ii) and
10 *Ps. putida* (iii) in neutralized beef extract and tomato serum model media A (□, 0.00%
11 sugars), B (▣, 1.16% sugars), C (▤, 2.32% sugars), D (▥, 5.80% sugars), and E (▦,
12 11.60% sugars) containing oregano (31.25 ppm) or thyme (62.5 ppm). Different letters
13 signify statistical differences between values ($p < 0.05$).

14

15 Fig. 3. Lag phase (λ) of *L. monocytogenes* (i), *Lb. sakei* (ii) and *Ps. putida* (iii) in
16 neutralized beef extract and tomato serum model media A (□, 0.00% sugars), B (▣,
17 1.16% sugars), C (▤, 2.32% sugars), D (▥, 5.80% sugars), and E (▦, 11.60% sugars)
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19 differences between values ($p < 0.05$).

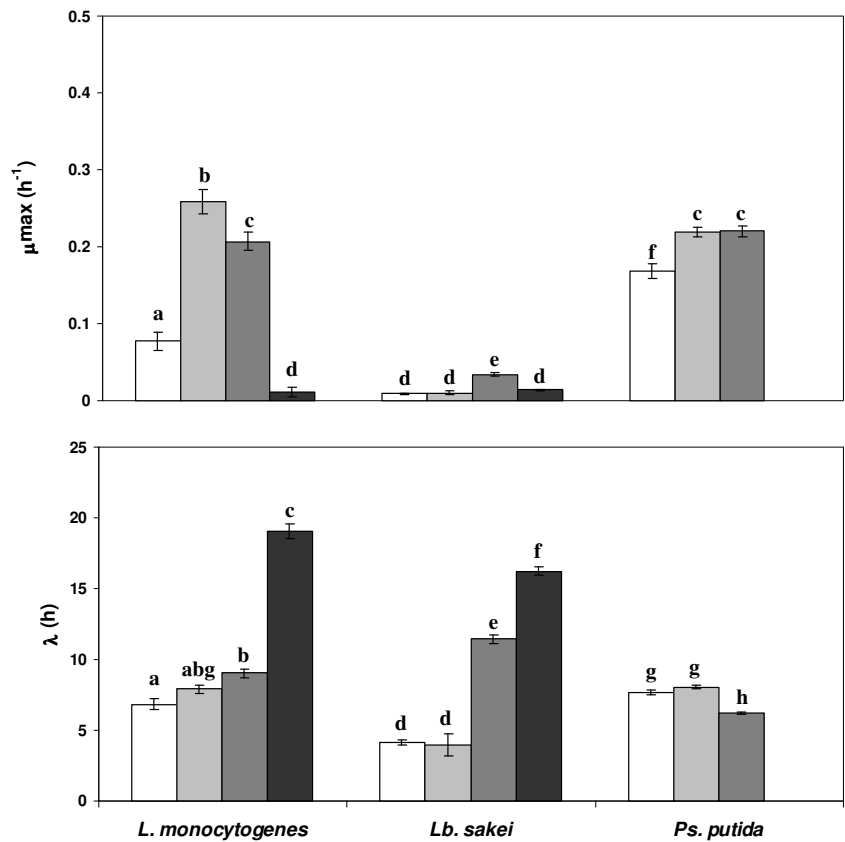
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16 Fig.1. Maximum specific growth (μ_{max}) rate and lag phase (λ) of *L. monocytogenes*
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19 pH 4.43). Different letters signify statistical differences between values ($p < 0.05$).

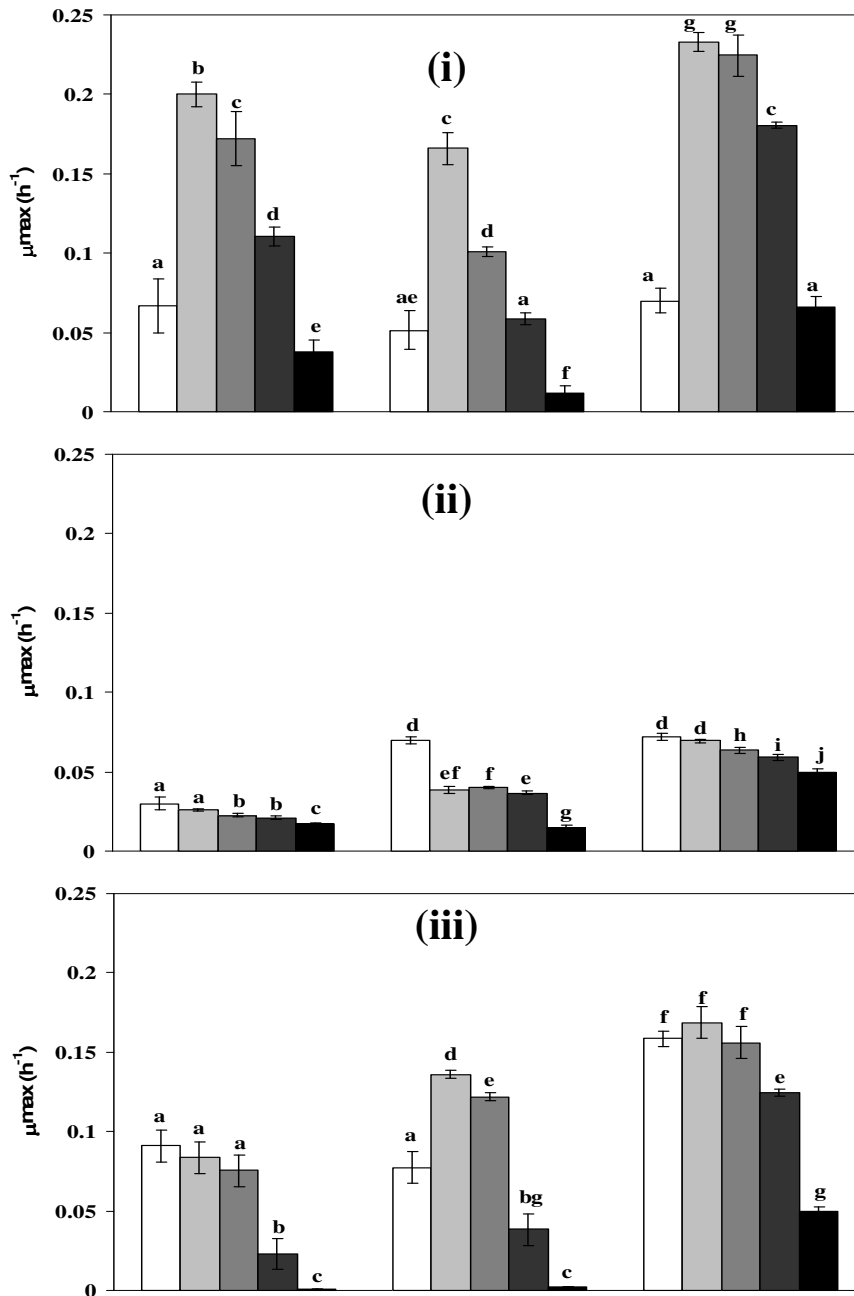


Fig. 2. Maximum specific growth rate (μ_{max}) of *L. monocytogenes* (i), *Lb. sakei* (ii) and *Ps. putida* (iii) in neutralized beef extract and tomato serum model media A (\square , 0.00% sugars), B (\square , 1.16% sugars), C (\square , 2.32% sugars), D (\blacksquare , 5.80% sugars), and E (\blacksquare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

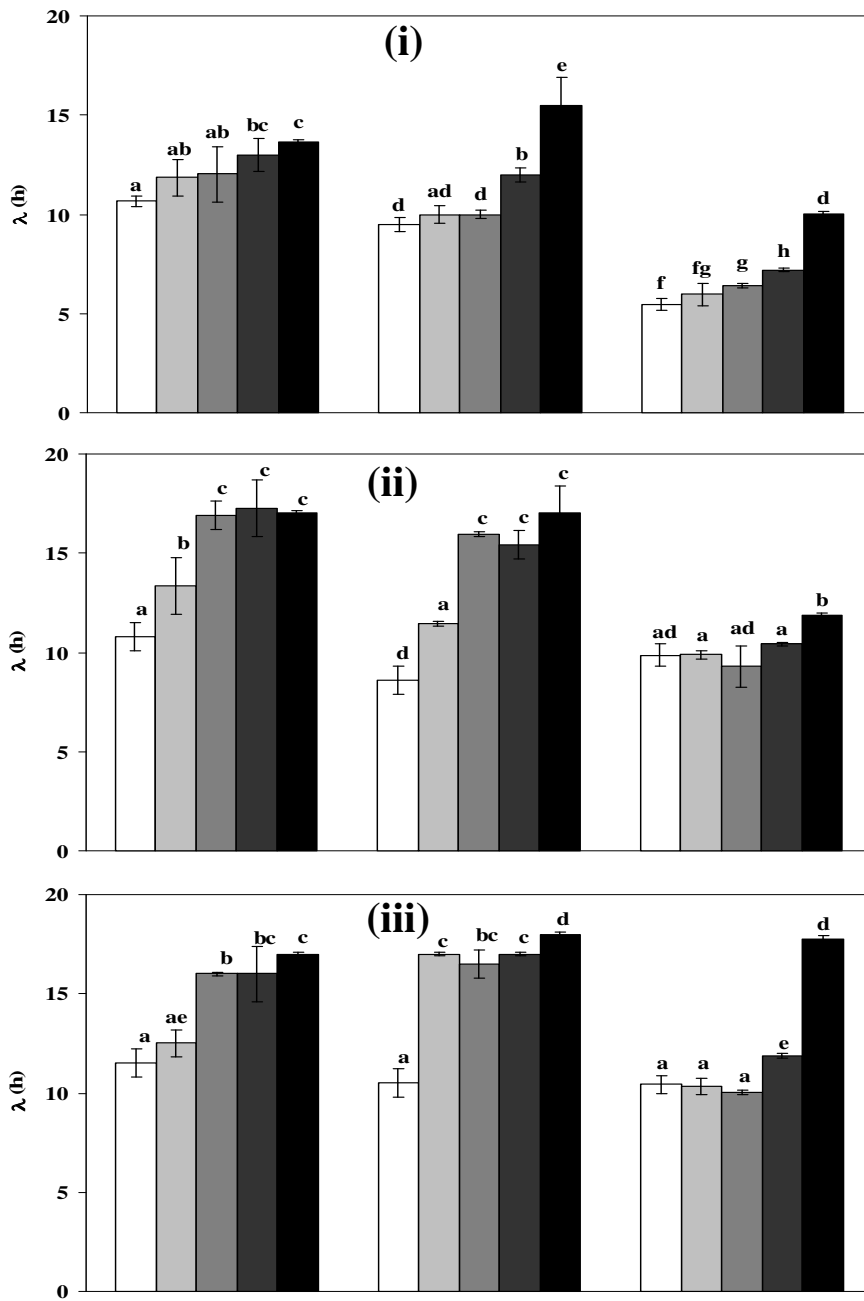


Fig. 3. Lag phase (λ) of *L. monocytogenes* (i), *Lb. sakei* (ii) and *Ps. putida* (iii) in neutralized beef extract and tomato serum model media A (\square , 0.00% sugars), B (\square , 1.16% sugars), C (\square , 2.32% sugars), D (\blacksquare , 5.80% sugars), and E (\blacksquare , 11.60% sugars) containing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

Table 1

Microorganisms used in this study

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

^aBE: Beef extract^bTS: Tomato serum^cRatios 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	μ_{\max}^b	λ	μ_{\max}	λ	μ_{\max}
<i>E. aerogenes</i>	5.57 ± 1.01 ^c	0.136 ± 0.017	7.65 ± 1.72	0.025 ± 0.002	ND	ND
<i>L. innocua</i> 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
<i>L. monocytogenes</i> IL323	6.72 ± 0.32	0.325 ± 0.008	17.46 ± 1.34	0.032 ± 0.008	ND	ND
<i>L. monocytogenes</i> NCTC1194	5.78 ± 0.08	0.352 ± 0.029	ND ^d	ND	6.38 ± 0.83	0.077 ± 0.012
<i>P. fluorescens</i>	5.86 ± 2.28	0.170 ± 0.027	9.58 ± 1.85	0.024 ± 0.002	6.18 ± 0.10	0.168 ± 0.009
<i>P. putida</i>	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
<i>L. innocua</i> 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
<i>P. fluorescens</i>				
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)				
<i>E. cloacae</i>	400	600	6,000	ND
<i>L. innocua</i> NCTC11288	200	200	ND ^a	2,500
<i>L. monocytogenes</i> IL323	200	200	ND	2,500
<i>L. monocytogenes</i> NCTC1194	200	200	ND	2,500
<i>P. fluorescens</i>	2,000	2,000	50,000	ND
<i>P. putida</i>	2,000	2,000	50,000	ND
(B)				
<i>E. cloacae</i>	250	250	2,000	ND
<i>L. innocua</i> NCTC11288	20	30	ND	250
<i>L. monocytogenes</i> IL323	20	30	ND	250
<i>P. fluorescens</i>	250	250	2,000	ND
(C)				
<i>L. innocua</i> NCTC11288	60	125	ND	500
<i>L. monocytogenes</i> NCTC1194	60	125	ND	500
<i>P. fluorescens</i>	1,500	2,500	12,500	ND
<i>P. putida</i>	1,500	2,500	12,500	ND

^aND, 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	<i>E. cloacae</i>		<i>P. fluorescens</i>		<i>L. innocua</i> NCTC11288		<i>L. monocytogenes</i> 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)

^aND, 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)	
<i>L. monocytogenes</i> 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	
<i>L. sakei</i> 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	
<i>P. putida</i>										
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.