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**Title: “The anti-microbial efficacy of plant essential oil combinations
and interactions with food ingredients”**

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**Running Title: Antimicrobial efficacy of plant EOs and interactions with food
ingredients**

1 **Abstract**

2 The objective of this study was to evaluate the efficacy of plant essential oils (EOs) in
3 combination and to investigate the effect of food ingredients on their efficacy. The EOs
4 assessed in combination included basil, lemon balm, marjoram, oregano, rosemary, sage
5 and thyme. Combinations of EOs were initially screened against *B. cereus*, *E. coli*, *L.*
6 *monocytogenes* and *P. aeruginosa* using the spot-on-agar test. The influence of varying
7 concentrations of EO combinations on efficacy was also monitored using *E. coli*. These
8 preliminary studies showed promising results for oregano in combination with basil,
9 thyme or marjoram. The checkerboard method was then used to quantify the efficacy of
10 oregano, marjoram or thyme in combination with the remainder of selected EOs.
11 Fractional inhibitory concentrations (FIC) were calculated and interpreted as synergy,
12 addition, indifference or antagonism. All the oregano combinations showed additive
13 efficacy against *B. cereus*, and oregano combined with marjoram, thyme or basil also had
14 an additive effect against *E. coli* and *P. aeruginosa*. The mixtures of marjoram or thyme
15 also displayed additive effects in combination with basil, rosemary or sage against *L.*
16 *monocytogenes*. The effect of food ingredients and pH on the antimicrobial efficacy of
17 oregano and thyme was assessed by monitoring the lag phase and the maximum specific
18 growth rate of *L. monocytogenes* grown in model media. The model media included
19 potato starch (0, 1, 5 or 10%), beef extract (1.5, 3, 6 or 12%), sunflower oil (0, 1, 5 or
20 10%) and TSB at pH levels of 4, 5, 6 or 7. The antimicrobial efficacy of EOs was found
21 to be a function of ingredient manipulation. Starch and oils concentrations of 5% and 10%
22 had a negative impact on the EO efficacy. On the contrary, the EOs were more effective
23 at high concentrations of protein, and at pH 5, by comparison with pH 6 or 7. This study

1 suggests that combinations of EOs could minimize application concentrations and
2 consequently reduce any adverse sensory impact in food. However, their application for
3 microbial control might be affected by food composition, therefore, careful selection of
4 EOs appropriate to the sensory and compositional status of the food system is required.
5 This work shows that EOs might be more effective against food-borne pathogens and
6 spoilage bacteria when applied to ready to use foods containing a high protein level at
7 acidic pH, as well as lower levels of fats or carbohydrates.

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9 **Key words: essential oils, antimicrobial, synergy, food ingredients, food application**

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1 **1. Introduction**

2 Essential oils (EOs) are aromatic and volatile oily liquids obtained from plant material.
3 They are normally formed in special cells or groups of cells, found in leaves and stems,
4 and commonly concentrated in one particular region such as leaves, bark or fruit
5 (Oussalah et al., 2006). Although the antibacterial properties of EOs have been long
6 recognized, the recent interest in alternative naturally derived antimicrobials has lead to a
7 renewed scientific interest in these substances. Many *in vitro* studies report a high
8 efficacy of EOs against food-borne pathogens and spoilage bacteria (Smith-Palmer et al.,
9 1998; Hammer et al., 1999; Elgayyar et al., 2001; Dorman and Deans, 2002). However, a
10 higher concentration of EO is needed to achieve the same effect in food as *in vitro* (Burt,
11 2004). If EOs are expected to be widely applied as antibacterials, the organoleptical
12 impact should be considered as the use of naturally derived preservatives can alter the
13 taste of food or exceed acceptable flavour thresholds (Hsieh et al., 2001; Nazer et al.,
14 2005). Thus, combinations of plant extracts may help to minimise concentrations and
15 consequently reduce sensory impact. Furthermore, these combinations may also control
16 some bacteria that are known to show consistently high resistance to plant antimicrobials,
17 such as *Pseudomonas* spp. (Hammer et al., 1999; Holley and Patel, 2005). Although
18 some studies have concluded that whole EOs have a greater antibacterial activity than the
19 major components mixed (Gill et al., 2002; Mourey and Canillac, 2002), the combination
20 of these major components with other components that have a weaker activity can
21 achieve a synergistic effect (Ultee et al., 2000).

22 In general, the efficacy of many added and naturally occurring antimicrobials may be
23 reduced by certain food components (Glass and Johnson, 2004). It is supposed that high

1 levels of fat and/or protein in foodstuffs protect bacteria from the action of EOs (Aureli et
2 al., 1992; Pandit and Shelef, 1994; Tassou al., 1995). Carbohydrates in foods do not
3 appear to protect bacteria from the antimicrobial effects of EOs (Shelef et al., 1984). Gill
4 et al. (2002) suggested that the greater availability of nutrients in foods compared to
5 laboratory media may enable bacteria to repair damaged cells faster. In this respect not
6 only the intrinsic properties of the food are important but extrinsic determinants, such as
7 temperature or characteristics of bacteria, can affect bacterial sensitivity (Burt, 2004).
8 Since most foods are mainly composed of water, carbohydrates, fats, proteins and NaCl,
9 it is important to analyse the influence of these components on the antimicrobial activity
10 of any proposed antimicrobial compound (Devlieghere et al., 2004).

11 Recent studies have shown that plant extracts are useful for reduction of pathogens
12 associated with chicken frankfurters (Mytle et al., 2006) or cooked beef (Ahn et al.,
13 2007). On the contrary, some authors recorded very low antimicrobial activity or no
14 effect against *E. coli* O157:H7 or *Salmonella* when EOs were applied to ground beef
15 (Uhart et al., 2005) or ready-to-cook chicken (Firouzi et al., (2007), respectively. The
16 application of plant EOs for control of foodborne pathogens and food spoilage bacteria
17 requires the evaluation of a number of aspects; the effects on organoleptic properties,
18 evaluation of the range of activity against the organisms of concern to a particular
19 product as well as food compositional effects on activity. Optimal application in real food
20 systems depends on these factors, therefore, the application of EOs in food should
21 incorporate studies to determine and quantify the effect of food ingredients on their
22 antimicrobial activity. This work aims to bridge *in vitro* studies with practical application
23 of EOs through assessing the effects of a range of concentrations of main food

1 components based on meat and vegetable substrates prior to application in complex food
2 systems. Thus, the objectives of this study were to evaluate and quantify the effect of a
3 range of plant EOs in combination, against four pathogens, *B. cereus*, *E. coli*, *L.*
4 *monocytogenes* and *P. aeruginosa*, and to determine interactive effects with protein,
5 carbohydrate, oil and pH levels in order to optimise applications in food.

6

7 **2. Material and methods**

8 *2.1. Essential oils*

9 The essential oils (EOs) selected for this study and their composition are indicated in
10 Table 1. The selection was based on a balance between reported antimicrobial efficacy,
11 sensory properties and presence of different components in the EOs. The EOs were
12 obtained from Guinness Chemical Ltd. (Portlaoise, Ireland) and were CO₂ soluble SE-
13 extracts from leaves.

14

15 *2.2. Bacteria*

16 The bacteria used in this study were *Bacillus cereus* ATCC 11778, *Escherichia coli*
17 ATCC 25922, *Listeria monocytogenes* IL323 and *Pseudomonas aeruginosa* ATCC
18 27853. The product isolated *Listeria* strain was kindly provided by the Department of
19 Life Sciences, University of Limerick, Ireland. All cultures were maintained at -70°C in
20 20% glycerol and grown in Tryptic Soy Broth (TSB, pH 7.2, Scharlau Chemie) at 37°C
21 for 24 hours to obtain sub-cultures. Working cultures were prepared from sub-cultures
22 and grown under optimal conditions for each bacterium for 18 hours. Working cultures

1 were adjusted to the required concentration of 10^6 CFU/ml using the McFarland standard
2 (Biomerieux Inc.).

3

4 2.3. Synergy studies

5 Combinations of EOs were qualitatively assessed using the spot-on-agar test. The
6 effect of varying concentrations on efficacy of EO combinations was determined using *E.*
7 *coli* ATCC 25922 as a test organism. Fractional inhibitory concentration (FIC) indices
8 were calculated using the checkerboard method to quantify the potential synergy of
9 oregano, marjoram or thyme in combination with the remainder of selected EOs. Assays
10 were performed in duplicate and then replicated.

11

12 2.3.1. Spot-on-agar test

13 The spot on agar test was performed based on previous work (Cintas et al., 1998) but
14 with modifications. Ten μ l of EO extracts diluted in ethanol at 10% (v/v) were spotted
15 onto TSA (1.2%) plates seeded with 10^6 CFU/ml of the indicator strain. Combinations
16 were initially assessed in a 1:1 ratio. Spotted plates were then incubated at 37°C for 18 h.
17 Ethanol was used as control and inhibition zones around the wells were measured in
18 millimetres.

19

20 2.3.2. Effect of varying concentrations on efficacy of EO combinations against *E. coli*

21 The influence of varying concentration of EO combinations on efficacy was assessed
22 against *E. coli* using 96-well micro titer plates (Sarstedt Ltd). The first row of each plate
23 contained 100 μ l of the EO extract or the EO combination (1:1) diluted in TSB. The range

1 of initial concentrations for each of the EOs is indicated in Table 2. Wells containing
2 EOs in the first row were then diluted two fold along each column. At least 2 columns
3 were used for controls. Positive controls contained growth media inoculated with the
4 organism under investigation. Negative controls contained EOs and sterile growth media
5 only. Finally, 100 μl of media containing 2×10^6 CFU/ml of the bacterium were added to
6 all wells. The plates were then placed in a microplate spectrophotometer (PowerWave,
7 Biotek) set at 37°C . The absorbance readings were taken at 600 nm every 30 minutes for
8 an 18 hours incubation period and kinetic curves were analysed.

9

10 2.3.3. Checkerboard Method

11 The checkerboard method was performed using 96-well microtitre plates as described
12 previously (Moody, 2003; Schelz et al., 2006), to obtain the FIC index. The microplate
13 assay was arranged as follows: EO_A was diluted two-fold along the x-axis, whilst EO_B
14 was diluted two-fold along the y-axis. The final volume in each well was 100 μl
15 comprising 50 μl of each EO dilution. Subsequently, 100 μl of media containing 2×10^6
16 CFU/ml of the indicator strain were added to all wells. The plates were then incubated at
17 37°C for 18 h. The FIC indices were calculated as $\text{FIC}_A + \text{FIC}_B$, where FIC_A and FIC_B
18 are the minimum concentrations that inhibited the bacterial growth for EOs A and B,
19 respectively. Thus, FICs were calculated as follows: $\text{FIC}_A = (\text{MIC}_A \text{ combination} / \text{MIC}_A$
20 $\text{alone})$ and $\text{FIC}_B = (\text{MIC}_B \text{ combination} / \text{MIC}_B \text{ alone})$. The results were interpreted as
21 synergy ($\text{FIC} < 0.5$), addition ($0.5 \leq \text{FIC} \leq 1$), indifference ($1 < \text{FIC} \leq 4$) or antagonism
22 ($\text{FIC} > 4$). The concentrations used for oregano, marjoram and thyme, either alone or in
23 combination with the remainder of the EOs, are shown in Table 3.

1 2.4. *Interactive effects of food ingredients and pH*

2 2.4.1. *Food ingredients and pH assays*

3 The effect of food ingredients and pH on the antimicrobial efficacy of EOs was
4 performed using a range of model media and *Listeria monocytogenes* IL323 as indicator
5 strain. The plant EOs were fixed factors: oregano (30 ppm) and thyme (60 ppm) were
6 independently assessed. Model media were comprised of the following: (i) water soluble
7 starch from potato (0, 1, 5 or 10%, Sigma-Aldrich Ireland Ltd) in TSB; (ii) beef extract
8 (1.5, 3, 6 or 12%, Scharlau Chemie) in deionized water; and (iii) sunflower oil (0, 1, 5 or
9 10%) in TSB. Model media containing starch or beef extract were autoclaved prior to use.
10 For the oil model media, the sunflower oil was autoclaved separately and then added to
11 sterile TSB. Filter-sterilized Tween 80 (Merck) was added at 0.1% to facilitate mixing
12 and to stabilize the emulsion. The pH of each model media was adjusted to 7.2. To
13 determine the effect of pH on EO efficacy TSB was adjusted to pH 4, 5, 6 or 7 with 1 N
14 HCl solution.

15 The growth of *L. monocytogenes* in each model medium with oregano or thyme was
16 monitored using 96 well-microplates. 200 µl of each medium containing 2×10^6 CFU/ml of
17 the *Listeria* strain were added to wells of 96 well-microplates, which were assessed in
18 microplate spectrophotometer as described above. Positive controls contained model
19 media inoculated with the organism under investigation. Negative controls contained EOs
20 and sterile model media only. The survival curves of *Listeria monocytogenes* IL323 in
21 model media were monitored at 600 nm over a 24 hour period.

22

23

1 *2.5 Kinetic analysis*

2 The kinetic curves were analyzed by the KC4 software (Biotek) calculating the
3 increase in lag phase (λ) and the maximum specific growth rate (μ_{max}). Statistical
4 analysis on data was performed using SPSS 15.0 (SPSS Inc., Chicago, U.S.A). Data
5 represents the means of experiments performed in duplicate and replicated at least twice.
6 Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level.

7

8 **3. Results**

9 *3.1. Synergy studies*

10 *3.1.1. Spot on agar test and effect of varying concentrations on efficacy of EO*
11 *combinations*

12 All the EO combinations were evaluated by the spot-on-agar-test. Only oregano in
13 combination with thyme showed a greater efficacy than when assessed individually
14 (results not shown). EO mixtures were also assessed monitoring the effect of varying
15 concentrations on *E. coli* (Table 2). Some combinations resulted in lag phase extensions
16 and reductions in the μ_{max} at half the concentrations of the individual EOs. The lag
17 phase duration of *E. coli*, when exposed to oregano in combination with basil, was
18 significantly increased by 7.44h with respect to the increase recorded by oregano alone (p
19 < 0.05). The combination of oregano with lemon balm resulted in a 3.55h extension of
20 the lag phase by comparison with lemon balm alone. When oregano was combined with
21 marjoram, the reduction in the maximum specific growth rate achieved was
22 approximately 3 fold higher than that with the EOs alone ($p < 0.05$). Combining oregano
23 with sage or thyme increased the lag phase of *E. coli*, by comparison with the individual

1 EOs, but when oregano was combined with rosemary, there was no benefit compared to
2 the EOs alone.

3

4 *3.1.2. Checkerboard method*

5 The quantitative effects of oregano, marjoram or thyme in combination with the other
6 EOs are described in terms of FIC indices. The FIC's of oregano in combination with the
7 other EOs are shown in Table 4. None of these combinations displayed a synergistic
8 activity against the bacteria used in this study. However, oregano combined with all the
9 other EOs had additive effects against *B. cereus*, but no additive or synergistic effects
10 were recorded for oregano based combinations against *L. monocytogenes*. The
11 combinations of oregano with marjoram and oregano with thyme had additive effects
12 against *E. coli* and *P. aeruginosa*, respectively, while oregano in combination with basil
13 had a useful additive activity against both Gram-negative organisms.

14 The FIC's for both marjoram and thyme combinations against *L. monocytogenes* are
15 shown in Table 5. A similar trend was noted for both EOs, where additive effects were
16 observed in combinations with basil, rosemary and sage.

17

18 *3.2. Interaction with food ingredients and pH*

19 The lag phase (λ) and the maximum specific growth rate (μ_{max}) of *L. monocytogenes*
20 IL323 grown in different model media are indicated in Tables 6 and 7, respectively.

21

22

23

1 3.2.1. *Effect of protein*

2 To investigate the effect of proteins on the antimicrobial activity of oregano and
3 thyme, growth experiments with *L. monocytogenes* were performed in beef extract at 4
4 different concentrations (1.5, 3, 6, and 12%). The lag phase of *L. monocytogenes* grown
5 in beef extract containing oregano was longer than the control at protein concentrations of
6 6% and 12% ($p < 0.05$). The antimicrobial activity of thyme was increased in high
7 protein concentrations, leading to a significantly longer lag phase from 3% to 12% of
8 protein, with respect to the control ($p < 0.05$). In the control culture, a longer lag phase
9 and a lower growth rate were observed at protein concentrations of 1.5% and 3% ($p <$
10 0.05). The growth rate of *Listeria* grown in beef extract with thyme was higher than the
11 control at protein concentrations of 3% and 6% ($p < 0.05$).

12

13 3.2.2. *Effect of starch*

14 Four different concentrations of potato starch (0, 1, 5 and 10%) were tested to
15 determine the influence of carbohydrates on the efficacy of oregano and thyme. The lag
16 phase of *L. monocytogenes* grown in starch model media containing oregano or thyme
17 decreased in either 5% or 10% starch concentration. Low concentrations of this
18 carbohydrate had a positive influence on the EO antimicrobial activity, with higher lag
19 phases by comparison with the control ($p < 0.05$). In general, the growth rate of *L.*
20 *monocytogenes* decreased at higher starch concentrations. There was no significant
21 difference in the growth rate of *Listeria* within starch model media at any concentration
22 whether it contained EOs or not ($p < 0.05$).

23

1 3.2.3. *Effect of sunflower oil*

2 To assess the effect of oil on the EO antimicrobial activity, the growth of *L.*
3 *monocytogenes* was monitored in model media containing sunflower oil concentrations of
4 0, 1, 5 and 10%. When EOs were included in the oil media, *L. monocytogenes* had a
5 shorter lag phase at higher oil concentrations ($p < 0.05$). The growth rate decreased at
6 10% oil. The addition of EOs to the varying concentrations of oil media did not
7 significantly effect the growth rate by comparison with controls.

8

9 3.2.4. *Effect of pH*

10 The effect of pH on the EO antimicrobial activity was evaluated using TSB at pH 4, 5,
11 6 and 7 (Tables 6, 7). *L. monocytogenes* did not grow at pH 4. The lag phase of *Listeria*
12 grown in pH model media with EO was longer than that recorded in EO free controls ($p <$
13 0.05), especially at pH 5, however, the lag phase was greatest at pH 5 in control media
14 also. The growth rate of *L. monocytogenes* increased at higher pH values, regardless of
15 presence or absence of EOs.

16

17 **4. Discussion**

18 Since higher concentrations of plant EOs are generally required when added to
19 food, the application of EOs in food may be limited due to changes in organoleptic and
20 textural quality of food or interactions of EOs with food components (Devlieghere et al.,
21 2004). Accordingly, a challenge for practical application of EOs is to develop optimised
22 low dose combinations to maintain product safety and shelf-life, thereby minimising the

1 undesirable flavour and sensory changes associated with the addition of high
2 concentrations of EOs.

3 In this work, combinations of selected EOs (basil, lemon balm, marjoram, oregano,
4 rosemary, sage and thyme) were tested against *B. cereus*, *E. coli*, *L. monocytogenes* and
5 *P. aeruginosa*. Preliminary studies showed that the combination of oregano with thyme
6 had a greater efficacy than the EOs separately against the four pathogens. Furthermore,
7 the combinations of oregano with lemon balm and basil increased the lag phase of *E. coli*
8 by 3.55h and 7.44h, respectively, by comparison with oregano alone. When oregano was
9 combined with marjoram, the maximum specific growth rate was reduced 3 times, with
10 respect to both EOs individually. There were important additive effects found when FIC
11 calculations from the extended study of oregano, marjoram and thyme in combination
12 with the remainder of the EOs were performed. Although none of the combinations
13 showed clear synergistic effects, combining EOs selected in this study, at lower
14 concentrations than required for the EOs alone, has potential for practical application to
15 extend the shelf-life of selected foods.

16 Burt (2004) suggested that the minor components present in the EOs extracts are more
17 critical to the activity than EOs main components mixed, and may have a synergistic
18 effect or potentiating influence. In many cases the result was an “additive effect”. All the
19 oregano combinations were additive against *B. cereus*. The following EO combinations
20 also showed additive effects: oregano in combination with basil or thyme against *E. coli*
21 and *P. aeruginosa*, oregano combined with marjoram against *E. coli*, and marjoram and
22 thyme mixed with basil, rosemary or sage against *L. monocytogenes*. These results can be
23 explained considering the efficacy of the main components of EOs individually. In

1 general, EOs possessing the strongest antibacterial properties contain a high percentage
2 of carvacrol and/or thymol, such as oregano or thyme (Elggayar et al., 2001; Dorman and
3 Deans, 2002; Burt, 2004; Oussalah et al., 2006). Since hydroxyl groups and allylic side
4 chains enhance EO efficacy (Burt, 2004), linalool and 4-thujanol, which are the main
5 components of basil and marjoram, respectively, may have contributed to the promising
6 antimicrobial activity achieved with their combinations. Previous studies reported the
7 high antimicrobial activity of linalool or basil against Gram-negative bacteria (Elggayar
8 et al., 2001; Oussalah et al., 2006). Acetate moieties in EO compounds may also
9 positively influence the activity of marjoram (Dorman and Deans, 2000). The main
10 components of rosemary and sage, which are camphor and eucalyptol, possess oxygen
11 functions in their structure and these functions are known to increase the antimicrobial
12 properties of terpenoids (Naigre et al., 1996) A major component of sage, β -
13 caryophyllene, also has a high antimicrobial activity against Gram-positive bacteria
14 (Longaray Delamare et al., 2005). As antimicrobial activity depends not only on chemical
15 composition but also on lipophilic properties, the potency of functional groups or aqueous
16 solubility (Dorman and Deans, 2000) and the mixture of compounds with different
17 biochemical properties may increase EO efficacy.

18 However, the mechanism of action as well as EO composition deserves to be studied
19 in more detail in order to elucidate why combinations of EOs with a strong individual
20 antimicrobial efficacy such as oregano and thyme, did not show synergistic effects, and
21 why on the other hand combinations of two EOs with individually moderate activity
22 resulted in enhanced effects in combination, such as marjoram, basil, rosemary or sage.
23 Lambert et al., (2001), reported that carvacrol and thymol in combination were additive

1 against *S. aureus* and *P. aeruginosa*. Nazer et al., (2004), found that thymol in
2 combination with other aromatic compounds led to improved inhibition, but no real
3 synergistic effect was demonstrated between compounds against *Salmonella*. As plant
4 EOs possess similar composition, their combinations may exhibit addition rather than a
5 synergistic effect. As a result, combinations with other compounds containing different
6 chemical structures may improve the EO efficacy. For example, synergism between
7 carvacrol and its precursor *p-cymene* has been noted (Ultee et al., 2000). It appears that *p-*
8 *cymene*, a very weak antibacterial, swells bacterial cell membranes to a greater extent
9 than carvacrol does, so *p-cymene* probably enables carvacrol to be more easily
10 transported into the cell. Lin et al., (2004) reported a 1 Log cycle reduction of *L.*
11 *monocytogenes* using an oregano and cranberry EO combination (ratio, 75:25) and
12 although the reduction was 6 log₁₀CFU higher with the addition of lactic acid, the authors
13 suggested that synergistic effects did not occur with the EOs tested. Thus, EOs may be
14 employed in combination with other food preservation hurdles in order to control the
15 growth of pathogens and spoilage in food at low doses. In this context, some authors
16 proposed that the combination of essential oil constituents with other natural
17 preservatives, such as bacteriocins or fatty acids, promoted their efficiency against food-
18 borne pathogens (Yamazaki et al., 2004; Grande et al., 2007).

19 Recently, some studies have shown successful or potential applications of EOs and
20 their compounds, alone or in combination with other preservation methods, in order to
21 reduce or control the presence of foodborne pathogens and spoilage microorganisms on
22 food produce, such as fruit (Raybaudi-Massilia et al., 2006; Martinez-Romero et al.,
23 2007), fish (Mahmoud et al., 2006), meat (Mytle et al., 2006; Ahn et al., 2007; Ghalfi et

1 al., 2007; Solomakos et al., 2008), or milk (Cava et al., 2007). However, in some cases
2 the EO activity decreased considerably when added to a complex food system. For
3 example, Firouzi et al., (2007) found that oregano and nutmeg were effective against *E.*
4 *coli* O157:H7 in a broth system, but had no effect in ready-to-cook chicken. Uhart et al.,
5 (2005), also observed that when in direct contact, spices inactivated *S. typhimurium*
6 DT104, but that the activity decreased when applied to ground beef. Thus, an important
7 aspect for the optimised application of plant EOs is the evaluation of efficacy within food
8 products or in model systems that closely simulate food composition, prior to application
9 on real food. In this study four different model media were prepared to assess and
10 quantify the effect of food components on the antimicrobial efficacy of oregano and
11 thyme against *L. monocytogenes*.

12 The presence of high concentrations of protein promoted the growth of *L.*
13 *monocytogenes*, however the efficacy of oregano and thyme was also greater at these
14 higher concentrations of protein. The beef extract culture medium was constituted mainly
15 of peptones, which may have displayed hydrophobic properties with consequent
16 interactions with EOs to facilitate their dissolution in this medium. Baranauskien et al.,
17 (2006), reported that proteins usually possess a high binding capacity for flavor volatile
18 compounds. However, other studies have shown that milk proteins are limiting factors for
19 antimicrobial efficacy (Pol et al., 2001, Smith-Palmer et al., 2001, Devlieghere et al.,
20 2004).

21 The antimicrobial activity of EOs was very high at pH 5. Previously, it was also
22 observed that the inhibitory effect of plant extracts was greater at acidic pH values (Del
23 Campo et al., 2000; Hsieh et al., 2001). The susceptibility of bacteria to EOs appears to

1 increase with lower pH values since the hydrophobicity of EOs increases at low pH,
2 consequently enabling easier dissolution in the lipids of the cell membrane of target
3 bacteria (Juven et al., 1994). The major efficacy of EOs at pH 5 was confirmed with the
4 lag phase and growth rate results for *Listeria* at this pH, which were longer and lower,
5 respectively, than at pH 6 or 7. Similar trends with regard to pH effects were observed
6 within control media without EOs to those containing EOs. As the pH was reduced, the
7 lag phase increased and the growth rate declined for all tests. Furthermore, the addition of
8 either Oregano or Thyme, particularly to media at pH 5, enhanced the reduction of the
9 growth rate and the extension of the lag phase.

10 High concentrations of sunflower oil had a negative influence on the antimicrobial
11 activity of oregano and thyme EOs. Singh *et al.* (2003) reported that thyme EO reduced
12 bacterial populations significantly in zero- and low-fat hotdogs, but not in full-fat
13 hotdogs. Cava et al. (2007) found that the antimicrobial activity of cinnamon and clove
14 EOs against *L. monocytogenes* was reduced in milk samples with higher fat content.
15 Similarly, Smith-Palmer et al. (2001) observed that EOs were less effective in full-fat soft
16 cheese than in low-fat soft cheese. Canillac and Mourey (2004) also observed that the
17 addition of dairy fat into a test medium reduced the antilisterial efficacy of *Picea excelsa*
18 EO. Previously, mint oil was found to exhibit little antibacterial effect against *L.*
19 *monocytogenes* and *S. enteritidis* in high fat products, whereas in low fat food the same
20 EO was much more effective (Tassou *et al.*, 1995). In this context, Glass and Johnson
21 (2004) reported that the antibotulinal effects of nisin and fatty acids were reduced by 20%
22 milk fat or soybean oil. Mejlholm and Dalgaard (2002) suggested that if the EOs

1 generally dissolve in the lipid phase there will be relatively less available to act on
2 bacteria present in the aqueous phase.

3 The EO efficacy was also reduced at high concentrations of starch, in contrast to the
4 general observation that carbohydrates in foods do not protect bacteria from the action of
5 EOs as much as fat and protein do (Shelef et al., 1984). Devlieghere et al. (2004) reported
6 a protective effect of carbohydrate for bacteria where starch at 30% had a negative impact
7 on the antimicrobial activity of chitosan. Ofman et al. (2004), also observed a negative
8 effect of tapioca starch on antimicrobial activity of preservatives.

9 Control of *L. monocytogenes* contamination, survival or growth in ready-to-eat (RTE)
10 meat is a major challenge confronting the food industry (Singh et al., 2003; Mytle et al.,
11 2006; Busatta et al., 2008). Organic acids, steam and hot water treatments are commonly
12 used as decontamination treatments, but *L. monocytogenes* can thrive in the environment
13 of meat processing facilities. Singh et al. (2003) suggested that the use of EOs in
14 conjunction with other preservation techniques such as chemical preservatives or low
15 temperature could develop a synergistic alternative to current methods. Based on the
16 results shown in this work, EO mixtures may be suitable for application within surface
17 decontamination protocols or inclusion as ingredients in ready to use raw or cooked
18 foods. This work shows a link between food composition and EO antimicrobial efficacy,
19 therefore careful selection of EO combinations taking food composition into account
20 prior to application development is important. Low dose combinations of EOs may be
21 useful for control of food safety in low fat RTE-meat based products where the sensory
22 characteristics could be desirable or in lower pH foods where a risk of pathogen survival

1 exists. Incorporation of organic acids may also enhance the efficacy of EO combinations
2 for preservation of quality and safety in real food systems for extended periods.

3

4 **5. Conclusions**

5 The antimicrobial efficacy of the EOs in this study was found to be a function of
6 ingredient manipulation. The antimicrobial activity of oregano and thyme against *L.*
7 *monocytogenes* was increased at higher protein concentrations and moderately acidic pH
8 conditions. Concentrations above 5% of potato starch or sunflower oil reduced EO
9 efficacy. Therefore, the application of EOs should be further investigated for control of
10 microbial safety and spoilage concerns in proteinaceous foods and/or foods with low pH
11 values, which may promote the antibacterial efficacy of EOs. The retention of anti-
12 microbial efficacy of EOs within suitable food systems should be evaluated alone as well
13 as taking hurdle effects of other preservation methods into account.

14 Combinations of plant EOs were assessed for synergistic activity, as this would allow
15 lower concentrations of EOs to be used, thereby achieving the twin aims of reducing any
16 undesirable organoleptic impact, as well as controlling food-borne pathogens and
17 spoilage bacteria in food. No synergy was found but addition occurred with a number of
18 combinations. Oregano and thyme were the most effective EOs applied individually, and
19 in combination they produced an additive effect against *B. cereus* and *P. aeruginosa*. The
20 combinations of oregano with marjoram and thyme with sage had promising efficacy
21 against *E. coli* and *L. monocytogenes* respectively. Thus, oregano combined with thyme
22 at low doses should be considered as a potential alternative for control of pathogens as
23 well as microbial spoilage issues, while the combinations of oregano with marjoram or

1 thyme with sage might be useful for targeted control of key Gram-negative or Gram-
2 positive bacteria, respectively.

3

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7

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Table 1

Essential oils selected for this study

Essential oil	Scientific name	Main components (%)
Basil	<i>Ocimum basilicum</i>	Linalool (42.3) Estragole (26.9) Eucalyptol (8.1)
Lemon balm	<i>Melissa officinalis</i>	Citral (Neral/Geraniol) (22.4/36.7) Caryophyllene (13.2)
Marjoram	<i>Origanum majorana</i>	4-Thujanol (36.2) Sabinene hydrate acetate (16.8) Terpinene-4-ol (8.7)
Oregano	<i>Origanum vulgare</i>	Carvacrol (68.5) Thymoquinone (12.1) p-Cymene (7.8)
Rosemary	<i>Rosmarinus officinalis</i>	Eucalyptol (39.6) Camphor (19) Alpha Pinene (4.8)
Sage	<i>Salvia triloba</i>	Eucalyptol (42.0) Camphor (12.0) Caryophyllene (7.2)
Thyme	<i>Thymus vulgaris</i>	Thymol (52.9) p-Cymene (34.0)

Table 2

Effect of EO combinations on growth parameters of *E. coli* ATCC 25922

EOs (Individual)		Lag phase ^a	Maximum specific growth rate ^b
		St. Dev.	St. Dev.
Oregano	300 ppm	3.84 ± 0.21	0.367 ± 0.042
Basil	10,000 ppm	4.86 ± 0.30	0.452 ± 0.005
Lemon balm	20,000 ppm	5.21 ± 0.43	0.401 ± 0.019
Marjoram	2,000 ppm	3.77 ± 1.01	0.406 ± 0.021
Rosemary	10,000 ppm	4.55 ± 0.70	0.480 ± 0.196
Sage	50,000 ppm	4.80 ± 1.61	0.432 ± 0.016
Thyme	600 ppm	3.29 ± 0.07	0.400 ± 0.006
EO combinations^c			
Oregano + Basil	(1/2)	11.28 ± 3.05	0.452 ± 0.004
Oregano + Basil	(1/4)	4.21 ± 0.19	0.449 ± 0.004
Oregano + Lemon balm	(1/2)	8.76 ± 0.18	0.312 ± 0.023
Oregano + Lemon balm	(1/4)	5.69 ± 0.18	0.407 ± 0.015
Oregano + Marjoram	(1/2)	4.05 ± 1.23	0.117 ± 0.043
Oregano + Marjoram	(1/4)	2.86 ± 0.34	0.404 ± 0.032
Oregano + Rosemary	(1/2)	5.84 ± 1.37	0.383 ± 0.018
Oregano + Rosemary	(1/4)	3.76 ± 0.81	0.420 ± 0.006
Oregano + Sage	(1/2)	8.50 ± 1.25	0.419 ± 0.019
Oregano + Sage	(1/4)	4.67 ± 0.51	0.425 ± 0.005
Oregano + Thyme	(1/2)	6.32 ± 0.57	0.376 ± 0.025
Oregano + Thyme	(1/4)	3.08 ± 0.17	0.395 ± 0.009

^a Lag phase is expressed in hours.^b Maximum specific growth rate is expressed in hours⁻¹.^c EO combinations were assessed at 50% (1/2) and 25% (1/4) of the individual concentrations.

Table 3

Concentrations^a of EOs used alone (A) or in combination (C) against the bacteria selected for this study

EOs	<i>B. cereus</i> ATCC 11778		<i>E. coli</i> ATCC 25922		<i>L. monocytogenes</i> IL323		<i>P. aeruginosa</i> ATCC 27853	
	A	C	A	C	A	C	A	C
Basil	20,000	10,000	20,000	10,000	20,000	10,000	200,000	100,000
Lemon balm	50,000	25,000	40,000	20,000	2,000	1,000	200,000	100,000
Marjoram	10,000	5,000	4,000	2,000	8,000	4,000	100,000	50,000
Oregano	500	250	400	200	200	100	4,000	2,000
Rosemary	20,000	10,000	20,000	10,000	10,000	5,000	200,000	100,000
Sage	200,000	100,000	200,000	100,000	5,000	2,500	200,000	100,000
Thyme	1,000	500	1,000	500	200	100	5,000	2,500

^a Concentrations are expressed in ppm

Table 4

FIC indices of oregano combinations

Combinations	<i>B. cereus</i> ATCC 11778		<i>E. coli</i> ATCC 25922		<i>L. monocytogenes</i> IL323		<i>P. aeruginosa</i> ATCC 27853	
	FIC	Stdev ^b	FIC	Stdev ^b	FIC	Stdev ^b	FIC	Stdev ^b
<i>Oregano</i> +								
Basil	0.83 (A) ^a	± 0.14	1.00 (A)	± 0.00	1.25 (I)	± 0.35	1.00 (A)	± 0.00
Lemon balm	0.86 (A)	± 0.18	1.17 (I)	± 0.34	1.25 (I)	± 0.43	1.38 (I)	± 0.12
Marjoram	0.75 (A)	± 0.15	0.83 (A)	± 0.30	1.18 (I)	± 0.40	1.75 (I)	± 0.35
Rosemary	0.79 (A)	± 0.20	1.83 (I)	± 0.29	1.50 (I)	± 0.00	1.50 (I)	± 0.00
Sage	1.00 (A)	± 0.00	1.38 (I)	± 0.18	1.75 (I)	± 0.35	1.50 (I)	± 0.00
Thyme	0.78 (A)	± 0.16	1.17 (I)	± 0.30	1.18 (I)	± 0.30	0.88 (A)	± 0.18

^a 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).

^b Standard deviation

Table 5

FIC indices of marjoram and thyme combinations against *L. monocytogenes* IL323

Combinations	Marjoram		Thyme	
	FIC	Stdev ^b	FIC	Stdev ^b
<i>Marjoram or thyme +</i>				
Basil	0.75 (A) ^a	± 0.51	0.94 (A)	± 0.44
Lemon balm	1.25 (I)	± 0.00	1.25 (I)	± 0.35
Marjoram	-		1.55 (I)	± 0.57
Oregano	1.18 (I)	± 0.40	1.18 (I)	± 0.30
Rosemary	1.03 (A)	± 0.54	1.06 (A)	± 0.62
Sage	1.00 (A)	± 0.25	1.00 (A)	± 0.00
Thyme	1.55 (I)	± 0.57	-	

^aResults are interpreted as synergy (**S**, FIC < 0.5), addition (**A**, 0.5 ≤ FIC ≤ 1), indifference (**I**, 1 < FIC ≤ 4) or

antagonism (**AN**, FIC > 4).

^b Standard deviation

Table 6

Lag phase (λ) of *L. monocytogenes* IL323 grown in model media containing oregano (30 ppm) or thyme (60 ppm)

Model media	Oregano		Thyme		Control ^c	
	λ (h) ^a	Stdev ^b	λ (h) ^a	Stdev	λ (h) ^a	Stdev
Beef extract						
1.5 %	7.39	± 0.96	9.15	± 2.57	8.01	± 2.63
3.0 %	7.81	± 1.22	11.79	± 1.33	6.14	± 0.08
6.0 %	10.75	± 2.31	10.94	± 2.51	6.48	± 0.20
12.0 %	10.79	± 1.53	9.75	± 2.16	6.33	± 0.37
Starch media						
0.0%	15.01	± 1.40	12.06	± 2.70	7.80	± 0.93
1.0%	13.96	± 3.66	11.69	± 2.08	7.04	± 0.17
5.0%	9.71	± 0.89	8.87	± 3.56	7.58	± 0.30
10.0%	8.84	± 1.52	8.43	± 2.38	8.04	± 0.75
Sunflower oil media						
0.0%	15.23	± 0.05	15.05	± 0.45	7.75	± 0.13
1.0%	14.21	± 0.33	12.54	± 0.06	7.31	± 0.10
5.0%	10.50	± 0.13	9.22	± 0.30	7.24	± 0.10
10.0%	9.17	± 0.14	9.47	± 0.03	7.33	± 0.18
pH						
TSB pH 4	0.00	± 0.00	0.00	± 0.00	0.00	± 0.00
TSB pH 5	12.43	± 0.44	16.13	± 1.28	9.55	± 1.83
TSB pH 6	7.70	± 0.73	9.48	± 1.51	6.75	± 1.57
TSB pH 7	9.50	± 0.09	10.80	± 0.95	6.88	± 1.10

^a Lag phase is expressed in hours.

^b Standard deviations are indicated beside each value.

^c *L. monocytogenes* grown in model media without any EO was used as the control.

Table 7

Maximum specific growth rate (μ_{\max}) of *L. monocytogenes* IL323 grown in model media containing oregano (30 ppm) or thyme (60 ppm)

Model media	Oregano		Thyme		Control ^c	
	μ_{\max} (h ⁻¹) ^a	Stdev ^b	μ_{\max} (h ⁻¹) ^a	Stdev	μ_{\max} (h ⁻¹) ^a	Stdev
Beef extract						
1.5 %	0.034	± 0.006	0.079	± 0.022	0.054	± 0.037
3.0 %	0.076	± 0.041	0.146	± 0.077	0.074	± 0.013
6.0 %	0.099	± 0.037	0.195	± 0.068	0.117	± 0.032
12.0 %	0.195	± 0.018	0.200	± 0.019	0.215	± 0.001
Starch media						
0.0%	0.185	± 0.058	0.250	± 0.021	0.238	± 0.040
1.0%	0.208	± 0.082	0.271	± 0.014	0.268	± 0.058
5.0%	0.142	± 0.032	0.164	± 0.020	0.147	± 0.008
10.0%	0.098	± 0.016	0.104	± 0.003	0.097	± 0.002
Sunflower oil media						
0.0%	0.240	± 0.015	0.231	± 0.008	0.279	± 0.008
1.0%	0.226	± 0.001	0.209	± 0.004	0.223	± 0.007
5.0%	0.208	± 0.012	0.235	± 0.005	0.200	± 0.018
10.0%	0.174	± 0.013	0.220	± 0.004	0.170	± 0.014
pH						
TSB pH 4	0.000	± 0.000	0.000	± 0.000	0.000	± 0.000
TSB pH 5	0.004	± 0.001	0.016	± 0.006	0.017	± 0.018
TSB pH 6	0.141	± 0.020	0.175	± 0.025	0.173	± 0.064
TSB pH 7	0.284	± 0.022	0.328	± 0.011	0.261	± 0.054

^a Maximum specific growth rate is expressed in hours⁻¹.

^b Standard deviations are indicated beside each value.

^c *L. monocytogenes* grown in model media without any EO was used as the control.