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Antimicrobial Activity of Essential Oil Components Against Potential Food Spoilage Microorganisms

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Abstract The antimicrobial activity of six essential oil components against the potential food spoilage bacteria *Aeromonas (A.) hydrophila*, *Escherichia (E.) coli*, *Brochothrix (B.) thermosphacta,* and *Pseudomonas (P.) fragi* at single use and in combination with each other was investigated. At single use, the most effective oil components were thymol (bacteriostatic effect starting from 40 ppm, bactericidal effect with 100 ppm) and carvacrol (50 ppm/100 ppm), followed by linalool (180 ppm/720 ppm), α-pinene (400 ppm/no bactericidal effect), 1,8-cineol (1,400 ppm/2,800 ppm), and α-terpineol (600 ppm/no bactericidal effect). Antimicrobial effects occurred only at high, sensorial not acceptable concentrations. The most susceptible bacterium was *A. hydrophila*, followed by *B. thermosphacta* and *E. coli*. Most of the essential oil component combinations tested showed a higher antimicrobial effect than tested at single use. Antagonistic antimicrobial effects were observed particularly against *B. thermosphacta*, rarely against *A. hydrophila.* The results show that the concentration of at least one of the components necessary for an antibacterial effect is higher than sensorial acceptable. So the use of herbs with a high content of thymol, carvacrol, linalool, 1,8-cineol, α-pinene or α-terpineol alone or in combination must be weighted against sensorial quality.

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Introduction

Microorganisms causing food spoilage are of high interest for food producers as well as for the sustainability of food consumption. The extension of shelf-life of perishable food products is therefore in the focus of retailers and consumers demands for prolonged best before date. Such extension is mainly achieved by technological measures and addition of synthetic food preservatives. However, the consumer prefers food with no artificial additives. Consequently there is an increasing demand for naturally preserved foods. Herbs are therefore of interest as alternative to conventional food preservatives [6, 17]. Herbs are products from dried parts of aromatic plants, which are used for flavoring foods and beverages [27]. In addition to their aromatic properties herbs show antimicrobial activities. In several studies thyme, oregano, rosemary, and basil showed antibacterial activities against food spoilage bacteria and antimycotic properties [1, 9, 13, 20, 24]. Aromatic and antimicrobial properties of herbs depend on the quantity and composition of their essential oils [14]. Genetically determined properties of the plant, environmental conditions, age of the plant, dehydration procedure, and storage conditions influence the quantity and composition of an essential oil [10]. Hence, the amount of essential oil in aromatic plants as well as the composition of essential oil is subjected to considerable variation limiting the prediction of their antibacterial properties.

Essential oils are variable mixtures of terpenoids, alcohols, aldehydes, phenolics, acids, and aliphatic hydrocarbons [22]. Especially terpenes are responsible for the aromatic and medicinal uses of herbs [13]. In several studies the phenols thymol, carvacrol, and eugenol showed a strong antimicrobial effect against foodborne pathogens [1, 4, 12, 18] and pathogens in the gastrointestinal tract [29].

Besides the phenolic compounds, alcohols such as linalool and α-terpineol possess antimicrobial properties [13, 18]. Hydrocarbons such as α-pinene show a comparable lower [13] or in the case of para-cymene and γ-terpineol no antimicrobial effect [1]. 1,8-cineol is comparable to α-pinene [5].

As to their antimicrobial activity synergistic and antagonistic effects between essential oil components were observed [5, 25]. Many studies have determined the antimicrobial properties of the whole essential oils or single essential oil components. But only few data concerning the interactions between different essential oil components are available [15, 17]. In this study the antimicrobial activity of the essential oil components thymol, carvacrol, linalool, 1,8-cineol, α-terpineol, α-pinene, and combinations of two of them against potential food spoilage bacteria were tested. Possible applications could be short and long ripened air dried raw sausage or smoked meat products with low temperature ripening. *Aeromonas* (*A.*) *hydrophila* and *Brochothrix* (*B.*) *thermosphacta* are typical Gram-negative and Gram-positive bacteria in meat and meat products. *E. coli* was chosen as representative for *Enterobacteriaceae* and *Pseudomonas* (*P.*) *fragi* as part of the pseudomonads.

Materials and Methods

Bacterial Strains and Culture Media

All bacterial strains were obtained from the German collection of microorganisms and cell cultures (DSMZ). The bacterial strains used were *Pseudomonas fragi* (DSM 3456, plant origin), *Brochothrix themosphacta* (DSM 20171, pork origin), *Aeromonas hydrophila* (DSM 30187^T, dairy origin), and *Escherichia coli* (DSM 10727, piglet). Stock cultures of the bacteria used were frozen at −80 °C. For experimental use, the bacterial strains were plated on Mueller–Hinton agar with sheep blood (Oxoid, Wesel, Germany) and incubated at 37 °C (*E. coli*), 30 °C (*A. hydrophila*) for 24 h or 25 °C (*B. thermosphacta*, *P. fragi*) for 48 h. Cultures for experiments were prepared by streak-plating the bacterial strain used on standard-I-nutrient agar (Merck, Darmstadt, Germany). Bacterial cell counts were also done on standard-I-nutrient agar.

Essential Oil Components and Preparation of Stock Broths

Thymol, carvacrol, linalool, 1,8-cineol, α-pinene, and α-terpineol were purchased from Sigma-Aldrich (Munich, Germany). Stock broths of each essential oil component were prepared by dissolving the compound in nutrient broth No. 2 (Oxoid, Wesel, Germany). Because of the hydrophobicity of essential oil components, Tween®80 (AppliChem, Darmstadt, Germany) or iso-propyl-alcohol (CG Chemikalien, Laatzen, Germany) were added to nutrient broth No. 2. Stock broths of thymol contained 1 % iso-propyl-alcohol, stock broths of carvacrol 0.4 % iso-propyl-alcohol. To emulsify linalool, 1,8-cineol, α-pinene, and α-terpineol 0.5 % Tween®80 was added to nutrient broth No. 2. The stock broths were filled in aliquots of 10–12 ml sterile Rotilabo® centrifuge tubes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and stored at −18 ± 2 °C.

Antimicrobial Assay

Initially strains were grown without the addition of essential oils to determine the number of cfu/ml for each test strain under optimal growth conditions. After the incubation (as described under section bacterial strains), a decimal dilution series was made. For confirmation the total cell count was determined immediately using the drop plate procedure according to the German standard method [2]. The results of these growth experiments were used to determine possible antimicrobial activity of the essential oils (Table 1). To test the antimicrobial activity of a single chemical compound the wells of a 96-well micro-well plate (Sarstedt, Nürmbrecht, Germany) were inoculated with 50 µl of bacterial suspension and were diluted with stock broth of the examined oil component. The bacterial suspension (containing ca. 10^6 cfu/ml) for inoculation of the micro-well plate was prepared by inoculating material of the appropriate bacterium in 7 ml brain–heart-infusion bouillon (Oxoid, Wesel, Germany). The bouillon was incubated for 20 h at 37 °C (*E. coli*), 30 °C (*A. hydrophila*) or 25 °C (*B. thermosphacta*, *P. fragi*). After the incubation, a decimal dilution series was made. For confirmation the total cell count was determined immediately using the drop plate procedure according to the German standard method [2]. Stock broths were prepared by an increasing addition of nutrient broth No. 2 without any additives. The lowest dilution concentration of the essential oil component was equal or higher as the usual concentration reached by the essential oil in the corresponding herb in food [3]. The concentration ranged between 4 and 100 ppm thymol, 2–200 ppm carvacrol, 360–1,200 ppm linalool,

Table 1 Number (*n*), arithmetic mean (*m*), root mean square deviation (*s*), lower confidence interval limit (lcI), and upper confidence interval limit (ucI) of control samples without any additives

Species	*n*	*m*	*s*	lcI	ucI
A. hydrophila	51	8.79	0.11	8.76	8.82
E. coli	42	8.74	0.12	8.71	8.78
B. thermosphacta	48	7.81	0.21	7.75	7.87
P. fragi	42	7.85	0.10	7.82	7.88

600–2,800 ppm 1,8-cineol, 340–1,200 ppm α-pinene, and 600–800 ppm α-terpineol. The lowest concentrations with commercially available compounds could be reached, when 1 g thyme, 0.5 g oregano, 2 g bay laurel, 2 g rosemary or 3 g marjoram are added to 1 kg of a food [3].

To examine the antibacterial activity of a combination of essential oil components, the wells were inoculated with bacterial suspension and the stock broths of the combined essential oil compounds. Each tested concentration of a single essential oil component and each combination of essential oil components was inoculated in seven wells per micro-well plate. Furthermore, a positive control (growth control) was prepared in 8 wells inoculated only with bacterial suspension and nutrient broth No. 2.

In addition, 8 wells of the plate were inoculated with bacterial suspension and nutrient broth No. 2 containing the appropriate amount of Tween®80 or iso-propyl-alcohol, which had been added to the stock broth used in the current test procedure. In each test procedure, three micro-well plates were independently inoculated and were subjected to the same test procedure simultaneously.

After inoculation, the micro-well plates were covered with optical clear, adhesive foil (Abgene, Epsom, United Kingdom) and incubated for 12 h at 25 °C (*B. thermosphacta, P. fragi*) or 7 h at 30 °C (*E. coli, A. hydrophila*) representing ripening temperatures of raw sausages. After incubation 100 µl of the content of one well were taken from each tested combination and the controls. Subsequently the aerobic viable cell count at 30 °C (*A. hydrophila*), 37 °C (*E. coli*), or 25 °C (*B. thermosphacta*, *P. fragi*) was determined using the drop plate procedure as described above. The limit of detection was 2.0×10^2 cfu/ml. If viable cell counts were lower than the detection limit, they were counted as 1.0×10^2 cfu/ml. For statistical evaluation the logarithm to the base 10 (lg) of the cell counts was taken.

Interpretation of Obtained Data and Statistics

A concentration of an essential oil component or a combination of essential oil components was classified as bacteriostatic, if the upper limit of their 95 % confidence interval (ucI) was lower than the lower limit of the 95 % confidence interval (lcI) of the related control. A concentration of an essential oil component or a combination of essential oil components was defined as not antimicrobial active, if their confidence intervals overlapped with the confidence interval of the appropriate control. A bactericidal effect was stated, if the viable cell count after incubation was lower than the number of bacteria inoculated. A synergistic effect of the combinations was stated, if the bacterial cell count was significantly ($P < 0.05$) less than the bacterial cell count after application of only one essential oil component. An antagonistic effect was stated, if the bacterial cell count was significantly higher than the bacterial cell count after application of only one essential oil component.

For enumeration each dilution was applied on two plates. The weighted average was calculated with the following formula from the colony count of the lowest and the next highest countable dilution:

$$\overline{c} = \frac{\sum c}{n_1 \cdot 1 + n_2 \cdot 0.1}$$

$\overline{c}$ is the weighted average of the colony count, $\sum c$ sum of the colony counts of all plates of both dilutions (the lowest and next highest countable dilution), n_1 number of plates of the lowest countable dilution, n_2 number of plates of the next highest countable dilution.

For presenting the results all total plate counts (cfu/ml or cfu/g) were transformed to logarithm (lg cfu/ml or lg cfu/g). The lowest dilution was 1.00×10^{-1}. This corresponded to a plate dilution of 5.00×10^{-3} with the drop plate method (0.05 ml per sector). The detection limit was 2.00×10^2 cfu/ml = 2.30 lg cfu/ml ($1/[5.00 \times 10^{-3}]$). In case of total plate counts below the detection limit further calculations were done with half of the detection limit.

For all calculations the program SAS® version 9.1.3 service pack 4 (Statistic Analysing Systems, SAS Institute®) was used. The whole set of original data for the single essential oil components and the combinations can be found in Rüben [26].

Results

Table 1 shows the results of the initial growth tests of the different strains without the addition of essential oils with their statistical parameter. Tables 2, 3, 4, 5 demonstrate the effect on the growth of the four tested strains with addition of selected essential oil component concentrations and combinations of essential oil components as well as synergistic or antagonistic effects.

Aeromonas hydrophila

Bactericidal effects against *A. hydrophila* (Table 2) could be shown for thymol (100 ppm), carvacrol (100 ppm), and linalool (720 ppm) as single component. All substances exhibited bactericidal effects at concentrations ranging from 40/50 ppm up to 1,400 ppm (Table 2). The lowest concentrations necessary for bacteriostatic or bactericidal effects could be attributed to thymol and carvacrol. Synergistic effects could be shown for most tested combinations against *A. hydrophila*. However for combinations of carvacrol and 1,8-cineol antagonistic effects have been observed (Table 2).

Table 2 Concentrations of *A. hydrophila* with addition of single substances and combinations of different essential oils (lower and upper limits of the 95 % confidence interval (lcI and ucI) of bacterial cell counts of single and combined essential oil concentrations tested [lg cfu/ml])

Concentration [ppm]	lcI and ucI single oil	lcI and ucI combined oils	S/A*
100 thymol^{bc}	2.72–3.11	2.00–2.00	S
50 carvacrol^{bs}	8.03–8.22		
40 thymol^{bs}	7.69–8.68	6.26–7.72	S
20 carvacrol	7.93–9.50		
40 thymol^{bs}	7.69–8.68	6.80–7.05	S
2 carvacrol	8.28–9.12		
40 thymol^{bs}	7.69–8.68	2.00–2.00	S
720 linalool^{bc}	5.73–5.84		
40 thymol^{bs}	7.69–8.68	7.48–8.12	–
360 linalool^{bs}	7.40–7.44		
4 thymol	7.71–10.73	1.92–2.14	S
720 linalool^{bc}	5.73–5.84		
40 thymol^{bs}	7.69–8.68	4.60–5.44	S
1,400 1,8-cineol	7.78–8.12		
40 thymol^{bs}	7.69–8.68	8.24–8.55	–
600 1,8-cineol	8.66–8.91		
4 thymol	7.71–10.73	6.12–6.32	S
1,400 1,8-cineol	7.78–8.12		
100 thymol^{bc}	2.72–3.11	2.00–2.00	S
800 α-pinene	8.40–8.46		
40 thymol^{bs}	7.69–8.68	8.25–8.76	–
1,200 α-pinene	8.26–8.62		
40 thymol^{bs}	7.69–8.68	8.38–9.19	–
800 α-pinene	8.40–8.46		
40 thymol^{bs}	7.69–8.68	5.79–6.32	S
600 α-terpineol^{bs}	7.85–8.57		
40 thymol^{bs}	7.69–8.68	6.63–7.11	S
400 α-terpineol	8.58–8.83		
4 thymol	7.71–10.73	7.78–8.18	–
600 α-terpineol^{bs}	7.85–8.57		
20 carvacrol	7.93–9.50	2.00–2.00	S
720 linalool^{bc}	5.73–5.84		
20 carvacrol	7.93–9.50	7.48–8.12	–
360 linalool^{bs}	7.40–7.44		
2 carvacrol	8.28–9.12	1.92–2.14	S
720 linalool^{bs}	5.73–5.84		
50 carvacrol^{bs}	8.03–8.22	3.94–4.38	S
1,400 1,8-cineol^{bs}	7.78–8.12		
50 carvacrol^{bs}	8.03–8.22	7.56–8.83	–
600 1,8-cineol	8.66–8.91		
20 carvacrol	7.93–9.50	4.67–5.99	S
1,400 1,8-cineol^{bs}	7.78–8.12		
100 carvacrol^{bc}	2.69–3.17	4.74–4.90	A
800 α-pinene	8.40–8.46		
50 carvacrol^{bs}	8.03–8.22	8.42–8.67	A
1,200 α-pinene	8.26–8.62		
50 carvacrol^{bs}	8.03–8.22	8.69–8.81	A
800 α-pinene	8.40–8.46		
20 carvacrol	7.93–9.50	8.71–8.77	A
1,200 α-pinene	8.26–8.62		
50 carvacrol^{bs}	8.03–8.22	6.39–6.91	S
600 α-terpineol^{bs}	7.85–8.57		
50 carvacrol^{bs}	8.03–8.22	6.89–7.70	S
400 α-terpineol	8.58–8.83		
20 carvacrol	7.93–9.50	7.71–8.01	–
600 α-terpineol^{bs}	7.85–8.57		

* *S/A* synergistic effect (S), antagonistic effect (A) or no combined effect (–) of combined concentrations ($P < 0.05$), *bs* bacteriostatic effect, *bc* bactericidal effect

Escherichia coli

No bactericidal effects could be confirmed against *E. coli* for the substances linalool, 1,8-cineol, α-pinene, and α-terpineol. For the latter two and for carvacrol also no bacteriostatic effect could be stated (Table 3). Thymol (at 100 ppm), linalool (1,200 ppm), and 1,8-cineol (2,800 ppm) exhibited bacteriostatic effects against *E. coli*. Only carvacrol (with 200 ppm) had a bactericidal effect. As for *A. hydrophila* also for *E. coli* a synergistic effect of combinations could be observed in most cases, whereas no antagonistic effects could be demonstrated (Table 3).

Brochothrix thermosphacta

Against *B. thermosphacta* only thymol (100 ppm) and α-pinene showed a bactericidal effect. On the other hand carvacrol (100 ppm), linalool (1,200 ppm), and 1,8-cineol (2,000 ppm) proved to be bacteriostatic (Table 4). No antimicrobial effect could be observed for α-terpineol. Synergistic effects of the different combinations were not as common as for *A. hydrophila* and *E. coli*. Thymol/α-terpineol, carvacrol/1,8-cineol, carvacrol/α-pinene, and carvacrol/α-terpineol had antagonistic effects in several combinations (Table 4).

Pseudomonas fragi

No bactericidal effects could be shown against *P. fragi* by the application of single essential oil components. Bacteriostatic effects were exhibited only by thymol (100 ppm), carvacrol (200 ppm) and 1,8-cineol (Table 5). However,

Table 3 Concentrations of *E. coli* with addition of single substances and combinations of different essential oils (lower and upper limits of the 95 % confidence interval (lcI and ucI) of bacterial cell counts of single and combined essential oil concentrations tested [lg cfu/ml])

Concentration [ppm]	lcI and ucI single concentration	lcI and ucI combined concentrations	S/A*
40 thymol	8.47–9.06	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
4 thymol	8.70–8.90	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
4 thymol	8.70–8.90	7.50–7.94	S
720 linalool	8.70–8.83		
40 thymol	8.47–9.06	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
4 thymol	8.70–8.90	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
4 thymol	8.70–8.90	7.50–7.94	S
720 linalool	8.70–8.83		
100 thymol[bs]	7.97–8.83	4.78–5.00	S
2,800 1,8-cineol[bs]	8.04–8.14		
100 thymol[bs]	7.97–8.83	6.60–6.78	S
2,000 1,8-cineol	7.97–8.92		
40 thymol	8.47–9.06	6.18–8.31	–
2,800 1,8-cineol[bs]	8.04–8.14		
100 thymol[bs]	7.97–8.83	7.65–8.20	S
1,200 α-pinene	8.28–9.01		
100 thymol[bs]	7.97–8.83	7.57–7.85	–
800 α-pinene	8.55–9.07		
40 thymol	8.47–9.06	8.71–8.84	–
1,200 α-pinene	8.28–9.01		
100 thymol[bs]	7.97–8.83	6.60–6.72	S
800 α-terpineol	8.69–8.92		
100 thymol[bs]	7.97–8.83	5.14–7.54	S
600 α-terpineol	8.64–8.78		
40 thymol	8.47–9.06	8.40–8.80	–
800 α-terpineol	8.69–8.92		
20 carvacrol	8.67–9.08	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
20 carvacrol	6.87–9.08	6.48–6.85	S
720 linalool	8.70–8.83		
2 carvacrol	8.73–9.00	2.00–2.00	S
1,200 linalool[bs]	7.74–7.92		
50 carvacrol	8.68–8.91	6.58–7.70	S
2,800 1,8-cineol[bs]	8.04–8.14		
50 carvacrol	8.68–8.91	7.96–8.02	S
2,000 1,8-cineol	7.97–8.92		
20 carvacrol	8.67–9.08	6.97–7.23	S
2,800 1,8-cineol[bs]	8.04–8.14		
200 carvacrol	3.88–4.41	2.00–2.00	S
1,200 α-pinene	8.28–9.01		
200 carvacrol[bc]	3.88–4.41	2.00–2.00	S
800 α-pinene	8.55–9.07		
100 carvacrol	8.37–8.92	8.74–9.00	–
1,200 α-pinene	8.28–9.01		
200 carvacrol[bc]	3.88–4.41	2.00–2.00	S
600 α-terpineol	8.64–8.78		
100 carvacrol	8.37–8.92	8.33–8.56	S
800 α-terpineol	8.69–8.92		
100 carvacrol	8.37–8.92	7.81–8.42	S
600 α-terpineol	8.64–8.78		

* *S/A* synergistic effect (S), antagonistic effect (A) or no combined effect (–) of combined concentrations ($P < 0.05$), *bs* bacteriostatic effect, *bc* bactericidal effect

Table 4 Concentrations of *B. thermosphacta* with addition of single substances and combinations of different essential oils (lower and upper limits of the 95 % confidence interval (lcI and ucI) of bacterial cell counts of single and combined essential oil concentrations tested [lg cfu/ml])

Concentration [ppm]	lcI and ucI single concentration	lcI and ucI combined concentrations	S/A*
100 thymol[bc]	5.34–5.70	2.07–3.28	S
50 carvacrol	7.60–8.15		
40 thymol	7.59–7.96	6.87–7.37	S
20 carvacrol	7.74–8.10		
40 thymol	7.59–7.96	6.94–7.47	S
2 carvacrol	8.01–8.20		
100 thymol[bc]	5.34–5.70	3.29–4.05	S
720 linalool	7.08–7.89		
40 thymol	7.59–7.96	1.28–4.40	S
1,200 linalool	6.10–6.13		
4 thymol	8.00–8.00	4.69–5.45	S
1,200 linalool	6.10–6.13		
100 thymol[bc]	5.34–5.70	4.60–5.14	S
2,000 1,8-cineol[bs]	7.22–7.42		
100 thymol[bc]	5.34–5.70	5.51–5.98	–
1,800 1,8-cineol	7.66–7.88		
100 thymol[bc]	5.34–5.70	4.65–4.96	S
400 α-pinene[bc]	5.43–6.14		
40 thymol	7.59–7.96	6.17–6.81	–
400 α-pinene[bc]	5.53–5.70		
100 thymol[bc]	5.34–5.70	6.16–7.36	A
800 α-terpineol	7.78–8.02		
100 thymol[bc]	5.34–5.70	6.64–7.34	A
600 α-terpineol	7.79–7.86		
40 thymol	7.59–7.96	7.58–8.22	–
800 α-terpineol	7.78–8.02		
50 carvacrol	7.60–8.15	3.89–4.28	S
1,200 linalool[bs]	6.10–6.13		
50 carvacrol	7.60–8.15	6.53–7.58	–
720 linalool	7.08–7.89		
20 carvacrol	7.74–8.10	2.85–3.50	S
1,200 linalool[bs]	6.10–6.13		
100 carvacrol[bs]	5.94–6.47	5.54–6.92	–
2,000 1,8-cineol[bs]	7.22–7.42		
100 carvacrol[bs]	5.94–6.47	5.59–6.39	–
1,800 1,8-cineol	7.66–7.88		
50 carvacrol	7.60–8.15	7.51–7.92	A
2,000 1,8-cineol[bs]	7.22–7.42		
100 carvacrol[bs]	5.94–6.47	6.74–7.30	A
340 α-pinene	7.50–8.02		
50 carvacrol	7.60–8.15	5.70–6.82	–
400 α-pinene[bc]	5.43–6.14		
100 carvacrol[bs]	5.94–6.47	7.64–7.88	A
800 α-terpineol	7.78–8.02		
100 carvacrol[bs]	5.94–6.47	7.58–8.11	A
600 α-terpineol	7.79–7.86		
50 carvacrol	7.60–8.15	7.55–8.12	–
800 α-terpineol	7.78–8.02		

* *S/A* synergistic effect (S), antagonistic effect (A) or no combined effect (–) of combined concentrations ($P < 0.05$), *bs* bacteriostatic effect, *bc* bacteriocidal effect

Table 5 Concentrations of *P. fragi* with addition of single substances and combinations of different essential oils (lower and upper limits of the 95 % confidence interval (lcI and ucI) of bacterial cell counts of single and combined essential oil concentrations tested [lg cfu/ml])

Concentration [ppm]	lcI and ucI single concentration	lcI and ucI combined concentrations	S/A*
100 thymol[bs]	7.11–7.61	7.60–7.76	–
1,200 α-pinene	7.76–7.94		
40 thymol	7.92–8.05	7.74–8.03	–
1,200 α-pinene	7.76–7.94		
100 thymol[bs]	7.11–7.61	6.81–7.00	S
1,200 linalool	7.61–7.82		
100 thymol[bs]	7.11–7.61	6.51–7.44	S
720 linalool	7.57–7.99		
40 thymol	7.92–8.05	6.50–7.09	S
1,200 linalool	7.61–7.82		
100 thymol[bs]	7.11–7.61	6.96–7.43	–
2,800 1,8-cineol[bs]	7.69–7.74		
40 thymol	7.92–8.05	7.65–7.81	–
2,800 1,8-cineol[bs]	7.69–7.74		
100 thymol[bs]	7.11–7.61	7.60–7.76	–
1,200 α-pinene	7.76–7.94		
40 thymol	7.92–8.05	7.74–8.03	–
1,200 α-pinene	7.76–7.94		
100 thymol[bs]	7.11–7.61	6.66–6.97	S
600 α-terpineol	7.63–8.20		
40 thymol	7.92–8.05	7.71–7.81	S
800 α-terpineol	7.75–7.89		
40 thymol	7.92–8.05	7.71–7.79	S
600 α-terpineol	7.63–8.20		
50 carvacrol	7.76–8.05	6.13–7.52	S
1,200 linalool	7.61–7.82		
50 carvacrol	7.76–8.05	7.85–7.85	–
720 linalool	7.57–7.99		
20 carvacrol	7.05–9.48	6.87–7.37	S
1,200 linalool	7.61–7.82		
200 carvacrol[bs]	6.24–7.43	2.00–2.00	S
2,800 1,8-cineol[bs]	7.69–7.74		
200 carvacrol[bs]	6.24–7.43	2.97–3.09	S
2,000 1,8-cineol	7.78–7.84		
100 carvacrol	7.53–8.01	7.56–7.84	S
2,000 1,8-cineol	7.78–7.84		
200 carvacrol[bs]	6.24–7.43	3.81–5.35	S
1,200 α-pinene	7.76–7.94		
200 carvacrol[bs]	6.24–7.43	3.99–4.17	S
800 α-pinene	7.44–8.10		
100 carvacrol	7.53–8.01	7.57–7.83	–
1,200 α-pinene	7.76–7.94		
100 carvacrol	7.53–8.01	6.84–7.29	S
800 α-terpineol	7.75–7.89		
100 carvacrol	7.53–8.01	6.74–7.20	–
600 α-terpineol	7.63–8.20		
50 carvacrol	7.76–8.05	7.19–8.25	S
800 α-terpineol	7.75–7.89		

* *S/A* synergistic effect (S), antagonistic effect (A) or no combined effect (–) of combined concentrations ($P < 0.05$), *bs* bacteriostatic effect

for most of the combinations of the single components synergistic effects were observed, but not for the combinations thymol/carvacrol, thymol/1,8-cineol and thymol/α-pinene (Table 5).

Discussion

A. hydrophila was the most susceptible bacterium against all tested substances, followed by *B. thermosphacta* and *E. coli*, whereas *P. fragi* was the most resistant bacterium. *Pseudomonas* spp. often show a higher resistance toward essential oils [17]. Some studies show that Gram-negative bacteria are more resistant to essential oils [19, 30] others claim the same for Gram-positive bacteria [18]. In our study the Gram-negative bacteria were in both categories. The six essential oil components showed antibacterial activity against the tested bacteria only in high concentrations. These concentrations were at least 10-fold the concentration, which represent the concentrations usually used in commercial products (i.e., 1 g/kg thyme, 0.5 g/kg oregano, 2 g/kg bay laurel, 2 g/kg rosemary or 3 g/kg marjoram) [3].

When considering the amounts of the single components used and technologically achievable in food [3], thymol was the most effective antimicrobial substance against all tested bacteria, followed by carvacrol, linalool, α-pinene, 1,8-cineol, and α-terpineol, which was consistent with other studies regarding thymol and carvacrol [4, 13]. Inconsistent with studies of Dorman and Deans [13] and Cosentino et al. [10], linalool was much more antibacterial effective than α-terpineol. The weak antimicrobial properties of α-terpineol in our study might be explained in part by the use of 0.5 % Tween®80, which was needed to emulsify α-terpineol in nutrient broth.

Considering the combinations of essential oils, thymol and carvacrol were the most effective combination against all tested bacteria with technologically achievable concentrations [3], e.g., the growth of *B. thermosphacta* and *A. hydrophila* was inhibited by this combination. In other studies, combinations of thymol and carvacrol showed a high antimicrobial effectivity as well [7, 17]. Carvacrol and thymol respectively combined with linalool showed a synergistic antibacterial effect against all bacteria tested depending on their concentration. The highest increase of antibacterial activity was observed against *E. coli*. Sivropoulou et al. [28] assumed a synergistic effect between the phenolic compounds and linalool, when they tested the antimicrobial activity of three different oregano oils against *Salmonella* Typhimurium, *P. aeruginosa* and *Bacillus subtilis*. Carvacrol, and thymol, respectively, combined with α-terpineol led to a synergistic antimicrobial effect against *A. hydrophila*, *E. coli*, and *P. fragi* but reduced the antibacterial effect against *B. thermosphacta*. Thymol and α-terpineol are the main constituents of marjoram oil [11].

Antagonistic antimicrobial effects were observed particularly against *B. thermosphacta*, rarely against *A. hydrophila*. The results showed that even when combined, the concentration of at least one of the components is higher than sensorial acceptable [16], even if a synergistic effect exists. This is in line with observations from studies regarding the inhibition of other bacteria, such as *L. monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *S.* Typhimurium, *Staphylococcus aureus*, where concentrations necessary for inhibition were also higher than those acceptable in taste [8, 10, 18].

In addition, the necessary concentration of essential oils and their components to achieve an antimicrobial effect in foods is much higher than under in vitro conditions. The presence of salt, proteins, fat, carbohydrates, and a low a_w-value cause a decrease of the antimicrobial properties of essential oils [17]. Consequently, an extension of shelf-life of foods using herbs with a high content of thymol, carvacrol, linalool, 1,8-cineol, α-pinene or α-terpineol alone or in combination is a challenge [23]. The use of herbs might however be successful in the scope of the hurdle principle [4]. Hurdles have been successfully applied with essential oils in combination with wax and prolonged shelf life of citrus fruits [24].

It has been claimed that herbs possess better antimicrobial properties than herb extracts and essential oils better antimicrobial properties than their single components [27]. Therefore secondary components seem to influence the antimicrobial activity of herbs or essential oils [6, 27]. Besides the major components, which can reach an amount of 85 % percent of oil, essential oils contain about 60 further components. Furthermore, also the development of bacterial antibiotic resistance to essential oils should be considered. However, so far no indications exist that resistance is provoked by essential oils [5, 21].

Thymol, carvacrol, linalool, α-pinene, 1,8-cineol, and α-terpineol or a combination of them did not exhibit a relevant antimicrobial effect against the tested potential food spoilage organisms. Therefore, the concept of preservation only with natural components without further processing like salting cannot guarantee the safety of a product with a prolonged shelf-life. Inclusion of the herbs in the concept of the hurdle-theory (i.e., the application of different strategies to reduce spoilage microorganisms or pathogens e.g. through reduced a_w- or pH-value) could be more effective. In general, herbs or combinations of herbs with a large amount of thymol and carvacrol and a large amount of linalool, 1,8-cineol, α-pinene or α-terpineol at the same time may possess better antibacterial properties.

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