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Research Note

Synergistic Effect of Thymol and Carvacrol Combined with Chelators and Organic Acids against *Salmonella* Typhimurium

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ABSTRACT

To identify synergistic combinations of different food additives, the antimicrobial effects of thymol and carvacrol against *Salmonella* Typhimurium were assessed alone and in combination with various other preservatives including EDTA, acetic acid, lactic acid, and citric acid. Overall, growth of *Salmonella* Typhimurium was significantly inhibited in Mueller-Hinton broth containing thymol, carvacrol, EDTA, acetic acid, lactic acid, or citric acid at concentrations of 400 mg/liter, 400 μl/liter, 300 mg/liter, 0.2% (vol/vol), 0.2% (vol/vol), and 0.2% (wt/vol), respectively. The combination of different antimicrobials such as thymol or carvacrol with EDTA, thymol or carvacrol with acetic acid, and thymol or carvacrol with citric acid all resulted in significantly reduced populations of *Salmonella* Typhimurium. In samples treated with combinations, these antimicrobials had synergistic effects compared with samples treated with thymol, carvacrol, EDTA, acetic acid, or citric acid alone. However, the combined use of lactic acid with thymol or carvacrol did not produce a synergistic effect against *Salmonella* Typhimurium. Thus, some chelators or organic acids can be used as food preservatives in combination with thymol and carvacrol to reduce the concentrations needed to produce an adequate antimicrobial effect.

Food safety is an important public health issue *(30). Salmonella* Typhimurium is a major concern to public health and represents one of the most important *Salmonella* serovars implicated in gastroenteritis outbreaks worldwide *(2, 21).*

There is growing interest in the development of novel combinations of natural antimicrobials and other food preservation systems to improve the quality and safety of food. Thymol and carvacrol are two major components of oregano essential oil that exhibit strong antimicrobial activity *(15).* However, their use in foods as preservatives is often limited because of flavor and aroma considerations. Thymol and carvacrol would be ideal for use in food if they could produce the desired antibacterial effect low enough concentrations to minimize undesirable changes in flavor and/or aroma. Various synergistic manipulations have been suggested to achieve this goal, including mild heat treatment, refrigeration, acid, reduced redox potential, low water activity, and different additives. These approaches are being widely employed as hurdles, as described by Leistner *(16)* and can result in a stable and safe product without loss of sensory quality.

The mechanisms underlying the effects of thymol and carvacrol have received considerable attention. Thymol is structurally very similar to carvacrol, with the hydroxyl group at a different location on the phenolic ring. Both agents appear to increase cell membrane permeability *(15).* Thymol and carvacrol are able to destroy the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing permeability of the cytoplasmic membrane to ATP *(11, 12).* In studies with *Bacillus cereus,* carvacrol has formed membrane channels between the fatty acid chains of the phospholipids *(27).* This distortion of the physical structure causes expansion and destabilization of the membrane and increases membrane fluidity, which in turn increases passive permeability and allows ions to leave the cytoplasm *(25, 26).* The phenolic ring (destabilized electrons) is very important in the antibacterial activity of aromatic molecules *(26).* For these reasons, thymol and carvacrol have a strong antibacterial effect against *Salmonella* Typhimurium.

Several studies have focused on the synergistic effects of thymol or carvacrol in combination with other preservative systems, such as refrigeration *(29),* high hydrostatic pressure *(13),* pH extremes *(19), p*-cymene *(27),* and nisin *(20).* Chelators such as EDTA also have been reported to be synergistic with other antimicrobials *(6, 8, 10, 28).* Organic acids such as acetic, lactic, and citric acid have been used historically to control the growth of microorganisms and delay food spoilage. The antimicrobial activity of these organic acids can be enhanced when used in conjunction with other food preservatives or heat *(9, 17).*

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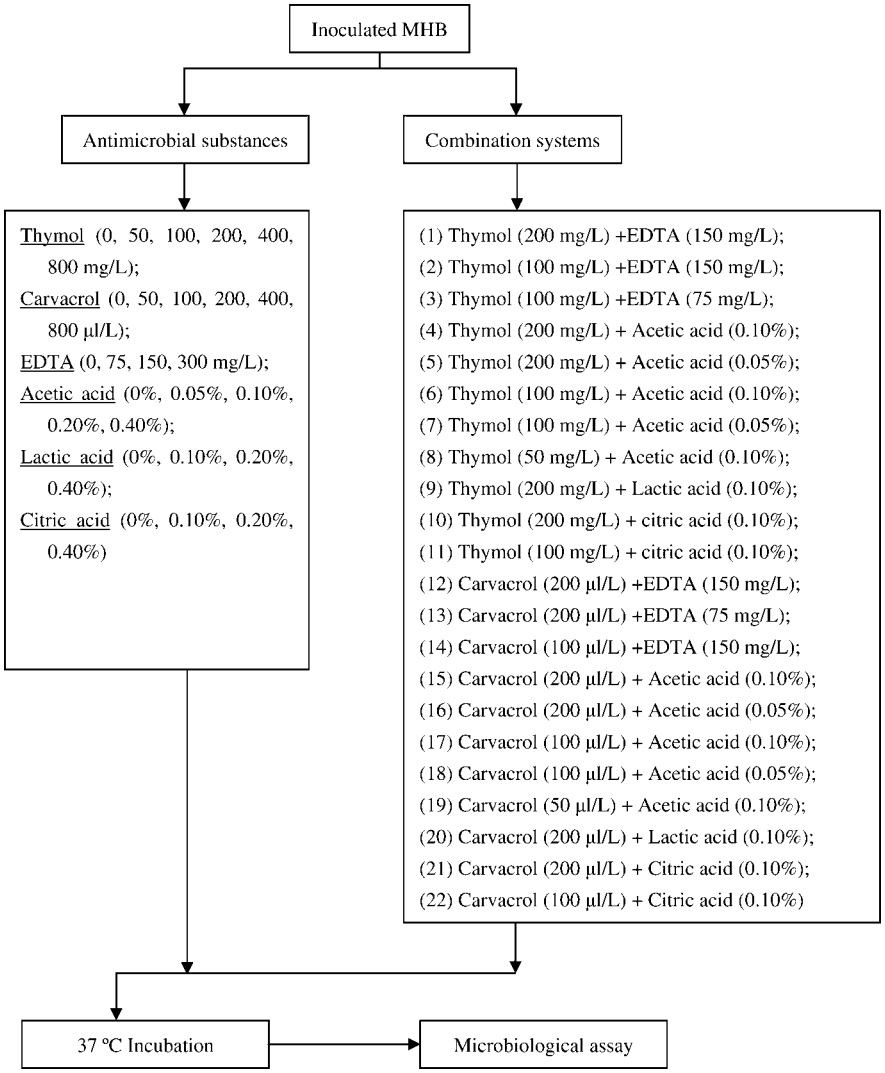

FIGURE 1. *Flow chart and treatment diagram of antimicrobial system application protocols.*

The objective of this study was to determine the effect of thymol and carvacrol alone and in combination with various chelators and organic acids on the growth of *Salmonella* Typhimurium and to identify the synergistic combinations.

MATERIALS AND METHODS

Chemicals. Thymol and carvacrol were purchased from Sigma Chemical Co. (St. Louis, Mo.). Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), nutrient broth (NB), nutrient agar (NA), peptone, EDTA, acetic acid, lactic acid, and citric acid were obtained from Beijing Chemical Co (Beijing, China). All of these chemicals were of analytical quality.

Microbial strains. *Salmonella* Typhimurium CGMCC 1.1174 was used to investigate antibacterial activity (China General Microbiological Culture Collection Center, Beijing, China). The suspension was transferred to NB and cultured overnight at 37°C. Dilutions were prepared in 0.10% sterile peptone water (wt/vol) and inoculated onto NA to determine cell numbers.

Determination of antibacterial activity. Six antibacterial agents were used in 22 combinations (Fig. 1). Thymol, carvacrol, EDTA, acetic acid, lactic acid, and citric acid were added to MHB from stock solutions to obtain the desired concentrations. The *Salmonella* population in all inoculated samples was 5×10^5 CFU/ml, as confirmed by direct plating. Samples were incubated at 37°C and analyzed for concentrations of *Salmonella* and for pH 24 h later; each experiment was conducted at least three times.

Analysis of samples. Serial dilutions were prepared in 0.10% sterile peptone water. *Salmonella* Typhimurium populations were enumerated by plating on MHA followed by 24 h of incubation at 37°C. The logarithmic difference in population (DP) of the test strain was calculated using the following formula *(5)*:

$$\log \mathrm{DP} = \log(N/N_0) = (\log N) - (\log N_0)$$

where N and N_0 represent the bacterial populations at time t and time 0, respectively.

The effect of the combination (EC) was calculated using the formula

$$\mathrm{EC} = \left|\log \mathrm{DP_I} - \mathrm{DP_{II}}\right|$$

where $\mathrm{DP_I}$ and $\mathrm{DP_{II}}$ represent the DP of the combined and the single use, respectively *(24)*.

Statistical analysis. An analysis of variance (ANOVA) was performed using a mixed model procedure with a randomized complete block design and repeated measures. Each experiment was repeated at least three times. Colony counts were converted into logarithmic values, and significance was expressed at $P \leq 0.05$ or 0.01. For each treatment, the data from the independent replicate trials were analyzed with SAS (SAS version 9.0, SAS Institute, Cary, N.C.).

TABLE 1. *pH values for samples incubated with* Salmonella *Typhimurium*

Treatment	pH of MHB containing test chemicals	
	Initial	Final[a]
Untreated	7.17	7.24
Thymol (mg/liter)		
50	7.21	7.00
100	7.19	6.95
200	7.18	7.03
400	7.19	7.16
800	7.15	7.15
Carvacrol (μl/liter)		
50	7.19	7.11
100	7.19	6.97
200	7.19	7.00
400	7.20	7.14
800	7.18	7.13
EDTA (mg/liter)		
75	7.17	7.11
150	7.12	6.93
300	7.08	6.93
Acetic acid (%)		
0.05	6.07	6.59
0.10	5.36	5.44
0.20	4.74	4.74
0.40	4.36	4.33
Lactic acid (%)		
0.10	6.05	6.20
0.20	4.77	4.90
0.40	4.06	4.10
Citric acid (%)		
0.10	5.63	5.78
0.20	4.84	4.85
0.40	4.20	4.20

[a] Samples were incubated at 37°C for 24 h.

Determination of the synergistic effect. The synergistic effect of the antimicrobial combinations on *Salmonella* Typhimurium growth was determined based on three principles: the decrease in populations (>90%), significant differences ($P \leq 0.05$), and the decrease in EC value (>2). Thus, when (i) the DP was <0.1 (log DP < −1), (ii) there was a significant difference (one-way ANOVA) between the combinations and the individual treatments, and (iii) there was a 2-log decrease in CFU for the combined compared with the most effective single agent after 24 h *(24),* the combinations of various agents were considered to have significant antibacterial activity. Only when the combination met each of the three requirements was a synergistic effect recorded.

RESULTS

Antibacterial activity of various agents. Addition of thymol and carvacrol did not change the pH of the growth medium (Tables 1 and 2). The individual antibacterial activities of thymol, carvacrol, EDTA, acetic acid, lactic acid, and citric acid against *Salmonella* Typhimurium were tested to determine the effective antibacterial concentrations of each component.

TABLE 2. *pH values for combination treatments incubated with* Salmonella *Typhimurium*

Combination treatment	pH of MHB containing test chemicals	
	Initial	Final[a]
Thymol (200 mg/liter) + EDTA (150 mg/liter)	7.09	7.04
Thymol (100 mg/liter) + EDTA (150 mg/liter)	7.11	7.07
Thymol (100 mg/liter) + EDTA (75 mg/liter)	7.18	7.00
Thymol (200 mg/liter) + acetic acid (0.10%)	5.20	5.29
Thymol (200 mg/liter) + acetic acid (0.05%)	6.20	6.19
Thymol (100 mg/liter) + acetic acid (0.10%)	5.40	5.48
Thymol (100 mg/liter) + acetic acid (0.05%)	6.07	5.98
Thymol (50 mg/liter) + acetic acid (0.10%)	5.34	5.41
Thymol (200 mg/liter) + lactic acid (0.10%)	5.93	5.98
Thymol (200 mg/liter) + citric acid (0.10%)	5.63	5.67
Thymol (100 mg/liter) + citric acid (0.10%)	5.62	5.64
Carvacrol (200 μl/liter) + EDTA (150 mg/liter)	7.12	7.07
Carvacrol (200 μl/liter) + EDTA (75 mg/liter)	7.18	7.12
Carvacrol (100 μl/liter) + EDTA (150 mg/liter)	7.11	7.03
Carvacrol (200 μl/liter) + acetic acid (0.10%)	5.24	5.32
Carvacrol (200 μl/liter) + acetic acid (0.05%)	6.16	6.13
Carvacrol (100 μl/liter) + acetic acid (0.10%)	5.43	5.47
Carvacrol (100 μl/liter) + acetic acid (0.05%)	6.03	5.85
Carvacrol (50 μl/liter) + acetic acid (0.10%)	5.36	5.38
Carvacrol (200 μl/liter) + lactic acid (0.10%)	5.97	5.89
Carvacrol (200 μl/liter) + citric acid (0.10%)	5.60	5.65
Carvacrol (100 μl/liter) + citric acid (0.10%)	5.67	5.62

[a] Samples were incubated at 37°C for 24 h.

The selected concentrations of these agents and the log DP for every concentration are shown in Figure 2. Samples treated with thymol (50 mg/liter), carvacrol (200 μl/liter), EDTA (150 mg/liter), acetic acid (0.20%), lactic acid (0.20%), and citric acid (0.20%) were significantly different ($P \leq 0.05$) compared with the untreated samples.

Although thymol had a significant inhibitory effect at 50, 100, and 200 mg/liter, some growth of *Salmonella* was still seen during 24 h of incubation; growth also was seen in samples treated with 200 μl/liter carvacrol. Therefore, the combined inhibitory effects on *Salmonella* Typhimurium were determined for thymol (50, 100, and 200 mg/liter) and carvacrol (50, 100, and 200 μl/liter) with EDTA (75 and 150 mg/liter), acetic acid (0.05 and 0.10%), lactic acid (0.10%), or citric acid (0.10%).

Inhibition of *Salmonella* Typhimurium using various combinations of preservatives. Twenty-two combinations of antimicrobial agents were assessed for inhibition of *Salmonella* Typhimurium in MHB during 24 h of incubation at 37°C (Table 3). The log DP values were lower than −1 for samples treated with combinations 1, 2, 4, 5, 6, 9, 10, 12, 15, 17, and 21, which indicated significant antibacterial activity. The one-way ANOVA of the 22 combinations revealed that all except three combinations (7, 18, and 20) had significant antibacterial activity. The EC values for combinations 1, 2, 4, 6, 10, 12, 15, 17, and 21 revealed a 2-log decrease in CFU for these combination treatments compared with the most effective single agent.

TABLE 3. *Effect of combined antimicrobial treatments on* Salmonella *Typhimurium growth in MHB stored at 37°C*

Treatment no.	Antimicrobials	Log DP	ANOVA *P* values[a]		EC	
			I[b]	II[c]	I	II
1	1. Thymol (200 mg/liter) 2. EDTA (150 mg/liter)	−3.19	0.0017**	0.0020**	3.79	4.00
2	1. Thymol (100 mg/liter) 2. EDTA (150 mg/liter)	−1.30	<0.0001**	0.0015**	2.45	2.11
3	1. Thymol (100 mg/liter) 2. EDTA (75 mg/liter)	0.7156	0.0460*	0.0126*	0.44	1.18
4	1. Thymol (200 mg/liter) 2. Acetic acid (0.10%)	−3.67	0.0008**	0.0081**	4.27	4.28
5	1. Thymol (200 mg/liter) 2. Acetic acid (0.05%)	−1.21	0.0074**	0.0113*	1.55	2.54
6	1. Thymol (100 mg/liter) 2. Acetic acid (0.10%)	−2.82	<0.0001**	0.0001**	3.98	3.43
7	1. Thymol (100 mg/liter) 2. Acetic acid (0.05%)	0.57	0.0595	0.0148*	0.58	1.01
8	1. Thymol (50 mg/liter) 2. Acetic acid (0.10%)	−0.12	<0.0001**	0.0085**	1.55	0.73
9	1. Thymol (200 mg/liter) 2. Lactic acid (0.10%)	−1.09	0.0025**	0.0045**	1.69	2.64
10	1. Thymol (200 mg/liter) 2. Citric acid (0.10%)	−2.81	<0.0001**	<0.0001**	3.44	4.31
11	1. Thymol (100 mg/liter) 2. Citric acid (0.10%)	−0.03	0.0351*	0.0074**	1.18	1.49
12	1. Carvacrol (200 μl/liter) 2. EDTA (150 mg/liter)	−3.93	<0.0001**	0.0001**	4.44	4.74
13	1. Carvacrol (200 μl/liter) 2. EDTA (75 mg/liter)	−0.25	0.0062**	0.0011**	0.76	2.14
14	1. Carvacrol (100 μ/liter) 2. EDTA (150 mg/liter)	−0.89	0.0082**	0.0114*	2.06	1.71
15	1. Carvacrol (200 μl/liter) 2. Acetic acid (0.10%)	−3.83	0.0013**	0.0080**	4.34	4.43
16	1. Carvacrol (200 μl/liter) 2. Acetic acid (0.05%)	−0.57	0.0172*	0.0069**	1.26	2.33
17	1. Carvacrol (100 μl/liter) 2. Acetic acid (0.10%)	−3.27	0.0003**	0.0009**	4.34	3.70
18	1. Carvacrol (100 μl/liter) 2. Acetic acid (0.05%)	0.15	0.0522	0.0043**	1.29	1.44
19	1. Carvacrol (50 μl/liter) 2. Acetic acid (0.10%)	−0.15	<0.0001**	0.0005**	1.45	0.75
20	1. Carvacrol (200 μl/liter) 2. Lactic acid (0.10%)	0.37	0.6791	0.0982	0.15	1.18
21	1. Carvacrol (200 μl/liter) 2. Citric acid (0.10%)	−2.84	0.0001**	<0.0001**	3.35	4.30
22	1. Carvacrol (100 μl/liter) 2. Citric acid (0.10%)	−0.27	0.0264*	0.0003**	1.69	1.72

[a] Some differences were significant (*$P \leq 0.05$) and some were highly significant (**$P \leq 0.01$).
[b] Antimicrobial 1 versus antimicrobial 1 + antimicrobial 2.
[c] Antimicrobial 2 versus antimicrobial 1 + antimicrobial 2.

The following nine combination treatments had a synergistic effect that could help reduce the effective doses of thymol and carvacrol: 1, thymol (200 mg/liter) plus EDTA (150 mg/liter); 2, thymol (100 mg/liter) plus EDTA (150 mg/liter); 4, thymol (200 mg/liter) plus acetic acid (0.10%); 6, thymol (100 mg/liter) plus acetic acid (0.10%); 10, thymol (200 mg/liter) plus citric acid (0.10%); 12, carvacrol (200 μl/liter) plus EDTA (150 mg/liter); 15, carvacrol (200 μl/liter) plus acetic acid (0.10%); 17, carvacrol (100 μl/liter) plus acetic acid (0.10%); and 21, carvacrol (200 μl/liter) plus citric acid (0.10%).

The results indicate that there was a similar synergistic effect when thymol and carvacrol were combined with acetic acid or citric acid, and the synergistic effect of thymol combined with EDTA was stronger than that of carvacrol with EDTA. The lowest concentration of thymol combined with EDTA that exerted a synergistic effect was 100 mg/liter.

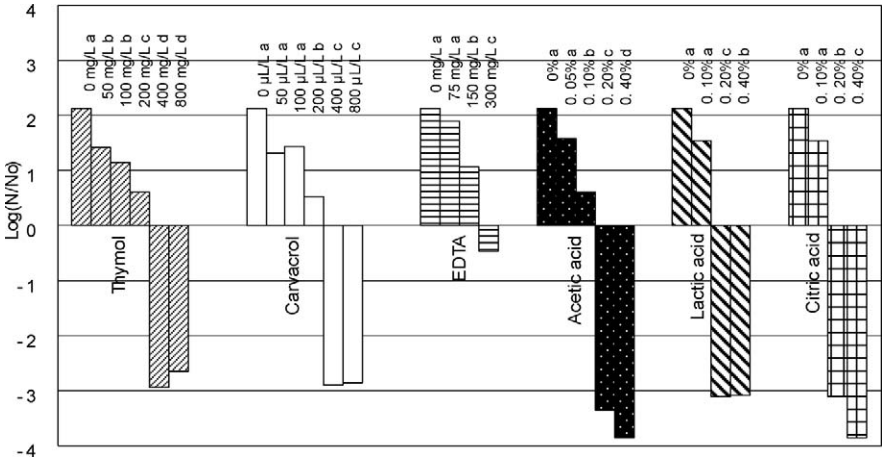

FIGURE 2. *Effect of thymol, carvacrol, EDTA, acetic acid, lactic acid, and citric acid on populations of* Salmonella *Typhimurium in medium incubated at 37°C for 24 h. Within the same group, means with different letters are significantly different (*$P \leq 0.05$*).*

DISCUSSION

Thymol and carvacrol, the two major components of oregano essential oil *(15),* are legally registered flavorings or foodstuffs in the European Union and the United States. According to the results in Figure 2, these two components exhibited a strong antibacterial effect, but their application in foods has been limited by their strong flavor. Thus, the aim of this study was to reduce the concentrations of thymol and carvacrol needed for effective inhibition of *Salmonella* Typhimurium by combining these antimicrobials with other food preservation agents, such as chelators or organic acids.

The combination of thymol or carvacrol with EDTA had a synergistic effect on *Salmonella* Typhimurium (Table 3). The synergism of preservatives with EDTA has also been reported by other researchers *(10, 28).* Outer membrane permeability can be altered by treatment with EDTA, resulting in increased sensitivity to nisin *(10, 28).* In addition, several studies have been focused on the synergistic effect of thymol or carvacrol in combination with other preservation agents. Thymol and carvacrol exhibit a synergistic effect with high hydrostatic pressure against *L. monocytogenes (13).* Synergism between carvacrol and its biological precursor (*p*-cymene) has been demonstrated against vegetative *B. cereus* cells *(27).* The combined use of nisin (0.15 μg/ml) and carvacrol or thymol (0.30 mmol/liter or 45 μg/ml) resulted in a larger decline in viable *B. cereus* counts than that observed when the antimicrobials were used individually *(20).*

Inhibitory activity of the combinations of thymol (100 mg/liter) plus EDTA (150 mg/liter) and of carvacrol (100 μl/liter) plus EDTA (150 mg/liter) reached those of 400 mg/liter thymol alone and 400 μl/liter carvacrol alone. The antibacterial mechanisms of thymol and carvacrol have been previously discussed. *Salmonella* Typhimurium is resistant to antibiotics, lysozyme, and detergents because of the presence of lipopolysaccharides in the outer membrane. Lipopolysaccharides are strongly anionic and are stabilized by Ca^{2+} and Mg^{2+}. However, chelating agents such as EDTA can disrupt the outer membrane, resulting in increased sensitivity to other antimicrobial agents such as those compounds found in essential oils *(18).* We hypothesized that the observed synergy was the result of enhanced sensitivity of *Salmonella* Typhimurium to thymol or carvacrol in the presence of EDTA.

The four combinations of thymol or carvacrol with acetic acid also had strong synergistic activity. In the presence of acetic acid, the antibacterial activity from the combinations of thymol (100 mg/liter) plus acetic acid (0.10%) and of carvacrol (100 μl/liter) plus acetic acid (0.10%) reached those of 400 mg/liter thymol and 400 μl/liter carvacrol alone. These combination treatments could achieve the desired antibacterial effect at concentrations low enough to minimize undesirable changes in flavor.

The synergistic effect of thymol or carvacrol with acetic acid can be explained by the dissociation of most antibacterial agents into ionic forms in solution. An increase in H^+ forces the equilibrium towards the molecular form *(1).* The addition of certain organic acids increases the concentration of H^+, moving thymol or carvacrol in the molecular state. The molecular form of thymol or carvacrol is freely permeable across the plasma membrane and thus is able to enter the cell. Therefore, addition of an organic acid such as acetic acid allows thymol or carvacrol to exist in a form that can enter the bacterial cell and exert its antimicrobial activity. Inhibition of bacterial growth by weak acids is due to several factors, including membrane disruption *(3, 7, 23),* inhibition of essential metabolic reactions *(14),* and stress on intracellular pH homeostasis *(3, 4, 22).*

The synergistic effects of thymol and carvacrol with citric acid may be related to the fact that citric acid is a weak organic acid that works by a mechanism similar to that of acetic acid. Citric acid also is able to chelate Ca^{2+} and Mg^{2+} ions as does EDTA.

According to the antibacterial results from these single-agent and combination treatments, the effective antibacterial concentrations of thymol and carvacrol when combined with acetic acid could be reduced from 400 mg/liter and 400 μl/liter to 100 mg/liter and 100 μl/liter, respectively. Consequently, thymol and carvacrol combined with acetic acid could serve as a food preservatives. However, further work is needed to confirm and extend the present findings by evaluating different bacterial species under various experimental conditions.

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