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EVALUATION OF EFFECT OF THYMOL COMBINED WITH LACTIC ACID OR SODIUM LACTATE ON PSYCHROPHILIC BACTERIA AND *SALMONELLA* SPP. ON CHICKEN DRUMSTICK

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Abstract

This study was carried out to evaluate the combined antimicrobial effect of thymol with lactic acid or sodium lactate on *Salmonella* and psychrophilic bacteria on chicken drumstick. Drumstick samples were artificially contaminated with *Salmonella* spp., then sprayed with sterile 0.85% NaCl solution (control), and thymol (0.25% w/v) with lactic acid (2% and 4% v/v) or sodium lactate (2% and 4% v/v), alone or in combination, for 30 s. The samples were stored at 4°C and analyzed on days 0, 2 and 4 for *Salmonella* and psychrophilic bacteria. Thymol alone did not show antibacterial effect on *Salmonella* and psychrophilic bacteria on chicken drumstick when compared with the control group during storage period. Spray with 4% lactic acid + thymol reduced *Salmonella* and psychrophilic bacteria by 1.4 and 1.8 $\log_{10}$ CFU/ml on day 0, respectively. A significant decrease in the number of *Salmonella* and psychrophilic bacteria was observed in the samples sprayed with 4% lactic acid and 4% lactic acid + thymol on days 2 and 4 when compared to the control ($P<0.05$). The combinations of thymol with lactic acid or sodium lactate did not show synergistic or additive effect on *Salmonella* and psychrophilic bacteria present on chicken drumstick with skin.

Key words: chicken drumstick, thymol, lactic acid, *Salmonella*, psychrophilic bacteria

Salmonella Typhimurium and *Salmonella* Enteritidis are two of the most common serovars within the *Salmonella* gastroenteritis outbreaks and continue to be important pathogens for the poultry industry. These pathogens have a large economic impact because of illness, medical cost, loss of productivity etc. (Freitas et al., 2010; Lee et al., 2014; Mani-López et al., 2012; Oladunjoye et al., 2013).

Sodium lactate and lactic acid are affirmed as GRAS (generally recognized as safe) by the FDA and can be added directly to the various foods to control microbial growth and to extend the shelf life of food products (Bolton et al., 2014; Burfoot and Mulvey, 2011; Smaoui et al., 2012). Herbs and spices, and their constituents have been used as flavoring agents in foods since the earliest history, and it is well estab-

lished that many have antibacterial activity. There are successful review papers on the subject in the latest literature (Bajpai et al., 2012; Jayasena and Jo, 2013; Prakash et al., 2015; Seow et al., 2015). Thymol is one of the phenolic compounds obtained from plants like *Origanum vulgare* and *Thymus vulgaris,* and is classified as GRAS (Tajkarimi et al., 2010; Hyldgaard et al., 2012). The mode of antibacterial action of thymol is not fully understood, but it is believed that thymol alters the physical and chemical properties of cytoplasmic membrane of bacteria, and this may change the permeability of the cell membrane and cause the leakage of ions and other cell contents (Burt, 2004; Xu et al., 2008; Hyldgaard et al., 2012; Vergis et al., 2015; Calo et al., 2015). There are many published studies, which were conducted both in microbiological media and in food environments, related to the antimicrobial efficacy of thymol on wide range of organisms (Lambert et al., 2001; Singh et al., 2003; Bagamboula et al., 2004; Xu et al., 2008; Chavan and Tupe, 2014; Makhal et al., 2014) and *Salmonella* Typhimurium (Nazer et al., 2005; Zhou et al., 2007 a; 2007 b).

It is well known that the concentrations of essential oils used to obtain sufficient antimicrobial efficiency in food products should be markedly higher than those used in the laboratory media. On the other hand, increasing the concentration of essential oils has a negative impact on the sensory quality of the food. Therefore, combinations of essential oils with other preservation methods have been used to minimize the application concentrations required. There has been conducted much successful research related to the combinations of thymol with the other antimicrobial agents and methods (Nazer et al., 2005; Mahmoud et al., 2006; Zhou et al., 2007 a; Over et al., 2009; Corbo et al., 2009; Oladunjoye et al., 2013; Ilhak and Guran, 2014; Kim and Rhee, 2016).

As mentioned above, it has been suggested that thymol is able to disintegrate the outer membrane of bacteria and that it increases the permeability of the cytoplasmic membrane (Lambert et al., 2001; Xu et al., 2008). If so, taking into account the antibacterial effect of thymol, it may facilitate the diffusion of lactic acid or sodium lactate into the cellular cytoplasm. Although there are many studies on the efficacy of essential oils combined with the other antimicrobial agents on foodborne pathogens and spoilage bacteria in food products, there is no published research as regards the efficacy of combination of thymol with lactic acid and sodium lactate on *Salmonella* and spoilage flora in complex food, such as chicken drumstick with skin. The objective of this study was to investigate whether the combinations of thymol and lactic acid or sodium lactate have an antimicrobial effect against *Salmonella* and psychrophilic bacteria on chicken drumstick with skin.

Material and methods

Chemicals

Thymol and sodium lactate (50%) were purchased from Sigma Chemical Co. (St. Louis, MO). L (+) lactic acid solution (88–92%) was obtained from Sigma-Aldrich (Seelze, Germany). Peptone water, tryptic soy broth (TSB) and plate count agar were

purchased from Merck (E. Merck, Darmstadt, Germany). Xylose lysine deoxycholate agar (XLD) was obtained from Difco (Sparks, MD).

Preparation of bacterial inocula

Mixture of two *Salmonella* Typhimurium (NCTC 12416 and NCTC 74) and one *Salmonella* Enteritidis (RSKK 92 (Refik Saydam National Public Health Agency-Turkey)) strains were used in this study. Each strain was grown in 10 ml of tryptic soy broth at 37°C for 18 h. Then, the cultures were centrifuged at 4,192 × g for 10 min at 5°C, and the pellets were washed with 0.1% sterile peptone water before re-centrifuging to remove organic residues. The supernatant was decanted and the pellets of each strain were re-suspended in an aliquot of 0.1% sterile peptone water. These suspensions were then combined in a single tube and completed to 10 ml with 0.1% sterile peptone water. This combined *Salmonella* inoculum (approximately 8.3 $\log_{10}$ CFU/ml) was used immediately.

Inoculation of drumsticks, and treatments

For each of three replicate trials, 64 chicken drumstick samples with skin (weighing 110–140 g each) were purchased from a local supermarket on the day of the experiments (double samples were used for each treatment on each sampling day). The samples were transported to our laboratory within 20 minutes and stored at 4°C. Before the inoculation procedure, two randomly selected drumstick samples were taken and used for the analysis of indigenous *Salmonella* spp. For the inoculation, 0.5 ml combined *Salmonella* suspension was spread on the drumstick sample by a sterile disposable hockey stick spreader. After inoculation, the drumsticks were kept for 10 min at room temperature to allow for bacterial attachment, and two samples were taken and used for the detection of inoculation level of *Salmonella* spp. Then, the drumstick samples were mist sprayed (rotating all surface) with approximately 30 ml (for each one) of solution using a spray bottle from a distance of 15 cm for 30 s. Decontamination treatments were as follows; 1 – Control (sprayed with sterile 0.85% NaCl), 2 – 2% Lactic acid, 3 – 2% Lactic acid + 0.25% Thymol, 4 – 4% Lactic acid, 5 – 4% Lactic acid + 0.25 Thymol, 6 – 2% Sodium lactate, 7 – 2% Sodium lactate + 0.25 Thymol, 8 – 4% Sodium lactate, 9 – 4% Sodium lactate + 0.25% Thymol, and 10 – 0.25% Thymol, with each of the 10 groups consisting of 6 drumsticks. Thymol was dissolved in 2 ml of 1% ethanol before added to the treatment solutions. Spray bottles including thymol (alone or in combination) were vigorously shaken to disperse thymol in the solution before they were used.

After these treatments, the chicken drumsticks were drained for 1 min and stored individually in sterile stomacher bags at 4°C for 4 days. During the study, a total of 192 drumstick samples were used.

Microbiological sampling

Microbiological sampling was performed on days 0 (after the spraying treatment), 2 and 4. On each sampling day, two drumstick samples of each group were used. A 100 ml of 0.1% sterile peptone water was added to sterile stomacher bag containing the drumstick sample, and the drumstick was rinsed by manually massaging

for 1 min. After that procedure, a 1 ml solution was taken from the rinsing solution and serially diluted in 0.1% sterile peptone water and surface plated on xylose lysine deoxycholate agar for enumeration of *Salmonella*. Characteristic colonies were counted after the plates were incubated at 35°C for 24–36 h. Plate count agar was used for the enumeration of psychrophilic bacteria, and colonies were counted after the plates were incubated at 4°C for 10 days.

Determination of pH of drumstick samples

On each sampling day, after microbiological analysis of the sample was completed, the pH of the rinse solution of the sample was measured with pH meter (Selecta pH 2001, J.P. Selecta, s.a, Barcelona, Spain).

Statistical analyses

Three independent replicates of the study were conducted. Microbiological data were converted to $\log_{10}$ CFU/ml and subjected to analysis of variance (ANOVA) appropriate to replicates × treatment groups × sampling days to determine fixed effects and interactions between variables. Least squares means were separated using Fisher's least significant difference test (LSD) according to general linear model (GLM) procedure of Statistical Analyses System (SAS Institute, Carry, NC). Statistical significance level was expressed as $P \leq 0.05$.

Results

Mean counts of *Salmonella* and psychrophilic bacteria of the chicken drumstick samples sprayed with various chemicals were shown in Tables 1 and 2, respectively. No indigenous *Salmonella* spp. was detected on drumstick samples used in the study.

The mean inoculation level of *Salmonella* on the drumstick samples (unsprayed sample) was 5.3 $\log_{10}$ CFU/ml (Table 1). There was a significant reduction in the number of *Salmonella* in the control group (sprayed with sterile 0.85% NaCl) compared to unsprayed sample ($P<0.05$). During four days of storage, the number of *Salmonella* spp. in the control samples continued to slowly decrease and dropped to 3.9 $\log_{10}$ CFU/ml, but no significant difference was observed between storage days ($P>0.05$). Immediately after the treatments, the most pronounced reduction in the number of the pathogen was provided by combination of 4% lactic acid + 0.25% thymol by 1.4 $\log_{10}$ CFU/ml. However, there were no significant differences between the treatments and control sample ($P>0.05$). On days 2 and 4 of storage, the combination of 4% lactic acid + 0.25% thymol had the best antimicrobial efficacy on the *Salmonella* spp. compared to control by reduction of 0.9 and 1.0 $\log_{10}$ CFU/ml, respectively ($P<0.05$).

Spraying 4% sodium lactate resulted in 1.1 and 1.3 $\log_{10}$ CFU/ml reductions in the numbers of *Salmonella* (Table 1) and psychrophilic bacteria (Table 2), respectively. The antimicrobial effect of sodium lactate insignificantly increased with increasing concentration (from 2% to 4% sodium lactate). Compared to the control

group, spraying with 4% sodium lactate + 0.25% thymol or 4% sodium lactate alone had significant antibacterial effect neither on *Salmonella* nor psychrophilic bacteria on chicken drumstick with skin (P>0.05).

Table 1. The mean numbers of *Salmonella* spp. of the drumstick samples sprayed with various chemicals and stored at 4°C (log_{10} CFU/ml ± Sd)

Prior to spray (inoculation level) 5.3 A±0.2			
Treatment groups	Days		
	0	2	4
Control (0.85% NaCl)	4.3 Bx±0.2	4.0 Bx±0.2	3.9 Bx±0.3
2% Lactic acid	4.2 Bx±0.3	3.7 BCx±0.2	3.6 BCx±0.2
2% Lactic acid + Thymol	4.2 Bx±0.2	3.7 BCx±0.1	3.6 BCx±0.2
4% Lactic acid	4.0 Bx±0.3	3.2 Cy±0.2	2.9 Cy±0.3
4% Lactic acid + Thymol	3.9 Bx±0.2	3.1 Cy±0.2	2.9 Cy±0.2
2% Sodium lactate	4.4 Bx±0.2	3.9 Bx±0.2	3.8 Bx±0.1
2% Sodium lactate + Thymol	4.2 Bx±0.2	3.7 BCxy±0.2	3.5 BCy±0.1
4% Sodium lactate	4.2 Bx±0.3	3.5 BCy±0.3	3.4 BCy±0.3
4% Sodium lactate + Thymol	4.1 Bx±0.2	3.6 BCxy±0.2	3.2 BCy±0.2
Thymol	4.4 Bx±0.3	4.0 Bx±0.2	3.8 B±0.3

A, B, C – the numbers in the same column with the different letters are significantly different (P≤0.05).
x, y – the numbers in the same row with the different letters are significantly different (P≤0.05).

Table 2. The mean numbers of psychrophilic bacteria of the drumstick samples sprayed with various chemicals and stored at 4°C (log_{10} CFU/ml ± Sd)

Prior to spray (unsprayed sample) 4.5 A±0.2			
Treatment groups	Days		
	0	2	4
Control (0.85% NaCl)	3.2 Bx±0.16	5.2 BCy±0.16	7.6 Bz±0.3
2% Lactic acid	3.0 Bx±0.17	4.9 BCy±0.19	7.3 BCz±0.2
2% Lactic acid + Thymol	3.0 Bx±0.19	5.0 BCy±0.17	7.3 BCz±0.2
4% Lactic acid	2.7 Bx±0.21	4.7 Cy±0.20	6.8 Cz±0.3
4% Lactic acid + Thymol	2.7 Bx±0.20	4.7 Cy±0.14	6.8 Cz±0.2
2% Sodium lactate	3.3 Bx±0.19	5.2 BCy±0.18	7.4 BCz±0.1
2% Sodium lactate + Thymol	3.3 Bx±0.16	5.1 BCy±0.23	7.5 BCz±0.1
4% Sodium lactate	3.2 Bx±0.20	5.0 BCy±0.20	7.4 BCz±0.3
4% Sodium lactate + Thymol	3.2 Bx±0.16	4.9 BCy±0.17	7.4 BCz±0.2
Thymol	3.3 Bx±0.19	5.5 By±0.20	7.9 Bz±0.3

A, B, C – the numbers in the same column with the different letters are significantly different (P≤0.05).
x, y z – the numbers in the same row with the different letters are significantly different (P≤0.05).

The average number of psychrophilic bacteria on the drumstick sample (unsprayed sample) was 4.5 log_{10} CFU/ml (Table 2). A significant reduction of 1.3 log_{10} CFU/ml was observed after spraying with the sterile 0.85% NaCl (control sample)

(P<0.05). During four days of storage, the number of psychrophilic bacteria in the control sample continued to increase and reached 7.6 $\log_{10}$ CFU/ml. When compared with the control group, the combination of 4% lactic acid + 0.25% thymol and 4% lactic acid treatments were more effective in reducing the number of psychrophilic bacteria on the drumstick on days 0, 2 and 4 by 0.5, 0.5 and 0.8 $\log_{10}$ CFU/ml, respectively. There were differences between the samples sprayed with 4% lactic acid (with or without thymol combination) and control group in psychrophilic bacteria numbers on day 4 (P<0.05). However, there was no difference between 4% lactic acid and 4% lactic acid + thymol.

Table 3. The mean pH levels of the drumstick samples sprayed with various chemicals and stored at 4°C (Mean pH±SD)

Treatment groups	Days		
	0	2	4
Control (0.85% NaCl)	6.81 Ax±0.07	6.92 ABxy±0.06	7.12 Ay±0.06
2% Lactic acid	6.13 Bx±0.07	6.64 By±0.09	6.82 BCDy±0.10
2% Lactic acid + Thymol	6.16 Bx±0.08	6.71 ABy±0.10	6.92 ABCy±0.06
4% Lactic acid	4.52 Cx±0.06	6.45 BCy±0.09	6.71 CDy±0.09
4% Lactic acid + Thymol	4.47 Cx±0.07	6.33 Cy±0.06	6.59 Dy±0.08
2% Sodium lactate	6.78 Ax±0.06	6.99 Ax±0.11	7.08 ABx±0.05
2% Sodium lactate + Thymol	6.77 Ax±0.07	6.99 Ax±0.10	6.99 ABCx±0.08
4% Sodium lactate	6.64 Ax±0.08	6.92 ABxy±0.06	7.03 ABy±0.07
4% Sodium lactate + Thymol	6.75 Ax±0.06	6.86 ABx±0.06	7.01 ABCx±0.08
Thymol	6.78 Ax±0.09	6.89 ABx±0.09	7.12 Ax±0.06

A, B, C, D – the numbers in the same column with the different letters are significantly different (P≤0.05).
x, y – the numbers in the same row with the different letters are significantly different (P≤0.05).

The changes in the pH level of the drumstick samples treated with various chemicals were presented in Table 3. The pH level of the control samples (sprayed with the sterile 0.85% NaCl) was 6.81 on day 0. In control group and all treatment groups, the pH level continuously increased during storage. The pH values of the drumstick samples sprayed with sodium lactate (with or without thymol combination) were between 6.64 and 6.78 depending on the samples after the application. The pH values of the drumstick samples treated with lactic acid (with or without thymol combination) were between 4.47 and 6.16 depending on the lactic acid concentrations. However, on day 2 of storage, the pH levels of the drumstick samples treated with lactic acid increased, and they were found to be 6.33 and 6.71 depending on the lactic acid concentrations.

Discussion

In the present study, 0.85% sterile NaCl (control group) spraying decreased both psychrophilic bacteria and *Salmonella* spp. numbers by 1.3 and 1.0 $\log_{10}$ CFU/ml

on the drumstick sample when compared with unsprayed sample, respectively. Apparently, these reductions are due to removal of loosely attached bacteria from the skin. During storage, there was a little decrease (0.4 $\log_{10}$ CFU/ml) in the number of *Salmonella* spp. in the control samples, but there was a significant increase in the number of psychrophilic bacteria from initially 3.2 $\log_{10}$ to 7.6 $\log_{10}$ CFU/ml after 4 days of storage ($P<0.05$). Probably, increasing number of psychrophilic bacteria may have caused the decrease in the *Salmonella* number, due to competition. On the other hand, Zhou et al. (2007a) reported that 50 mg/L thymol was effective on *Salmonella* Typhimurium in Muller Hinton agar incubated at 37°C for 24 hours. It has been well known that the level of essential oils required for antibacterial efficiency in a food matrix can be considerably higher compared to microbiological media. Because of that, thymol was used at a very high concentration (2500 mg/L) in the present study. However, spraying the drumstick samples with 0.25% thymol alone did not show antibacterial effect on *Salmonella* and psychrophilic bacteria when compared to the control group. It can be said that use of thymol less than 0.25% concentrations to decontaminate drumstick samples would be ineffective as regards antimicrobial effects. On the other hand, immediately after treatments, the scent of thyme was detected in the groups treated with thymol. On day 2, a slight smell of thyme was detected in the samples. On day 4, no smell of thyme was perceived from samples.

Spraying 4% lactic acid resulted in 1.3 and 1.8 $\log_{10}$ CFU/ml reductions in the numbers of *Salmonella* (Table 1) and psychrophilic bacteria (Table 2), respectively. As it was expected, the antimicrobial effect of lactic acid increased as its concentration increased (from 2% to 4% lactic acid). However, this increase in antimicrobial effect was of insignificant magnitude (0.2 and 0.3 log CFU/ml additional reduction for *Salmonella* and psychrophilic bacteria on day 0, respectively). When considering these reductions, we concluded that increasing the concentration of lactic acid from 2% to 4% does not provide a significant additional antimicrobial efficacy on chicken drumstick with skin ($P>0.05$).

It has been reported that the susceptibility of bacteria to the antimicrobial effect of essential oils, lactic acid or sodium lactate increases with a decrease in pH level of food (Tiwari et al., 2009; Mani-López et al., 2012). In the present study, immediately after the treatments, the groups treated with 2% or 4% lactic acid (with or without thymol combination) had low pH level compared to control and the other groups treated with sodium lactate ($P<0.05$). However, their pH levels increased and approached the pH levels of other treatment groups on day 2. Apparently, the buffering capacity of the chicken skin and fat content of the product may have caused an increase in the pH level of the samples surface. It has been noted that thymol has more inhibitory effect on Gram-negative bacteria at pH 5.5 than at 6.5 (Vergis et al., 2015). In our study, the pH level of the samples (except the groups treated with 4% lactic acid with or without thymol on day 0) was close or higher than pH 6.5. It is possible that the bactericidal or inhibitory effect of thymol, sodium lactate or lactic acid on psychrophilic bacteria and *Salmonella* spp. may be restricted due to the high pH level of the drumstick.

On the other hand, it has been known that to remove pathogens from the feather follicles and folded areas on poultry skin is difficult (Lee et al., 2014; Nagel et al.,

2013). Therefore, the little reductions in the numbers of bacteria observed in this research may be attributed to the physical structure of drumstick skin surface which may protect the bacteria from the effect of antimicrobials. And also, chicken meat with skin is composed of proteins, carbohydrates, fat and so on. The interaction between thymol, lactic acid or sodium lactate and all these components may affect the action of the antimicrobials. Probably, bacteria may have been protected by the high pH, high level of fat and protein content of the drumstick skin. This situation may explain the lack of synergistic or additional effect of antimicrobials used in this research.

Considering the results obtained from the present study, it can be said that chicken drumstick with skin is a complicated food to evaluate the possible effect of combining thymol with lactic acid or sodium lactate. In the present study we used spray method for 30 s and applied approximately 30 ml solution per drumstick. It may be speculated that longer spraying time, use of large amounts of solution, and a higher concentration of thymol could be more effective on *Salmonella* spp., and psychrophilic bacteria on chicken drumstick. However, the use of higher concentrations of thymol does not seem possible because of its negative impact on flavor of food. Generally, dipping chicken/chicken parts into the chemical solutions (dipping method) is considered to be more effective against the bacteria than the spraying method. However, a spraying system in the processing line can be more ideal for the poultry industry since it does not require large space. Hence, the mode of application of antimicrobials to chicken meat with skin should be optimized.

The results of this research indicated that combining thymol with lactic acid or sodium lactate could not show synergistic or additional antimicrobial effect on *Salmonella* spp., and indigenous psychrophilic bacteria on the surface of chicken drumstick with skin. Although spraying with 0.25% thymol + 4% lactic acid gave the highest reduction in the number of *Salmonella* and psychrophilic bacteria, its effect was not satisfactory. Further studies are needed to find more suitable combination of essential oils with food-grade antimicrobials and mode of application against the foodborne pathogens and food spoilage bacteria on chicken meat.

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