THE EFFECTS OF THYMOL AND LACTIC ACID AGAINST *CAMPYLOBACTER JEJUNI* AND THE AMOUNT OF BIOGENIC AMINES IN BROILER BREAST MEAT

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Abstract. The aim of this study was to evaluate the effects of thymol and lactic acid on *Campylobacter jejuni* in association with the amount of biogenic amines of broiler breast meat during 4 days of storage at +4°C temperature. Treatment with 1% thymol had a significant impact on *Campylobacter jejuni* counts in broiler meat. The counts of *Campylobacter jejuni* were reduced by 0.41 log CFU ($P \geq 0.05$) and 0.66 log CFU ($P \leq 0.05$) on broiler breast meat treated with 0.5% and 1% thymol solution, respectively, compared with an untreated sample contaminated with *Campylobacter jejuni*. In addition, treatment of broiler meat with 0.5% thymol solution reduced the levels of cadaverine, tyramine and spermine, compared with the control sample ($P \leq 0.05$) during 96 hours of storage at +4°C temperature. Besides, the amount of cadaverine and spermine was similarly reduced by 1% thymol solution and 3% lactic acid solution, respectively ($P \leq 0.05$). There was no D-lactic acid isomer formed in the samples treated with 0.5% and 1% thymol solution and 3% lactic acid solution ($P \leq 0.05$). Therefore, 1% thymol solution could be used not only as the decontamination measure against *Campylobacter jejuni* but may also reduce the amount of biogenic amines and inhibit the production of D-lactic acid isomer on broiler breast meat.

Keywords: campylobacter, thymol, lactic acid, biogenic amines, pH

Introduction

Campylobacteriosis is one of the most frequent diarrheal foodborne illnesses worldwide with *Campylobacter jejuni* being the most prevalent cause of human campylobacteriosis (Nichols et al., 2012). Poultry meat is usually referred as the main source of human infection (Eideh and Al-Qadiri, 2011; Nichols et al., 2012). Reducing campylobacter counts on poultry, especially broiler meat, could lower the incidence rate of human campylobacteriosis (Ganan et al., 2012). Therefore, various decontamination techniques like ozonation, freezing, steam pasteurisation and the use of chemical agents are considered as possible control measures for the reduction of human infection (Whyte et al., 2001; Boysen and Rosenquist, 2009).

Chemical treatment of fresh meat is not authorised for use in the European Union with an exception for lactic acid, which is approved for decontamination of bovine carcasses (Commission Regulation (EU) N. 101/2013). Many chemical compounds are being discussed as possible decontaminants of poultry meat: 2% lactic acid solution, acidified sodium chlorite, trisodium phosphate and others. They could be applied by spraying with pressure, immersing the carcass meat during cooling or injecting the solution under the skin (EFSA, 2011a). While lactic acid has already been approved to use on bovine carcasses, thymol is rarely considered as a possible control measure for campylobacters on poultry. However, thymol (5-methyl-2-iso-propylphenol) is a natural antimicrobial agent from thyme and thyme oil. It has demonstrated a significant antimicrobial activity against both gram-positive and gram-negative bacteria (Yu et al., 2010). Nonetheless, thyme extract (thymol) is one of the essential oils used to improve taste properties of products and to extend their validity (Tsigarida et al., 2000; Burt, 2004).

Currently, published results on the effect of bioactive substances are controversial: for example, Lecompte et al. (2009) determined that 5% of lactic acid did not have a significant impact on campylobacter counts on poultry meat, while Riedel et al. (2009) discovered that 2.5% lactic acid solution was effective against campylobacters. Nonetheless, the activity of microorganisms in acidic environment creates conditions favourable for the formation of biogenic amines (Karovičová and Kohajdova, 2005; Suzzi and Gardini, 2003). Identification of biogenic amines in fresh and processed meat products is important because it could indicate potentially harmful changes for humans in treated meat and be considered as a chemical indicator of production conditions or undesirable microbiological contamination in food products (Bover-Cid et al., 2006; Ruiz-Capillas et al., 2004). Therefore, the evaluation of the amount of biogenic amines in meat is relevant when organic acids are used to reduce the counts of bacteria. However, it is determined that components of spices, such as thymol, may inhibit the formation of biogenic amines (Singh et al., 1999). Therefore, the aim of this study was to evaluate the effects of thymol and lactic acid on the counts of campylobacter in association with the amount of biogenic amines of broiler breast meat during 4 days of storage at +4°C temperature.

Material and Methods

Sample preparation

The experiment was done with breast fillets obtained from broilers originating from the same batch. Broiler breast fillets were frozen for 2 weeks. Afterwards, breast fillet samples were held at 4°C for 1 day to defrost and were divided into 7 groups considering different treatments with bioactive substances (Table 1). All the tests were repeated 3 times. Broiler breast fillets were contaminated with *C. jejuni* strain assigned to Clonal Complex and Sequence Type 464, isolated from a broiler product during previous studies (Kudirkienė et al., 2013).

Table 1. **Details about the experiment design**

Sample group	Abbreviation used in this study
Control group, not treated	C
Contaminated by *C. jejuni* and immersed into distilled water for 3 minutes	CAM
Contaminated by *C. jejuni* and immersed into 1.5% lactic acid L+ for 3 minutes	CAM+1.5%LA
Contaminated by *C. jejuni* and immersed into 3% lactic acid L+ for 3 minutes	CAM+3.0%LA
Contaminated by *C. jejuni* and immersed into 1.5% lactic acid L+ and 0.5% thymol aqueous solution for 3 minutes	CAM+1.5%LA+0.5%T
Contaminated by *C. jejuni* and immersed into 0.5% thymol aqueous solution for 3 minutes	CAM+0.5%T
Contaminated by *C. jejuni* and immersed into 1.0% thymol aqueous solution for 3 minutes	CAM+1.0%T

Every sample was made from 4x10 g broiler breast fillet pieces under aseptic conditions. Six samples were contaminated by 10^8 CFU/g *C. jejuni*, and 1 sample (control) was not contaminated. For the attachment of bacteria, the samples were kept at a temperature of 4ºC for 1 hour. After 1 hour, the samples from No. 3 to No. 7 (Table 1) were immersed into solutions with different concentrations of lactic acid (L-(+)-Lactic acid, 50%, 69778, 1L, Sigma-Aldrich) and thymol (Thymol, 98%, 89838, 1L, Sigma-Aldrich) for 3 minutes. Sample No. 2 was treated only with distilled water, and sample No. 1 was used as a control (untreated). After 3 minutes, the samples were taken into another sterile bag for the storage period of 4 days.

During the storage, the samples were kept at 4ºC temperature. All the poultry meat samples were tested for the level of pH, total bacterial count, campylobacter counts, biogenic amines and lactic acid isomers periodically 1 hour, 4 hours, 24 hours, and 96 hours after the treatment.

Determination of total bacterial count. The effect of bioactive substances on the total bacterial count was determined by doing decimal dilutions in Buffered peptone water (REF 611014, Liofilchem, Italy) and plating on Plate count agar (REF 610040, Liofilchem, Italy) by the pour-plate method. The counts of aerobic bacteria were determined after 72 hours at 30ºC.

Detection of *C. jejuni* counts. The effect of bioactive substances was determined using Buffered peptone water (REF 611014, Liofilchem, Italy) for decimal dilutions and plating on Campylobacter blood free medium base (REF 610130, Liofilchem, Italy) with Campylobacter CCDA supplement (REF 81037, Liofilchem, Italy). The counts of *C. jejuni* in the broiler breast meat were determined at 37ºC after 48 h.

Detection of biogenic amines. A reversed-phase high-performance liquid chromatography method was used for the quantitative analysis of the biogenic amines: putrescine, cadaverine, histamine, tyramine, spermidine, and spermine. The biogenic amines were extracted from a homogenised sample with 0.4 mol/L perchloric acid. The extract was derivatised for 45 minutes by dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) solution in acetone at 40°C. The samples were filtered through 0.45 µm membrane filter, and 10 µL was injected into the chromatographic system. The analysis was performed using LiChro column CART® 125-4. The carrier phase – eluents: B – acetonitrile and A – ammonium acetate 0.1mol/L. The analysis lasted 28 minutes changing the content of the eluents during the first 19 minutes from 50% of B to 90% of B (from 50% of A to 10% of A, respectively), then leaving the content constant for 1 minute at 90% of B (10% of A); later, to ensure isolation of materials for another analysis, the eluent with the composition of 50% of B and 50% of A was being added to the chamber for 8 minutes. The flow rate of 0.9 mL/min did not change during analysis, and the column temperature was 40°C. UV detection was observed at 254 nm. Biogenic amines were identified by comparing the retention time of each amine in the chamber with the retention time of the respective reference material. The internal standard method of calculating the peak area for the defined amount of reference material was used to perform quantitative analysis.

Determination of lactic acid isomers. L(+)- and D(–)-lactic acid isomers were determined by the enzymatic method using D- and L-lactate dehydrogenases kit (K-DLATE, Megazyme, Poland).

pH was examined following the standard method ISO 2917:1999 using PP-15 pH meter (Sartorius Professional meter for pH Measurement, Germany).

Statistical analysis of the data was performed using SPSS 9.0 version and one-way ANOVA to determine differences between samples. The Bonferroni method was used to determine multiple comparisons. The Dunnet test was applied when the control group was present. The Student t test was used to determine average values of indicators, standard deviations and correlations of pH and biogenic amines. The difference was considered to be reliable when $P \leq 0.05$.

Results and Discussion

The formation of L+ and D- lactic acid isomers in broiler breast meat treated with thymol and lactic acid solutions

Fresh meat is a highly suitable environment for spoilage microflora and food-borne pathogens (Zhou et al., 2010). Post-mortem changes in meat occur as autolytic enzymatic spoilage (Dave and Ghaly, 2011). Therefore, meat becomes

softer, the number of peptides increases, and due to a better accessibility of nutrients, the growth of microorganisms intensifies even at low (5ºC) temperatures (Kuwahara and Osako, 2003). As a result of activity of different kinds of microorganisms (bacteria, fungi, yeast, cyanobacteria), lactic acid isomers (L+, D-) may be produced in meat (Abdel-Rahman et al., 2013).

Our study showed that the formation of L+ lactic acid was activated from 1 hour to 4 hours in the samples treated with 0.5% and 1% thymol solutions from 0.252±0.001% to 1.104±0.002% and from 0.252±0.001% to 1.112±0.002%, respectively. Later, during the 4−24-hour period, the amount of L+ lactic acid was stable, and the lowest amount was detected after 96 hours of storage, 0.015±0.003% and 0.016±0.002%, respectively. There was no D-lactic acid isomer formed in the samples treated with 0.5% and 1% thymol solutions and 3% lactic acid solution. These treatments prevented more intensive formation of lactic acid isomers as a result of meat spoilage with psychotropic lactic acid bacteria even at the storage at a low temperature (4ºC) (Johansson et al., 2011).

Furthermore, the formation of lactic acid D-isomer was considerable only in the control meat sample and in the sample treated with distilled water within 24 hours: 0.022±0.002% and 0.023±0.002%, respectively. These results indicate that no risk to human health is posed by using bioactive substances as D-lactic acid isomer is considered to be more cytotoxic in high concentrations than L+ lactic acid isomer, which does not affect negatively metabolic processes (Snijders et al., 2011).

The influence of pH on *Campylobacter jejuni* and total aerobic bacterial count in treated broiler breast meat samples

Quick and significant pH change might result in the reduction of campylobacter and total bacterial count (Maijala et al., 1995). This theory was tested by evaluating the influence of lower pH to the counts of bacteria. pH did not change considerably during the 96-hour period in different samples with thymol and lactic acid (mean±SD; 6.14±0.02 to 6.29±0.02), except for broiler breast fillets treated with 3% lactic acid solution (Fig. 1). The average pH value in this sample varied from 6.27±0.02 to 5.9±0.02, measuring 4 hours after the immersion, to 5.5±0.02 after 96 hours ($P \leq 0.05$). The highest pH value after 96 hours was determined in the control sample and the sample contaminated with *C. jejuni* and immersed into distilled water.

Therefore, higher reduction was expected in the broiler breast fillets sample treated with 3% lactic acid solution. However, the difference between the total bacterial counts in the tested samples was not significant compared with the control sample, regardless of the contamination of meat with campylobacter (Fig. 2). Thymol and lactic acid were likely to affect the total number of aerobic bacteria by temporarily suspending their growth. A decrease in pH in the broiler meat treated with 1.5% and 3% of lactic acid did not have any significant effect on the total aerobic bacterial count.

While our study showed that lactic acid solutions did not have a significant effect on the total bacterial count, it also did not considerably reduce campylobacter numbers. The opposite effect was determined with 1% of thymol, which reduced campylobacter numbers by 0.66 CFU_{10}log/g and was the most effective bioactive compound treatment in comparison with the untreated sample contaminated with *C. jejuni* ($P \leq 0.05$) (Fig. 3). 0.5% of thymol was less effective and reduced campylobacter numbers by 0.41 CFU_{10}log/g ($P \leq 0.05$), followed by 3% of lactic acid solution (0.3 CFU_{10}log/g). Meanwhile, the combination of 1.5% lactic acid and 0.5% thymol had no significant impact on campylobacter numbers.

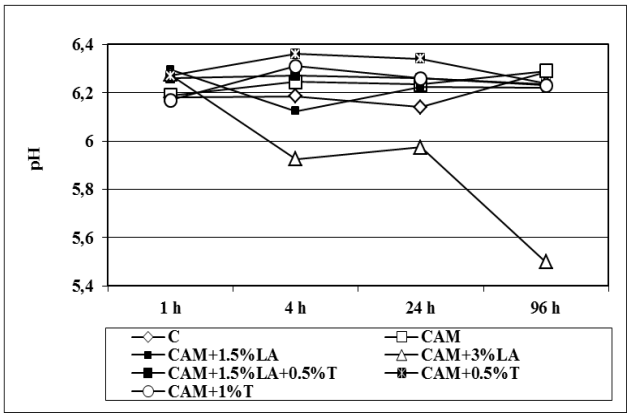

Fig. 1. **Variation of meat pH values during 4 days of storage at +4ºC**

Several studies have shown that thymol is a substance able to create a strong antibacterial effect (Adam et al., 1998; Dorman and Deans, 2000; Zheng and Wang, 2001; Shan et al., 2005; Zinoviadou et al., 2009). As a result of its hydrophobic properties, thymol interacts with the level of lipids of the bacterial membrane and increases permeability, thus, causing the loss of nucleic acids of cell components, such as ATP – adenosine triphosphate (Leistner et al., 1995).

The formation of biogenic amines in treated broiler breast meat samples

Biogenic amines are a metabolism product in animals, plants and microorganisms (Herrero-Fresno et al., 2012). Despite the natural origin of these low molecular weight organic bases, they can cause health disorders to sensitive individuals in case of high levels in food (Ladero et al., 2010). The formation of biogenic amines in food might be controlled by adding bioactive substances, supressing biogenic amines producing bacteria. Therefore, the appliance of lactic acid and thymol on broiler meat should be an effective way to reduce the counts of biogenic amines in meat.

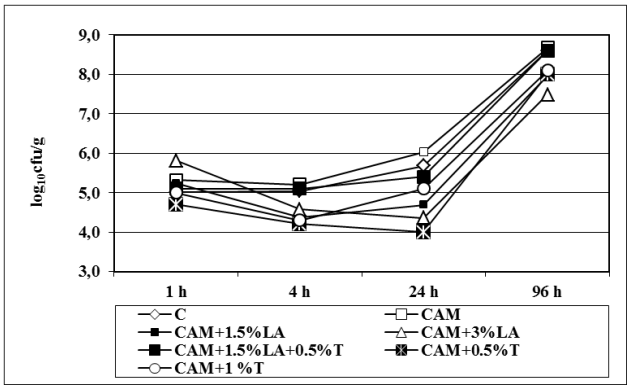

Fig. 2. **Variation of the total bacterial count in broiler breast meat during 4 days of storage at +4ºC**

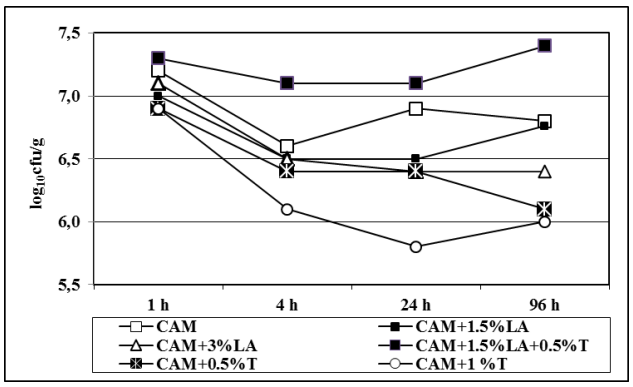

Fig. 3. **Variation of *C. jejuni* count on breast meat during 4 days of storage at +4ºC**

However, the formation of biogenic amines in our study was not intensive during the entire period of 96 hours after the treatment (Fig. 4). During the experiment, small quantities of putrescine, cadaverine, tyramine, spermidine and spermine were formed with the highest amounts of putrescine, tiramine and spermine in the broiler meat contaminated with *C. jejuni* and immersed into distilled water. These results show that the addition of bioactive substances did not induce the formation of biogenic amines in the treated broiler meat samples.

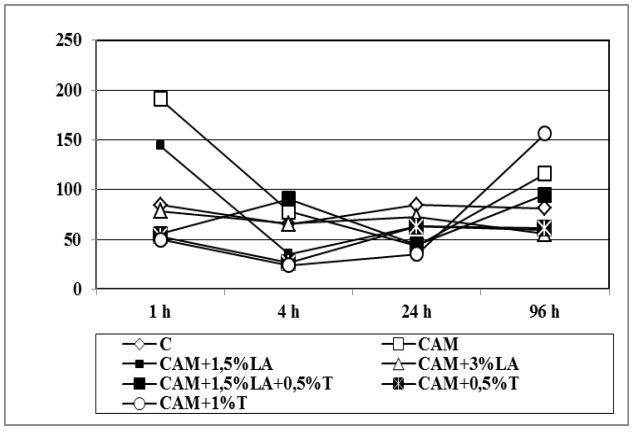

Fig. 4. **Variation of the total amount of biogenic amines in breast meat during 4 days of storage at +4ºC**

The intense formation of cadaverine was observed after 96 hours in the control sample (13.00±0.43 mg/kg) and in the sample contaminated with *C. jejuni* and immersed into 1.5% lactic acid solution (9.825±0.24 mg/kg). No cadaverine was found in the broiler meat that had been immersed into 0.5% and 1% thymol solutions and the amount of putrescine was reduced from 11.82±0.12 to 10.92±0.11 mg/kg by 1% thymol solution ($P \leq 0.05$). After 24 hours, the amount of tyramine decreased in the samples treated with 1.5% and 3% lactic acid solution, and after 96 hours in the samples treated with 0.5% thymol solution. No significant differences were determined between the amounts of tyramine in differently treated broiler meat samples.

Levels exceeding 50 mg/kg of histamine in foods may be harmful to healthy people, while individuals with histamine intolerance may experience adverse health effects even from food with a small amount of histamine (Byun and Mah, 2012). HDC (histidine decarboxylase) is the enzyme that bacteria and yeast use to create histamine from proteins in breast meat. HDC can remain in foods even after the death of bacteria (or yeast) that produced that enzyme (EFSA, 2011b). Fortunately, HDC can be inactivated by acids or phenolic compounds and acids mixes.

In our experiment, the highest amount of histamine was detected in the samples treated with CAM and CAM+1.5%LA after 1 hour of the experiment (Fig. 5). However, within the first 4 hours, the amount of histamine was reduced in all the samples. The lowest amount of histamine was detected in the broiler meat sample treated with 1.5% lactic acid solution after 96 hours of storage. Nonetheless, at this period, the formation of histamine was stimulated in the broiler meat samples treated with 1% thymol, and the level of histamine was higher compared with the control sample and the sample contaminated with *C. jejuni* and not treated with any bioactive substances (Fig. 5).

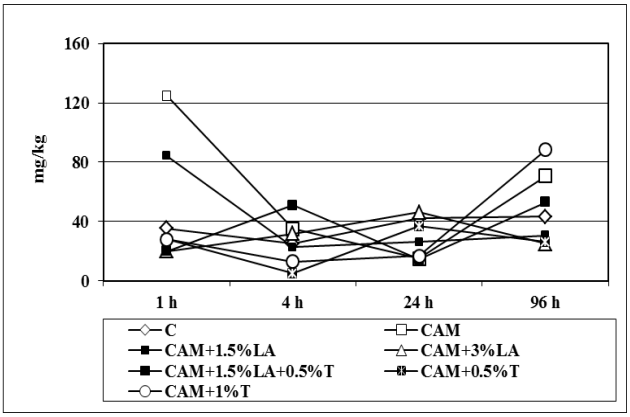

Fig. 5. **Variation of the amounts of histamine in broiler breast meat during 4 days of storage at +4ºC**

The largest amount of spermine (47 mg/kg) was detected in the meat samples treated with 1% thymol solution after 96 hours. Only 3% of lactic acid and 0.5% of thymol solutions had a significant effect on the amount of spermine ($P \leq 0.05$). The changes of the total amount of biogenic amines (Fig. 4) showed that 1% thymol and 0.5% thymol solutions effectively retarded the formation of biogenic amines only within the first 4 hours after the treatment. Higher levels of the total amount of biogenic amines were detected after 96 hours of storage in the broiler meat samples treated with 1% of thymol (156.19±0.32 mg/kg) and 1.5% lactic acid (59.45±0.16 mg/kg). Latorre-Moratalla et al. (2010), Mah and Hwang (2009) and Hu et al. (2007) suggest that lactic acid has a positive effect on the amount of biogenic amines in meat products. Our study showed that 1.5% lactic acid treatment reduced the formation of biogenic amines; however, no significant reduction was observed during the 96-hour period. Nonetheless, 3% of lactic acid solution and 0.5% of thymol solution significantly reduced the amount of spermine ($P \leq 0.05$) in the broiler meat but did not have a significant effect on the total amount of biogenic amines.

Correlation between tested meat properties and bacterial numbers in treated broiler breast meat samples

The highest negative correlation between pH and the total aerobic bacterial count during the 96-hour period was observed in the samples treated with the solution of 1.5% lactic acid and 0.5% thymol ($R = -0.959$). Not so strong correlation between pH and the total bacterial count was observed in the broiler meat treated with 0.5% thymol solution ($R = -0.819$) (Table 2).

The highest correlation between pH and campylobacter numbers was also observed in the samples treated with 1.5% lactic acid and 0.5% thymol solutions, $R = -0.837$ ($P \leq 0.05$). pH negatively correlated with the amount of putrescine, especially in the broiler meat samples treated with 0.5% and 1% thymol solutions, and with tyramine in the samples treated with 1.5% lactic acid and 0.5% thymol solutions, when $R = -0.990$ ($P \leq 0.05$). In marinated samples, pH and concentration of phenolic compounds are the most important factors in influencing the formation of biogenic amines and other physicochemical factors including a_w, storage temperature and additives (Komprda et al., 2009). These parameters should be investigated further to determine the optimal marinating process leading to the reduced biogenic amines formation.

Table 2. **Correlation coefficients R between pH and average values of biogenic amines, campylobacter numbers and total bacterial count during 96 h storage at +4ºC**

	PUT	CAD	HIS	TYR	SPD	SP	TBA	CAM	TBC
C	-0.493	0.879	0.274	0.734	0.177	-0.836	-0.039	-	0.865
CAM	-0.540	-0.475	-0.440	-0.583	0.097	0.179	-0.458	-0.699	0.823
CAM+1.5%LA	0.934	0.624	0.807	0.996	0.627	0.721	0.897	0.245	0.229
CAM+3.0%LA	0.719	0.793	-0.046	-0.917	0.706	0.193	0.970	0.776	-0.585
CAM+1.5%LA+0.5%T	-0.364	0.301	-0.329	-0.990	0.154	-0.646	-0.351	-0.837	-0.959
CAM+0.5%T	-0.836	-	-0.382	-0.776	0.355	-0.901	-0.577	-0.377	-0.819
CAM+1.0%T	-0.938	-	-0.318	-0.205	0.099	-0.167	-0.312	-0.558	-0.284
All R values were reliable when $P \leq 0.05$. PUT, Putrescine; CAD, Cadaverine; HIS, Histamine; TYR, Tyramine; SPD, Spermidine; SP, Spermine; TBA, Total amount of biogenic amines; CAM, Campylobacter jejuni numbers; TBC, Total bacterial count									

Conclusions

Thymol in comparison with lactic acid solutions significantly reduced campylobacter counts, D- lactic acid isomer formation, and was efficient in the reduction of biogenic amines. Therefore, 1% solution of this natural bioactive substance could be used as a decontamination measure against *Campylobacter jejuni* in broiler breast meat. After 96 hours of storage, the lowest amount of histamine was detected in the broiler meat samples treated with 1.5% lactic acid solution and the strong negative correlation with spermine was observed in the samples treated with 0.5% thymol solution ($R = -0.901$, $P \leq 0.05$). Even though the formation of some particular biogenic amines was stopped or delayed, the total amount of biogenic amines was similar in all the experimental broiler meat samples in comparison with the control meat sample during 96 hours of storage. Only 1.5% lactic acid treatment reduced the formation of biogenic amines; however, no significant reduction was observed during the 96-hour period.

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