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ANTIBACTERIAL EFFECT OF THYMOL ON POULTRY DRINKING WATER: *IN VITRO* AND *IN VIVO* STUDY

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ABSTRACT

Objective: This study sheds light on the antibacterial effects of thymol on reducing bacterial load in poultry drinking water and in improving zootechnical performances of animals. **Methodology:** *In vitro***,** the antibacterial activity of thymol, in different water samples used as drinking water in poultry farms, was carried out using the plate count method. Water samples were treated by increasing concentrations of thymol. *In vivo*, chicken were randomly assigned to three groups; (untreated and treated water with thymol (1 and 2 g l^{-1} of NP). The efficiency of water treatment was evaluated on the bacterial intestinal load and zootechnical performances of animals (Body weight, body weight gain, food intake and the consumption index). **Results:** The *in vitro* test showed an important antibacterial activity, depending on the different treatment concentrations ($p<0,05$; $p<0,01$; $p<0,001$), on different species (total mesophilic aerobic flora, coliforms, *staphylococcus, salmonella, C. perfringens*). The *in vivo* test showed that the total mesophilic aerobic bacteria and *C. perfringens* intestinal loads were significantly lower ($p<0,05$; $p<0,01$) in the groups treated with thymol. In addition, results show a significant increase ($p<0.05$; $p<0,01$; $p<0,001$) in the body weight of treated groups during the whole rearing period compared to the control. The body weight gain of animals in the treated groups was significantly ($p<0.05$; $p<0,01$; $p<0,001$) higher than the control. Treated groups represent a lower consumption index compared to the control. **Conclusion**: In addition to demonstrating a high antibacterial activity, this work offers an alternative solution to chemical biocides which are commonly used in poultry farming.

KEYWORDS: Water, poultry, thymol, antibacterial, intestinal flora, performance parameters.

INTRODUCTION

In recent years, animal feed has been affected by several regulations, such as the ban of antibiotics as growth promoters.[1] This led to a deterioration of animals zootechnical performances and the emergence of digestive disorders such as non-specific enteritis, which constitute a real economic problem for poultry farming.[2]

Moreover, bacteriological drinking water quality deterioration is a causal factor of disorders in farmed animals.[3] For this reason, it is necessary to control drinking water quality through the water composition, the type of the treatments performed and their effectiveness. Some active substances such as biocides are commonly used for the destruction and elimination of germs. However, they present a high risk for humans and ecosystems due to their proven toxicity.[4]

Our laboratory, which has extensively worked on essential oils and their major compounds, demonstrated the antimicrobial activity of these components.[5,6,7,8] These results suggested a potential use of these substances as alternatives to antibiotics in poultry drinking water.

The objective of this study is to evaluate *in vitro* the antibacterial activity of thymol and to evaluate *in vivo* the thymol's effect on intestinal bacterial load of chicks, as well as the improvement of their zootechnical parameters.

MATERIALS AND METHODS

In vitro test

The aim of the *in vitro* test was to evaluate the antibacterial activity of thymol in different water samples used as drinking water in poultry farms.

Sampling

Water samples were collected from surface water (Tank (T), water tower (WT) coming from a river and four different groundwater points (W1, W2, W3, and W4). They are used as drinking water in different poultry farms. They were collected in sterile bottles and transported directly to the laboratory in a cooler at 4°C. They were analysed within 24 hours of arrival.

Culture Media

Plate Count Agar (PCA) (Biokar) was used for the total mesophilic aerobic flora culture and the antibacterial test. Deoxycholate Lactose Agar (Biokar) was used for the coliforms culture and the antibacterial test. Cultures and antibacterial tests of *staphylococcus* were prepared using Chapman Mannitol Agar (Biokar). For the culture and the antibacterial test of *Salmonella*, Wilson-blair Agar (Biokar) was used. As for the culture and the antibacterial test of anaerobic bacteria (*Clostridium perfringens*), the medium Tryptone Sulfite Cycloserine Agar (TSC) (Biokar) was used.

Antibacterial agent

Thymol is the active principle of NP (15% of thymol), produced by the Industrial Laboratory of Veterinary Alternatives (LIAV, LLC) in Morocco. Thymol is was obtained from *Origanum compactum.* In addition to thymol, other excipients have been added to provide stability and solubility. Different concentrations of the thymol (1, 2 and 4 g/l of NP) were added to each water sample. A negative control was also prepared.

Antibacterial test

The antibacterial test was carried out using the plate count method. 1ml of each sample was placed in a sterile Petri dish (90 x 16 mm). The medium was then poured and perfectly homogenized. The inoculated dishes were incubated at 37°C for 24 hours for total mesophilic aerobic bacteria and total coliforms. Fecal coliforms were incubated at 44°C for 24 hours. Staphylococci were incubated at 37°C for 44 hours and *Salmonella* from 24 to 48 hours at the same temperature. *C. perfringens* anaerobic bacteria were incubated in anaerobiosis jar with a hydrogen and carbon dioxide mixture for 24 hours at 37°C.

In vivo test

The main objective of the *in vivo* test was to evaluate the efficiency of the water treatment with thymol on the bacterial intestinal load of the chicken and their performances.

Animals and breeding conditions

A disinfection of the cages was carried out before the beginning of the experiment. The chicks used in this study were one-day-old (approximately 37g). They were divided into groups of twelve and housed in separate cages. The photoperiod was adjusted on a daily basis to 12 hours of light and 12 hours of darkness.

The aeration was provided by a fan. At the beginning of the experiment, the ambient temperature was 32°C. It was reduced by 2 to 3°C each week to reach 23°C at the end of the experiment. Chicks were given *ad libitum* access to food and water. They were fed with maize-based food, free of antibiotics and antiparasitics. For sanitation, drinkers were cleaned daily.

Treatment of drinking water

The drinking water sample was taken from the distribution tank on a poultry farm (sample T). The bacterial analysis of the water (sample T) before any treatment showed that total mesophilic aerobic bacteria load was $2{,}3\ 10^5$ CFU/ml. After treatment with thymol, it was reduced to $1{,}6\ 10^4$ CFU/ml with 1 g/l and $3\ 10^3$ CFU/ml with 2 g/l of NP. The *C. perfringens* burden was 90 CFU/ml before treatment with the thymol and was reduced to 27 CFU/ml with the concentration 1 g/l and to 2 CFU/ml with the concentration 2 g/l of NP.

The animals were divided randomly into three experimental groups of 12 chicks each:
Group 1 (n = 20) control group: Animals that consumed untreated water.
Group 2 (n = 20): Animals that consumed treated water with thymol (1 g/l of NP).
Group 3 (n = 20): Animals that consumed treated water with thymol (2 g/l of NP).

Evaluation of bacterial intestinal load of animals

During the rearing period, the impact of the treated water with thymol on the bacterial intestinal load of animals was tested for total mesophilic aerobic bacteria and anaerobes bacteria in particular *C .perfringens.* Once a week (Day 1, Day 7, Day 14 and Day 21), 1 g of fresh feces sample from each group was collected and solubilized in 9 ml of physiological serum and dilutions were performed.

Culture media

Plate Count Agar (PCA) (Biokar) was used for the culture and the antibacterial test of total mesophilic aerobic bacteria. Tryptone Sulphite Cycloserine Agar (TSC) (Biokar) was used for the culture and the antibacterial test of *C. perfringens*.

Evaluation of bacterial intestinal load

The evaluation of the intestinal load was performed by the plate count method. For the culture and the antibacterial test of total mesophilic aerobic bacteria, sterile Petri dishes (90 x 16 mm) containing the PCA

were inoculated with 100 µl of the diluted feces samples and incubated at 37°C for 24 hours. For the culture and the antibacterial test of anaerobic bacteria, the inoculated dishes with 1 ml of the diluted feces samples were incubated for 24 hours at 37°C.

Performance parameters

The impact of the treatment of drinking water with thymol on the animals' intestinal bacterial load was evaluated by the animals' zootechnical parameters. Body weight, body weight gain, food intake and the consumption index were the studied parameters that we chose to include in this experiment.

Statistical analyses

The results are presented by the means and their standard error**.** The data was analyzed by the T-test using SigmaStat 4.0. The significance was verified for bacterial analysis of drinking water, intestinal load of animals and their zootechnical parameters. The significance level chosen for both tests is 5% at $P<0.05$.

RESULTS

***In vitro* test**

Figure 1 shows the variation of the bacterial load of the different tested samples.

Before any treatment, the bacterial tests showed an important load of total mesophilic aerobic bacteria for all tested samples. After treatment with thymol, a reduction was observed for both samples W3 and T ($p<0,001$) with the concentration 1g/l of NP. A significant decrease ($p<0.05$; $p<0,01$; $p<0,001$) was noted with the concentration of 2 g/l, for the samples W1, W3, W4, T and WT. The treatment by 4 g/l provides a notable reduction ($p<0,05$; $p<0,001$) of the total mesophilic aerobic bacterial load for W1 and T. A total inhibition of load was noticed for W2, W3, W4 and WT.

Samples W1, W3 and W4 were initially characterized by the absence of total coliforms load. Both samples T and WT were characterized by the presence of a high total coliform load. A significant reduction ($p<0.05$; $p<0,001$) of the load was observed with the concentration of 2 g/l for the three samples (W2, T and WT), while a total inhibition was observed with the concentration of 4 g/l.

As for the fecal coliform load, samples W1, W3 and W4 were distinguished by the initial absence of fecal contamination. Samples T and WT were characterized by the presence of an important fecal coliform load that decreases significantly ($p<0.01$) as we were increasing the thymol concentration. The sample WT load was completely inhibited with the concentration of 4 g/l. An inhibition of fecal coliform growth was observed with the concentrations 2 g/l and 4 g/l for sample W2.

For samples W1, W2, W3 and W4, an initial total absence of staphylococci was noted. Additionally, for both samples T and WT, a significant decrease ($p<0.05$; $p<0,01$) of the load was noticed for the concentrations 2 g/l and 4 g/l.

The presence of *Salmonella* was detected only in the samples T and WT. For both samples, a significant reduction ($p<0,05$) of the intestinal burden was obtained thanks to the increase of the treatment concentration, with a total inhibition at the level of 4 g/l.

Before treatment, we noticed an absence of *C. perfringens* load in samples W2 and W3. A low burden of *C. perfringens*, which was observed in sample W1, was totally inhibited after treatment with 1 g/l. A reduction of the bacterial load was obtained for sample W4 with the concentration 1 g/l, whereas a total inhibition was noticed for the concentrations 2 and 4 g/l. For the samples T and WT, the burden decreased as we increased the treatment concentration.

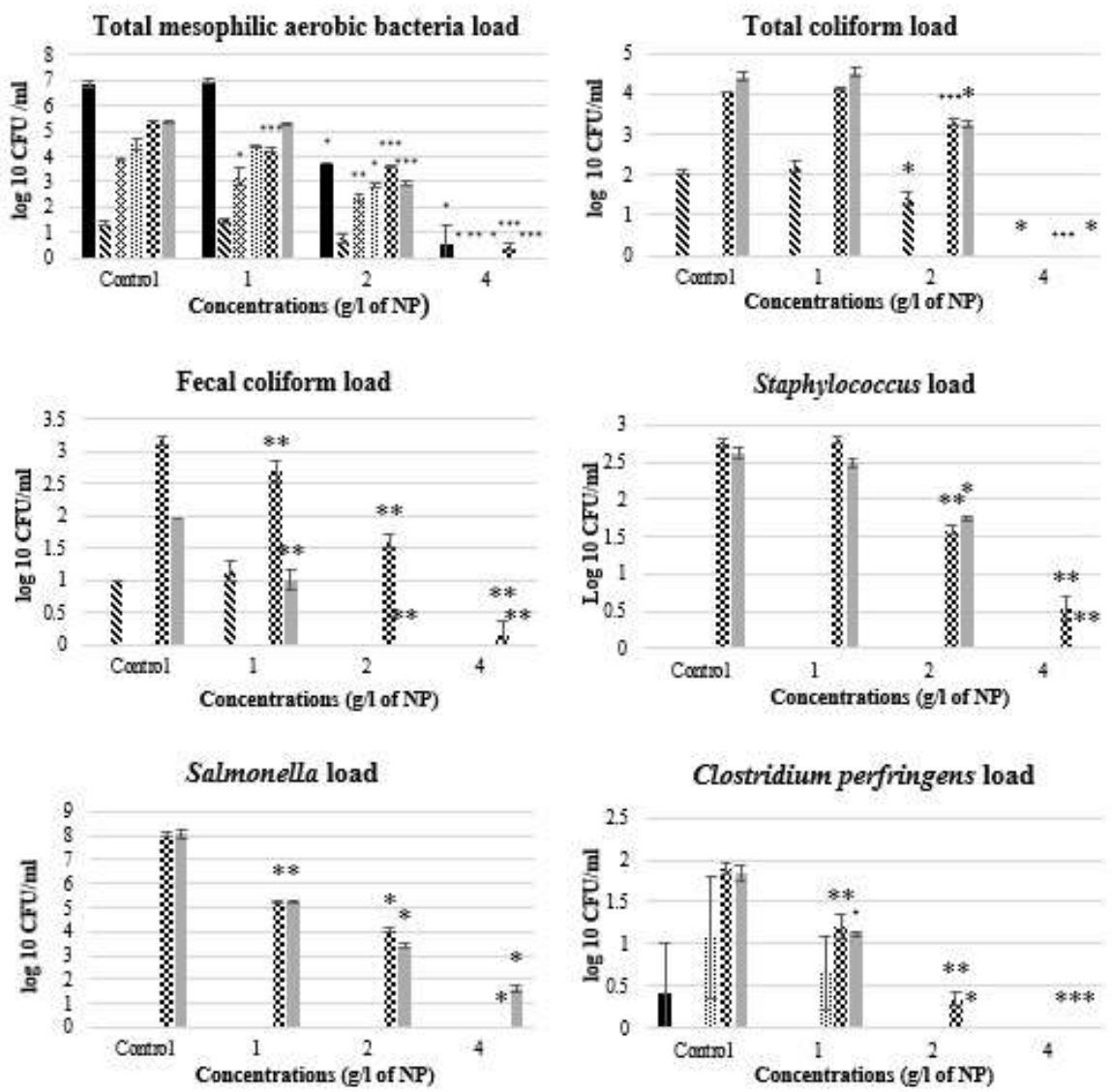

Figure 1: Variation in bacterial load depending on the thymol concentration.

W1, W2, W3, W4 (groundwater points), T (Tank), WT (water tower)
(Values are means (n=6) ± SEM (Standard error of the mean)) (indicate a significant difference between the control group and the treated group at (p<0.05) according to t-test; ** indicate a significant difference between the control group and the treated group at (p<0.01) according to t-test; *** indicate a significant difference between the control group and the treated group at (p<0.001) according to t-test)*

***In vivo* test**

Effect of the consumption of untreated water compared with treated water with thymol on the assessment of the bacterial intestinal burden

Table 1 shows the variation of the bacterial intestinal load for different groups of animals. At the beginning of the experiment (Day 1), the total mesophilic aerobic bacteria load of all groups was 2.33 10^{10} CFU/g. On Day 7, an increase of the load was noted in the group 1 (control). While the group 2 treated with thymol showed slightly lower values compared to the control, the group 3 showed a significant decrease ($p<0.01$). On the 14th and 21st days, this same group was distinguished by a higher reduction in the total mesophilic aerobic bacterial load compared to the group treated with 1g/l and to the control group (5 log units).

On Day 1, the *C. perfringers* intestinal load was 5 10^{8} CFU/g**.** All groups exhibited a similar load during the first 14 days with a slight superiority of the control. Day 21 stood out with a significant decrease in the bacterial load of the group 3 compared to the control one (2 log units). An increase of load was noticed in the control and group 2.

Table 1: Evolution of the bacterial intestinal load.

	Total mesophilic aerobic bacteria load (log 10 CFU/g)			***Clostridium perfringens* load (log 10 CFU/g)**		
	Group 1	***Group 2***	***Group 3***	***Group 1***	***Group 2***	***Group 3***
Day 1	10,36 ± 0,04			8,69 ± 0,00		
Day 7	11,04 ± 0,01	10,39 ± 0,06*	10,11 ±0,07**	9,15 ± 0,30	8,79 ±*	8,69 ± 0,00**
Day 14	11,12 ± 0,06	10,41 ± 0,01 *	6,66 ± 0,30**	9,36 ± 0,04	9,1 ± 0,26	9 ± 0,13**
Day 21	10,83 ± 0,06	10 ± 0,00*	7 ± 0,15**	9,91 ± 0,01	9,6 ± 0,00	7,56 ± 0,25 **

Group 1 (untreated water); Group 2 (treated water with thymol (1 g l^{-1} of NP)); Group 3 (treated water with thymol (2 g l^{-1} of NP)). (Values are means (n=6) ± SEM (Standard error of the mean)) (indicate a significant difference between the group 1 (control) and the treated group at (p <0.05) according to t-test; ** indicate a significant difference between the group 1 (control) and the treated group at (p <0.01) according to t-test)*

Effect of untreated water compared with treated water with thymol on the evolution of zootechnical parameters

Evolution of body weight

The effect of the treatment with thymol on the body weight evolution is shown in table 2. Results show a significant increase (p<0.05; p<0,01) in the weight of the group 2 during the whole rearing period compared to the control. This increase is more significant (p<0.05; p<0,001) after treatment with the concentration 2 g/l.

Evolution of the body weight gain, food intake and consumption index

The evolution in time of the body weight gain, food intake and consumption index of the different groups during rearing period is shown in table 2. Throughout the experiment, the body weight gain of animals in the two treated groups was significantly (p<0.05; p<0,01; p<0,001) higher than the control. Concerning the consumption index, the control represents a higher consumption index compared to treated groups.

Table 2: The effect of water treatment on body weight, body weight gain, food intake and consumption Index.

		Group 1	**Group 2**	**Group 3**
Body weight (g)	*Day 1*	37,16 ± 1,64	36,58 ± 3,01	36,83 ± 3,03
	Day 7	70,83 ± 6,33	80,41 ± 6,85*	86,63 ± 11,23*
	Day 21	214 ± 6,8	246,36 ± 25,12**	317 ± 7,6 ***
Body weight gain (g)	*Day 1- Day 7*	33,6 ± 7,04	43,8 ± 5,04**	49,8 ± 13,50**
	Day 7- Day 21	143 ± 3,43	166 ± 26,40*	230 ± 7,30***
Total body weight gain (g)		406	643	688
Food intake (g)	*Day 1- Day 7*	75	61,6	69
	Day 7 - Day 21	270	245	270
Consumption index	*Day 1- Day 7*	2,23	1,41	1,39
	Day 7 - Day 21	1,89	1,48	1,17

Group 1 (untreated water); Group 2 (treated water with thymol (1 g l^{-1} of NP)); Group 3 (treated water with thymol (2 g l^{-1} of NP)). Values are means (n=20) ± SD, (indicate a significant difference between the group 1 (control) and the treated group at (p<0.05) according to t-test; ** indicate a significant difference between the group 1 (control) and the treated group at (p<0.01) according to t-test; *** indicate a significant difference between the group 1 (control) and the treated group at (p<0.001) according to t-test).*

DISCUSSION

In the present work, we performed an *in vitro* assessment of the antibacterial activity using thymol. We also evaluated the effects of thymol on reducing the intestinal load of animals *in vivo*, particularly total mesophilic aerobic bacteria and *C. perfringens*.

***In vitro* test**

The microbiological analysis revealed the contamination of water by different micro-organisms which are total mesophilic aerobic bacteria, coliforms, staphylococci, *Salmonella* and anaerobic bacteria (*C. perfringens*). Drinking water samples were treated by increasing concentrations of thymol, which are 1, 2 and 4 g/l of NP. As a first step, we tested the thymol's effect on reducing total mesophilic aerobic bacterial load. The results have shown that both groundwater and surface water samples have an important total mesophilic aerobic bacterial load that varies between 10^4 and 10^7 CFU/ml. The treatment with thymol at 1 g/l significantly reduced this load from 10^7 to 10^4 or 10^5 (99% to 99.9% of reduction). Also, the total mesophilic aerobic bacterial load treated with a concentration 4 g l^{-1} had become almost undetectable. This inhibitory effect is explained by the fact that thymol belongs to one of the most effective terpenes against bacteria.[9,10,11] The efficiency of the thymol treatment was also confirmed on total coliforms, fecal coliforms, and staphylococci. For all these species, thymol yields a significant reduction of bacterial burden with a concentration of 1 g/l and a nearly total disappearance with a concentration of 4 g/l. Ouwehand *et al.*[11] tested the antibacterial activity of thyme essential oil on *E. coli* at relatively low doses (5 and 50 mg/l) and found that it causes a decrease in the *E.coli* load. The sensitivity of

staphylococci to thymol has been shown in several studies.[12,13]

Anaerobic bacteria *C. perfringens* and *Salmonella* are of great concern to farmers. Indeed, they cause serious diseases such as necrotic enteritis in chicks. Our water analysis showed that, among the six samples of drinking water, three were loaded with *C. perfringens* (between 30 and 81 CFU/ml) and two were loaded with *Salmonella* (1,1 10^8 and 1,3 10^8 CFU/ml). The results showed that thymol is effective on *C. perfringens*. As a matter of fact, the concentration 1 g/l led to a reduction of 90 % of the load, and with the concentration 2 g/l, germs became almost undetectable. As for the *Salmonella* load, the treatment by 1 g/l caused an important reduction. The effect of the treatment is dose-dependent. These results are similar to the ones reported by Broudiscou *et al*.,[9] Lee *et al*.[10] and Ouwehand *et al*.[11] who have shown that thymol inhibits the development of numerous pathogenic bacteria responsible for necrotic enteritis including *Salmonella* and *C. perfringens*. Our results showed that thymol, with the concentration 1 g/l of NP significantly reduces the load of all the analyzed germs. Therefore, thymol, at doses as low as 1 g/l of NP, can keep the bacterial load of tested waters low, which subsequently limits the occurrence of a subclinical infection. Also, this reduction can stimulate the immune system against the aforementioned germs. This will be verified in the *in vivo* test.

***In vivo* test**

For the *in vivo* test, the experiment with a control (group 1) receiving untreated water and two others receiving the same water treated with thymol (group 2 and group 3) showed that the total mesophilic aerobic bacteria and *C. perfringens* intestinal loads were significantly lower in the groups treated with thymol. We also noted that the effect after treatment with 2 g/l is more important than the one provided with 1 g/l. According to Jamroz *et al*.,[14] this intestinal load reduction is probably due to thyme, which has a phytobiotic effect on chickens and limits the adaptation of pathogenic microorganisms. Indeed, thyme proved to be a promoter of microbial balance in the intestines of the animals.

During our experiment, the animals in the control group consumed a similar quantity of food as the treated groups and yet showed worse zootechnical performances. This can be explained by the reduction of the bacterial load by thymol which affects intestinal integrity. With a balanced intestinal flora, food may be more easily absorbed, which explains the difference in growth between animals. A study conducted by Lee *et al*.[15] also showed that thymol increases the activity of chicken's intestinal amylase which improves chicken's growth through the increase of the digestibility of nutriments and the regulation of the intestinal microflora. Thymol could represent a natural alternative replacing chemical biocides currently used in poultry farming.

CONCLUSIONS

The results of these experiments lead to the conclusion that thymol exercises a significant antibacterial action on drinking water. This action causes a decrease of the total intestinal burden and has a significant positive effect on the zootechnical performance of the animals. The effect obtained on the bacterial load must be verified on other species such as protozoa, yeast and molds.

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CONFLICT OF INTEREST

No conflict of interest declared.

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