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Effect of thymol on planktonic and biofilm cells in drinking water: An anti-cryptosporidium effect

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ABSTRACT. There is a consensus that biofilm shows resistance to antimicrobial agents, especially in poultry farming. The current study assesses how thymol's antiparasitic properties affect the load of parasites, particularly *Cryptosporidium* oocysts load, in chicken drinking water. The first experiment used microscopic counting to evaluate *in vitro* the anti-cryptosporidium activity of NPEB (a thymol-based product) on drinking water samples. Thymol was added to samples in increasing doses (1, 2, and 4 g L^{-1} of NPEB). The anti-cryptosporidium efficacy *in vitro* was dose-dependent ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Moreover, the antibiofilm efficiency of the thymol-based product against protozoan biofilm (*Cryptosporidium* oocysts) was tested using an experimental arrangement simulating the water supply system in poultry farming. In order to do that, we conducted two preventive and curative tests utilizing two distinct product concentrations (1 and 2 g L^{-1}). A greater reduction was shown for the concentration 2 g $L^{-1,}$ which is in the order of three logarithmic units. The removed water from treated pipes with thymol (1 g L^{-1} of the product) showed a significant decrease ($p < 0.05$) in the curative study as compared to controls. However, after just 24 hours of treatment, the amount of 2 g L^{-1} treated pipes was significantly reduced ($p < 0.01$).

Keywords: thymol, protozoa, *Cryptosporidium* oocysts, biofilm, poultry farming, water supply.

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Introduction

Biofilms are multicellular communities held together by a self-produced extracellular matrix (Berlanga & Guerrero, 2016). Protozoans are an essential component of biofilms, which rapidly colonize new substrata and occur in high abundance in biofilms (Watson et al., 2015; Sikder et al., 2020). Generally, biofilms are structured by protozoan activities (feeding, movement) and by their lorica, stalks, and excretions.

In the poultry industry, biofilm is a persistent concern to drinking water that, without effective management, may pose a threat to flock health and performance in a variety of ways. Biofilm causes water contamination with hazardous microorganisms (Liu et al., 2016; Tasneem et al., 2018; Correa, 2020). However, it forms an slim adherence to pipelines and drinking water storage tanks causing clogged drinkers, damaged equipment, and reduced water flow. The parameters of drinking water (pH, ion concentration, etc.), distribution system characteristics, and breeding conditions in chicken farming, such as the high temperature of grow houses and water consumption of newborn chicks, all affect biofilm formation.

Breeders employ several disinfection procedures, which are typically carried out between production cycles, to get rid of the biofilm and the plankton (Maes et al., 2019). The susceptibility of biofilm cells to disinfecting agents is affected by several factors such as, the age of the biofilm, surface, and growth conditions (Abdallah et al., 2014; Corcoran et al., 2014; Kukhtyn et al., 2017; Tong et al., 2021).

Several studies demonstrate a wide antiparasitic activity of some essential oils and their major compound, especially phenolic compounds such as thymol (Tasdemir et al., 2019; Azadbakht et al., 2020). Studies on the antiparasitic effects of essential oils conducted in our laboratory both *in vitro* and *in vivo* have produced notable findings (Remmal et al., 2011; Tanghort et al., 2019).

The current study seeks to assess a natural alternative's antiparasitic effectiveness against protozoan plankton and biofilm developed in an experimental setup that mimics the water supply system in chicken

farming. The product, which is thymol-based, is used to replace the antibiofilm chemicals currently employed in the chicken industry.

Material and methods

Antiparasitic agent: Thymol was the antiparasitic substance utilized. It is the active ingredient in NPEB (15% of thymol), which is produced in powder form by the Industrial Laboratory of Veterinary Alternatives (LIAV, LLC) in Morocco. In addition to thymol, other excipients have been added to provide product stability and solubility of the product in water. The product was put into solution followed by stirring to promote its dissolution in water. After a contact time of about an hour is ready for use.

In vitro test: Thymol was tested *in vitro* in a variety of water samples used as drinking water in poultry farms for its anti-cryptosporidium effectiveness. Samples were taken from a water tower (WT), a tank (T) that were coming from a river and four separate groundwater points GP1, GP2, GP3, and GP4. They were gathered in sterile bottles, kept at 4°C in a fridge, and then brought straight to the laboratory. They were examined 24 hours after they arrived. Each water sample received additions of 1, 2, and 4 g L^{-1} of thymol-based product at various concentrations. Additionally, a negative control was prepared. Microscopy counting was used for the anti-cryptosporidium test. 10 µL of each sample was transferred to a Malassez chamber. The number of parasites was counted in 10 fields of view using standard techniques (Ryley, 1976), and the mean number of parasites per milliliter of the sample was calculated.

Experimental arrangement: Biofilm formation was assessed using an experimental arrangement composed of tank N°1, which contained 200 mL (already mounted) supplemented with 1.8 L of sterile water, and tank N°2 served as recovery. To favor biofilm formation, the ambient temperature was adjusted to 30°C using electric heating. The biofilm was formed experimentally on a 1 cm diameter and 2 m length PVC coiled pipe that links the two tanks. A tap installed at tank 1 allows the passage through the pipe of low water flow. The coiled pipe ensures water stagnation, protozoa fastening, and biofilm formation (Figure 1).

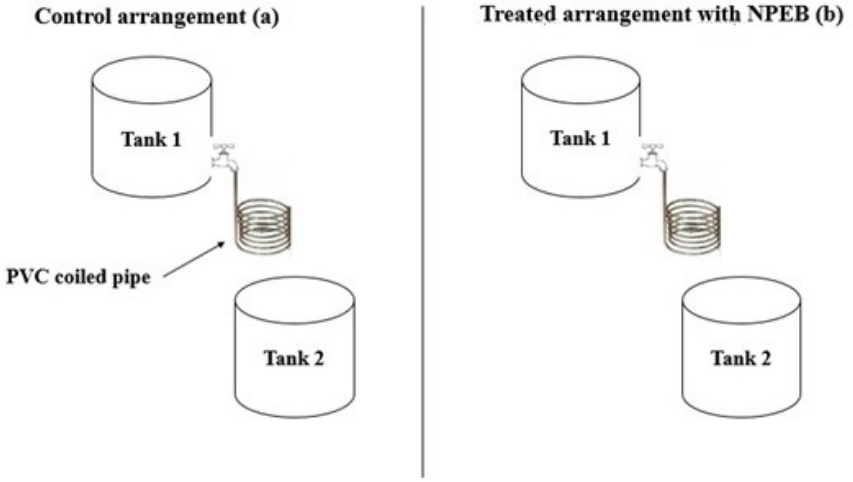

Figure 1. Organisms and growth conditions. Tank (1) contains 2 liters of aqueous nutrient solution contaminated by protozoa (6.2 10^7 cells mL^{-1}); Tank (2) is used to collect water from the tank (1); (a) Control arrangement: contains only water contaminated with protozoa; (b) Treated arrangement: contains water contaminated with protozoa +NPEB at 1 or 2 g L^{-1}.

Cryptosporidium oocysts were recovered from poultry droppings that have been diluted in physiological saline. A volume of 200 mL was centrifuged at 7,000 rpm for 10 min. The recovered pellet was diluted in a volume of 200 mL of physiological saline and the number of cells was counted in 10 rectangles of a Malassez chamber. The protozoan charge obtained was 6.2 10^7 cells mL^{-1}.

Treatment of biofilm

Preventive and curative essays have been performed using two concentrations of (NPEB). The preventive treatment consisted of preventing adhesion, protozoa development on pipes, and biofilm formation. For this, the NPEB has been incorporated in tank 1 in the permanent presence of protozoa. The duration of treatment was 21 days. Curative treatment consisted of treating the biofilms formed on pipes after a week of circulation of the water contaminated by protozoa. After a week, tanks and pipes were washed with sterile water and

supplied with 2 liters of water treated with NPEB. The treatment time was one week for the concentration of 1 g L^{-1} and 24 hours for the concentration of 2 g L^{-1}. For both essays, the control arrangement contained only water loaded with protozoa.

Effect of treatment with NPEB on adherence and biofilm formation

To get rid of any cells that were loosely attached or planktonic, pipes were given two rinses with sterile distilled water. A third rinse was carried out, followed by stirring, to recover the protozoa stuck to the pipe walls and a water sample was taken for analysis. The number of oocysts was counted over 10 rectangles of a Malassez chamber using 10 µl of the water sample.

Statistical analysis

Results are presented by means and their standard errors. The statistical software used for the evaluation of differences between groups was SigmaStat 4.0. The significance level chosen was 5% at $p < 0.05$.

Results

In vitro test: Anti-cryptosporidium effect of NPEB on drinking water samples

Table 1 displays the variation in the *Cryptosporidium* load of the several examined samples. The parasite test revealed a significant burden for all examined samples before any treatment. For samples GP2, GP3, GP4, and T after thymol treatment, a significant reduction was seen ($p < 0.05$, $p < 0.01$, and $p < 0.001$) with the concentration of 1 g L^{-1} of NPEB. The increase in treatment concentration resulted in a significant reduction ($p < 0.05$; $p < 0.01$; $p < 0.001$) of the *Cryptosporidium* load for all samples.

Table 1. Variation of the *Cryptosporidium* load in different samples depending on the thymol concentration.

	Parasitic load ($\log_{10}$ cells mL^{-1})					
	GP N° 1	GP N°2	GP N°3	GP N°4	Tank	Water tower
Control	5.72 ± 0.07	4.11 ± 0.00	5.18 ± 0.04	5.26 ± 0.01	4.12 ± 0.04	4.21 ± 0.1
1 g L^{-1} of NPEB	5.54 ± 0.08	4.04 ± 0.02*	4.97 ± 0.02**	4.9 ± 0.03***	3.25 ±0.03***	4.04 ± 0.01
2 g L^{-1} of NPEB	2.98 ± 0.08**	2.81 ± 0.09***	2.71 ± 0.01**	2.64 ± 0***	2.05 ±0.11***	2.9 ± 0.04*
4 g L^{-1} of NPEB	1.91 ± 0.05**	1.84 ± 0.06***	1.43 ± 0**	1.31 ±0.01***	1.69 ±0.14***	1.44 ± 0.01**

GP (Groundwater point); T (Tank), WT (Water tower); Values are means (n = 6) ± SEM (Standard error of the mean); Comparison with control: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Effect of preventive treatment with NPEB on the protozoa load

As Figure 2 shows, there is a significant reduction ($p < 0.05$; $p < 0.01$) in the protozoan load of treated pipes with thymol compared to the control pipes. A greater reduction was shown for the concentration 2 g L^{-1} which is in the order of three logarithmic units.

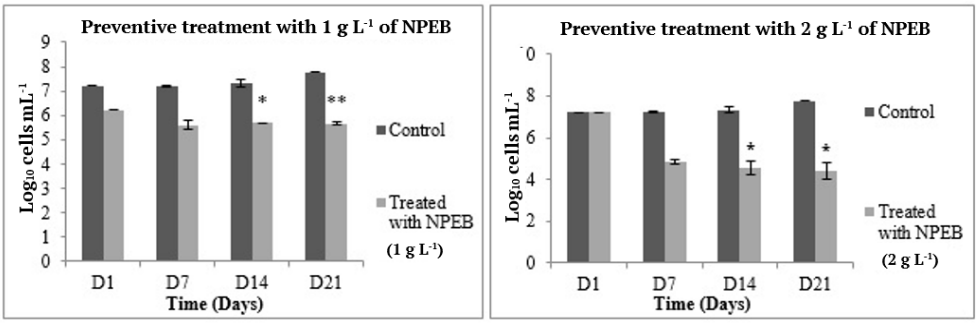

Figure 2. Protozoan load of treated pipes with thymol compared to the control pipes (preventive treatment). Values are means (n=3) ± SEM (Standard error of the mean); Comparison with control: *p < 0,05; **p < 0,01

Effect of curative treatment with NPEB on the protozoa load

Protozoan load evolution of treated pipes with thymol compared to untreated pipes is shown in the following Figure 3. For the various taken samples, a significant reduction ($p < 0.05$) was noted for the withdrawn water from treated pipes with 1 g L^{-1} of NPEB, compared to the controls. However, treated pipes with 2 g L^{-1} were significantly reduced ($p < 0.01$) after just 24 hours of curative treatment.

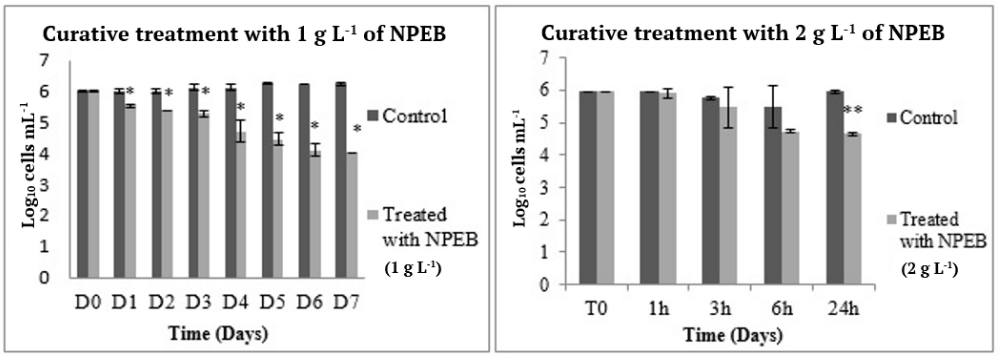

Figure 3. Protozoan load of treated pipes with thymol compared to the control pipes (curative treatment). Values are means (n=3) ± SEM (Standard error of the mean); Comparison with control: *p < 0,05; **p < 0,01

Discussion

Because of their biological traits, such as a cysts/oocysts resistant stage, a very strong wall, and a small size of the cellular body, protozoa are challenging to treat and control. According to a study conducted in 2012, they are to blame for a multitude of illnesses including severe gastroenteritis, persistent harm, and high morbidity (Fletcher et al., 2012).

This experience began with a preliminary analysis of deposits on the walls of tanks and pipes used in poultry farming. These results identified a variety of microorganisms including bacteria, fungi, and protozoa (investigation results). Even though protozoans may occur in very high abundances in biofilms in production houses, knowledge about substrate-associated protozoans' role is still deficient compared to a large number of publications linked to bacteria or fungi (Stojanov et al., 2017; Maes et al., 2019). The initial stage in this investigation was to confirm that thymol has an antiparasitic effect on protozoan cells by adding increasing doses of a thymol product (NPEB) to various watering water samples taken from different poultry farms.

Analyses of parasites showed that samples of both surface and groundwater were contaminated, with *Cryptosporidium* predominating. The *Cryptosporidium* load varies between 10^4 to 10^5 cells mL^{-1}. Thymol was applied to samples at three different concentrations, 1, 2, and 4 g L^{-1} of NPEB. Thymol treatment at 1 g L^{-1} greatly decreased this load, and the reduction was more significant with 2 g L^{-1} of NPEB. The fact that thymol is one of the most powerful antiparasitic effective explains this inhibitory action. Based on research done in 2019 witch tested the oocysticidic efficiency of thymol and discovered that it significantly reduces the number of *Cryptosporidium baileyi* and *Cryptosporidium galli* oocysts (88%) at a concentration of 0.5 mg mL^{-1} after just three hours of treatment, while no oocysts were visible at the concentration of 1 mg mL^{-1}. All oocysts from both *Cryptosporidium* species are eliminated at a dosage of 0.5 mg mL^{-1} after 24 hours of treatment (Tanghort et al., 2019).

In poultry production houses, microorganisms probably come from the water source whether underground or surface water (Daniels et al., 2016). Then they adhere to the inner walls of pipes and tanks, proliferate and contaminate animals during the breeding period. Several authors described the phenomenon of biofilm formation in drinking water systems (Puiu et al., 2017; Reuben et al., 2019). To fix this problem, we have developed an experimental arrangement to simulate animal watering conditions in poultry farming. Water circulates from one tank to another through a transparent PVC pipe, which allows a low flow of water rate. Therefore, the water was partially stagnant at the initial tank and pipes. Results showed a significant reduction when the initial tank containing approximately 10^7 cells mL^{-1} was treated with NPEB at a concentration of 1 g L^{-1}. The reduction is also more important when the product was used at a concentration of 2 g L^{-1} (about 2 logarithmic units). We observed that the addition of the product to the drinking water prevents protozoa development. Therefore, it was necessary to test the curative effect of NPEB. The obtained results show that 24 hours were enough to significantly reduce the protozoa load after treatment with a concentration of 1 g L^{-1}. After a week, the reduction was more important (approximately 2 logarithmic units). Due to the high concentration of thymol in the concentration of 2 g L^{-1} compared to 1 g L^{-1}, 1 day was sufficient to reduce the load with approximately 2 logarithmic units. Studies conducted in 2013 and 2019 explain the mechanism of action of essential oils and their major compounds on *Eimeria* species (Remmal et al., 2013). After treatment, morphological changes of *Eimeria* oocysts with cracked walls and debris were noted. Also identified the destructive action of thymol and carvacrol by measuring the release of the constituents of

Cryptosporidium oocysts absorbing at 273 nm (UV). At the concentration of 0.5 mg mL^{-1}, the number of oocysts was reduced with an important increase in absorbing material at 273 nm (UV) (Tanghort et al., 2019).

Treatment of biofilms is done during the disinfection programs of poultry production houses, in the absence of animals because of the toxicity of used products. However, thymol is an ideal solution, which can be used in the presence of animals. Based on previous in vivo studies conducted by our laboratory, both doses (1 and 2 g L^{-1}) are tolerated by animals and have no acute or chronic toxicity. In addition to exerting a disinfectant effect on the drinking water and the watering circuit, thymol plays a growth promotor role for animals (Sennouni et al., 2018; Hriouech et al., 2020).

To our knowledge, this is the first study focusing on the treatment of parasitic biofilms by a major compound of essential oils. Previous studies are mainly concerned with the treatment of bacterial and fungal biofilms (Didehdar et al., 2022; Miranda et al., 2022).

Conclusion

Results of these experiments lead to the conclusion that thymol may prevent protozoa adhesion and proliferation in drinking water systems and eliminate biofilms already formed. It can be used in poultry farming to disinfect water distribution systems, as well as due to play a growth promotor role for animals, by regulating their gut flora as has been demonstrated in previous studies.

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