ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2019.187.194

Research Article

A Comparative Study of the Anticlostridial Activity of Selected Essential Oils, Their Major Components and a Natural Product with Antibiotics

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Abstract

Background and Objective: The aim of this study was to evaluate the anticlostridial effect of natural promoter-volaille (NPV), selected essential oils (EOs) and their major components (MCs) as well as certain antibiotics on *Clostridium perfringens* strains responsible for necrotic enteritis in poultry. **Materials and Methods:** Anticlostridial activity was measured by determining the inhibition of bacterial growth. The microbroth dilution method was used to determine the minimal inhibitory concentration (MIC) for each compound. The minimum bactericidal concentration (MBC) value and the kinetics of action of the best products were tested. **Results:** Among the six essential oils tested, the oregano, thyme and clove essential oils were identified as being the most effective. The major components of these essential oils, namely, thymol, carvacrol and eugenol, showed the strongest bacterial activity at very low concentrations. NPV, a product made from the major components of essential oils, showed a high anticlostridial activity. For the eight antibiotics tested, enramycin showed the highest anticlostridial effect. A comparison of the bactericidal effect of the time course of enramycin and NPV demonstrated a high bactericidal activity. **Conclusion:** From these results it can be concluded that NPV could be an efficient natural alternative to antibiotics to fight and prevent some clostridial diseases in broiler chicken, since it has a destructive effect within only a few hours and at very low concentrations. Moreover, it reduces the risk of antimicrobial resistance associated with the overuse of antibiotics in the poultry industry.

Key words: Antimicrobial agents, *Clostridium perfringens*, essential oils, natural product, necrotic enteritis.

Received: January 10, 2019 Accepted: February 19, 2019 Published: March 15, 2019

Citation: A. Mzabi, M. Tanghort, H. Chefchaou, H. Moussa, N. Chami, F. Chami and A. Remmal, 2019. A comparative study of the anticlostridial activity of selected essential oils, their major components and a natural product with antibiotics. Int. J. Poult. Sci., 18: 187-194.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Necrotic enteritis (NE) is considered to be one of the most common infectious diseases for poultry. This disease affects the poultry industry worldwide and costs approximately six billion US dollars per year[1,2]. It is usually caused by *Clostridium perfringens (C. perfringens*), a gram-positive anaerobic spore-forming bacterium[3,4]. *Clostridium perfringens* is ubiquitous in soil and in the gastrointestinal tract of animals and humans[5]. It produces several extracellular toxins and enzymes that are responsible for serious diseases in animals, such as enterocolitis in horses and gangrenous dermatitis in poultry[6-9]. Professionals in the sector systematically use antibiotics in the feed to prevent avian necrotic enteritis. However, the use of low-dose antibiotics in poultry feed may increase the risk of developing some cross-resistance to other antibiotics[10]. Such use induces the selection of multidrug-resistant bacteria that complicates antibiotic therapy in sick animals and humans in the case of contamination[11,12]. For this reason, the European Union and other countries have banned the use of antibiotics in bird feed[13,14]. Following this ban, necrotic enteritis caused by *C. perfringens* has since re-emerged in broilers[15-17], hence the need to find alternative strategies to prevent and control the incidence and severity of this disease[18]. With increasing awareness of *C. perfringens*, efforts are being made to continue to develop new potent drugs that do not harm the environment and the health of the consumer. Attention is now being focused on plant extracts used in traditional medicine as the sources for new treatments[17-20].

The objective of this study was to evaluate the *in vitro* anticlostridial activity of six essential oils (EOs) and their major components and to compare the effect of NPV (a product containing some major components of EOs) with eight synthetic antibiotics against *C. perfringens*.

MATERIALS AND METHODS

Bacterial culture: *Clostridium perfringens* isolates were obtained from the intestine of diseased animals with clinical signs of necrotic enteritis. Stock cultures of *C. perfringens* isolates were prepared in brain and heart infusion broth (HIB, Oxoid Ltd., Basingstoke, UK) and were stored at -20°C. Prior to their use in experiments, bacteria were initially cultured overnight at 37°C in TSC agar (tryptose-sulfite-cycloserine agar, Biokar). A fresh culture of the different isolated strains was prepared to test their resistance to antibiotics and their virulence on one-day-old chicks (results not shown). The most virulent and resistant strain was retained for this study. One colony of the selected strain grew overnight at 37°C under anaerobic conditions in a brain and heart infusion broth (HIB). The inoculum size used was 10^6 CFU mL^{-1}. The bacterial growth was evaluated by the absorbance at 600 nm.

Effect of EOs and their major components on the growth of *C. perfringens*: All essential oils used in this study were purchased from Seema International. The essential oils used in the screening, as well as their major components, are summarized in Table 1. The eight major components of the essential oils used were isopulegol, carvacrol, carvone, eugenol, cineol, β-ionone, cinnamaldehyde and thymol. The components used in this study were purchased from Sigma-Aldrich (France).

All of the essential oils and their MCs were dispersed in a liquid medium containing 0.2% agar in pure water. This dispersion method was improved by Remmal *et al.*[21]. The anticlostridial activity of each essential oils or major components was determined using a microbroth dilution assay. Sterile 96-well microplates were used (Iwaki microplate, Asahi Techno Glass, Japan) and a doubling dilution of each antimicrobial agent was performed according to the method described by Elizondo *et al.*[22]. This dilution procedure resulted in a gradient of each tested agent concentration from 0.062-2 mg mL^{-1} across the plate. An overnight culture of bacteria growth in the HIB was inoculated in each well of the plate (10 µL per well). Then, the microplates were incubated in an anaerobic jar at 37°C overnight. Bacterial growth was determined by measuring the optical density at 600 nm.

The anticlostridial activity of the Eos and their MCs was determined in duplicate and expressed as the percentage of bacterial growth inhibition according to the following equation[23]:

Table 1: Essential oils used in the screening and their major compounds

Botanical names	Major components
Clove (*Eugenia caryophyllata*)	Eugenol: 72.9%, eugenyl acetate, 5.8%
Oregano (*Origanum compactum*)	Carvacrol: 30.5%, thymol: 27.5%, γ-terpinene: 18.2%
Thyme (*Thymus vulgaris*)	Thymol: 36.6%, p-cymene: 16.5%
Artemisia (*Artemisia absinthium*)	β-thujone: 64%, 1-8 cineole: 18%, p-cymene: 9,6%, sabinene: 7,8%
Rosemary (*Rosmarinus officinalis*)	1-8 cineole: 51.3%, alpha-pinene: 10%, camphor: 10.6%, bornyl acetate: 1.5%
Tea tree (*Melaleuca alternifolia*)	Terpinen-4-ol: 40%, γ-terpinene: 21.4%

$$I(\%) = \frac{OD_c - OD_i}{OD_c} \times 100$$

where, OD_c and OD_i represent the optical density of bacterial growth in the control and in treated wells, respectively.

Effect of antibacterial agents on the inhibition of bacterial growth: The action of five antibiotics, namely, avilamycin, flavomycin, enramycin, bacitracin methylene disalicylate (BMD) and florfenicol and three anticoccidials, namely, monensin, salinomycin and robenidine, purchased from Sigma Aldrich, was tested using a previously described method with EOs at the following concentrations: 0, 0.062, 0.125, 0.25, 0.5 and 1 mg mL^{-1}. These concentrations were prepared from 10 mg mL^{-1} of each antibacterial agent and were solubilized in HIB and filtered through 0.22 µm filters (Millipore, Bedford, MA).

Natural promoter-volaille (NPV) is a new product developed in our laboratory. It contains major compounds of EOs and excipients. Its anticlostridial activity was also tested by the broth method dilution.

Estimation of the cell constituent release after treatment with NPV or enramycin: The estimation of the cell constituent release was performed on washed bacteria. To prepare the washed bacteria, they were grown overnight at 37°C in 100 mL. The culture was harvested at 400 g for 25 min at 4°C. The supernatant was discarded and the cells were resuspended in PBS (phosphate-buffered saline; containing 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.13 g L^{-1} Na_2HPO_4, $2H_2O$ and 0.2 g L^{-1} KH_2PO_4). This operation was repeated twice. The washed bacterial suspension was adjusted to approximately 10^6 CFU mL^{-1}. The release at 260 nm of absorbing bacterial constituents was examined after the treatment of the bacteria as follows:

- Bacteria in PBS (control)
- Bacteria in PBS containing a variable concentration of NPV (ranging from 0.062-2 mg mL^{-1})
- Bacteria in PBS containing a variable concentration of enramycin (ranging from 0.062-2 mg mL^{-1})

According to the method described by Rhayour *et al.*[24], 100 mL of washed bacteria suspension was shaken and incubated with increasing concentrations of antibacterial agents for four hours at 37°C. Then, two 1.5 mL samples were removed and centrifuged at 12000×g for 5 min at 4°C. One milliliter of supernatant was decanted, diluted with PBS and used to measure UV absorption by spectrophotometer at 260 nm. A correction was made for the absorption of the suspending liquids containing the same concentration of NPV or enramycin centrifuged after 2 min of contact with the bacteria. Each test was repeated three times under the same conditions. The number of viable bacteria was determined on TSC agar plates using the drop count method described by Adams and Mead[25].

Test with variable duration of treatment: The concentration that demonstrated a maximum absorbing material release at 260 nm in the previous test was 0.25 mg mL^{-1} for NPV and 1 mg mL^{-1} for enramycin. These two concentrations were used to perform a time course test. To this effect, 100 mL of washed bacterial suspension (10^{10} CFU mL^{-1}) treated with NPV or enramycin was shaken and incubated for an increased duration ranging from 0-480 min at 37°C. Then, three samples of one milliliter were removed, diluted with PBS and used to perform a count of viable bacteria after different times of treatment. For the control, no agents were added.

Statistical analysis: The results were expressed as mean values±S.E.M (standard error of mean). Statistical analysis of the data was performed with a one way analysis of variance followed by Tukey's Multiple Comparison Test (ANOVA followed by Tukey's test) (Graph Pad Prism, version 5.03). Differences of $p<0.05$ were considered statistically significant.

RESULTS

Inhibition of *C. perfringens* growth by essential oils and their major compounds: The growth inhibition percentage (%ICM) results of the selected isolate in the presence of various EOs or MCs are presented in Table 2 and 3. The growth inhibition percentage varied with EOs and MCs and increased with the increasing concentrations used. For essential oils, oregano and clove showed an MIC value (100%) of 1 mg mL^{-1}. The clove EO was the most active, with an inhibition percentage of 58.39 and 95.38% at the concentrations of 0.25 and 0.5 mg mL^{-1}, respectively. The oregano EO was less active with a bacterial growth inhibition of 47.98 and 80.64% at the respective 0.25 and 0.5 mg mL^{-1} concentrations. The thyme EO completely inhibited the growth of the strain at a concentration of 2 mg mL^{-1}. The EOs of artemisia, rosemary and tea tree appeared to be significantly less active, with MIC values higher than 2 mg mL^{-1}.

Table 2: Growth inhibition percentage of *Clostridium perfringens* isolate (%I) after treatment with EOs

	Bacterial growth inhibition percentages (%)					
Essential oils	0.062 mg mL^{-1}	0.125 mg mL^{-1}	0.25 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}	2 mg mL^{-1}
Clove	20.81±0.11^{d}	33.42±0.25^{e}	58.39±0.45^{e}	95.38±0.34^{e}	100.00±0.00^{e}	100.00±0.00^{d}
Oregano	20.23±0.18^{cd}	28.04±0.43^{d}	47.98±0.01^{d}	80.64±0.21^{d}	100.00±0.00^{e}	100.00±0.00^{d}
Thyme	18.44±0.49^{c}	25.15±0.48^{c}	31.58±0.23^{a}	44.80±0.37^{c}	79.48±0.02^{d}	100.00±0.00^{d}
Artemisia	6.10±0.22^{a}	21.39±0.42^{b}	28.33±0.16^{c}	32.09±0.62^{a}	45.96±0.33^{c}	70.81±0.41^{c}
Rosemary	0.43±0.91^{b}	16.95±0.43^{a}	17.06±0.01^{b}	21.10±0.25^{b}	39.02±0.15^{b}	63.88±0.25^{b}
Tea tree	5.79±0.32^{a}	17.14±0.52^{a}	30.93±0. 48^{a}	33.40±0. 56^{a}	34.69±0.42^{a}	44.22±0.34^{a}

The values presented are the average of three repetitions (n = 3)±SD. The values followed by different letters are significantly different from each other at p<0.05

Table 3: Growth inhibition percentage of *Clostridium perfringens* isolate (%I) after treatment with MCs

	Bacterial growth inhibition percentages (%)					
Major compounds	0.062 mg mL^{-1}	0.125 mg mL^{-1}	0.25 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}	2 mg mL^{-1}
Thymol	73.27±0.11^{g}	93.82±0.01^{g}	100.00±0.00^{f}	100.00±0.00^{e}	100.00±0.00^{e}	100.00±0.00^{c}
Carvacrol	46.65±0.01^{f}	86.35±0.02^{f}	100.00±0.00^{f}	100.00±0.00^{e}	100.00±0.00^{e}	100.00±0.00^{c}
Eugenol	26.28±0.35^{e}	50.25±0.02^{e}	72.17±0.01^{e}	100.00±0.00^{e}	100.00±0.00^{e}	100.00±0.00^{c}
Cinnamaldehyde	31.70±0.36^{d}	55.67±0.01^{d}	63.91±0.02^{d}	85.05±0.02^{d}	91.75±0.01^{d}	100.00±0.00^{c}
Isopulegol	30.67±0.29^{d}	54.13±0.02^{c}	57.98±0.11^{c}	64.17±0.01^{c}	64.94±0.01^{c}	80.05±0.13^{b}
β-Ionone	35.82±0.31^{c}	53.78±0.41^{c}	61.13±0.20^{b}	75.00±0.01^{b}	76.03±0.01^{b}	79.86±0.25^{b}
Carvone	18.55±0.07^{b}	35.05±0.08^{b}	50.07±0.21^{a}	56.18±0.35^{a}	58.84±0.22^{a}	64.11±0.26^{a}
Cineol	24.19±0.12^{a}	42.78±0.04^{a}	49.93±0.33^{a}	55.71±0.41^{a}	59.15±0.53^{a}	62.60±0.83^{a}

The values presented are the average of three repetitions (n = 3)±SD. The values followed by different letters are significantly different from each other at p<0.05

Table 4: Growth inhibition percentage of *Clostridium perfringens* isolate (%I) after treatment with antimicrobial agents

	Bacterial growth inhibition percentages (%)				
Antimicrobial agents	0.062 mg mL^{-1}	0.125 mg mL^{-1}	0.25 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}
NPV	73.96±0.09^{g}	97.57±0.00^{g}	100.00±0.00^{g}	100.00±0.00^{f}	100.00±0.00^{g}
Enramycin	27.84±0.23^{f}	69.75±0.42^{f}	91.34±0.06^{f}	100.00±0.00^{f}	100.00±0.00^{g}
Florfenicol	33.72±0.15^{e}	54.80±0.17^{e}	83.44±0.95^{e}	100.00±0.00^{f}	100.00±0.00^{g}
BMD	11.89±0.12^{a}	28.93±0.57^{d}	37.42±0.43^{d}	59.83±0.12^{e}	67.48±0.01^{f}
Flavomycin	15.65±0.22^{d}	29.53±0.31^{d}	40.92±0.83^{a}	61.23±0.48^{e}	73.66±0.17^{e}
Avilamycin	4.98±0.48^{c}	14.23±0.85^{c}	24.55±0.20^{c}	33.45±0.17^{d}	43.41±0.22^{d}
Monensin	6.91±0.02^{b}	20.24±0.38^{b}	42.02±0.71^{a}	65.42±0.38^{c}	78.19±0.63^{c}
Robenidine	11.70±0.4^{a}	19.78±0.29^{b}	28.72±0.22^{b}	38.82±0.67^{b}	55.31±0.02^{b}
Salinomycin	10.10±0.62^{a}	32.97±0.11^{a}	38.29±0.93^{a}	44.14±0.45^{a}	47.87±0.01^{a}

The values presented are the average of three repetitions (n = 3)±SD. The values followed by different letters are significantly different from each other at p<0.05

For the major compounds, the results showed that thymol and carvacrol have a minimal inhibitory concentration (MIC) of 0.25 mg mL^{-1}. Thymol appears to be the most active antibacterial agent on the studied *C. perfringens* isolate. Indeed, at a concentration of 0.062 and 0.125 mg mL^{-1}, the bacterial growth was inhibited at the respective percentages of 73.27 and 93.82%. This inhibition was significantly lower in the presence of the same concentrations of carvacrol (46.65% for 0.062 mg mL^{-1} and 86.35% for 0.125 mg mL^{-1}). Eugenol completely inhibited growth at a concentration of 0.5 mg mL^{-1}. Cinnamaldehyde had anticlostridial activity with an MIC of 2 mg mL^{-1}. Isopulegol, β-ionone, carvone and cineol were the least active major compounds on the strain used.

Growth inhibition of *Clostridium perfringens* by different antimicrobial agents: The growth inhibition percentage of *C. perfringens* in the presence of the different concentrations of antimicrobial agents tested is summarized in Table 4. The results show that NPV had the lowest MIC (0.25 mg mL^{-1}) since it had a high inhibition percentage of 97.57%, even at half of the MIC (0.125 mg mL^{-1}). The MICs of enramycin and florfenicol were 0.5 mg mL^{-1}. The other tested antibiotics showed higher MIC values. Regarding the anticoccidial drugs used for this study, monensin proved to be the most effective, with a percentage of 78.19% at the concentration of 1 mg mL^{-1}. Robenidine and salinomycin were less active, with respective inhibitions of 47.87 and 55.31% at the same concentration. The results in Table 4 show that NPV and enramycin were the most active anticlostridial agents on the studied isolate in comparison with all of the tested antimicrobial agents. This justifies the choice of these two drugs in the rest of this study.

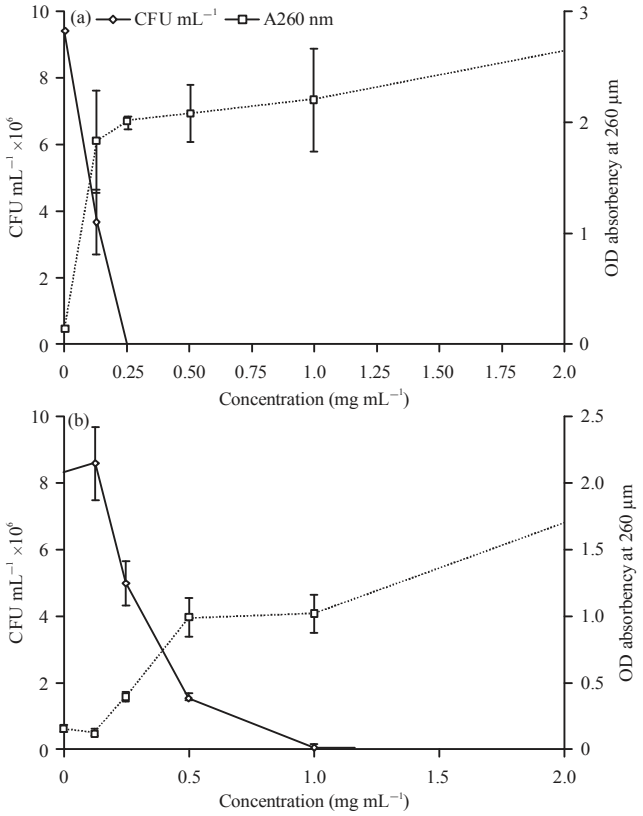

Fig. 1(a-b): The effect of (a) NPV or (b) Enramycin concentration on cellular mortality and the release of 260 nm absorbing material from *Clostridium perfringens*

Effect of NPV and enramycin on the release of 260 nm absorbing material and their time course: The anticlostridial activity of NPV and enramycin at different concentrations is reported in Fig. 1. Overall, the results show that the release of the absorbent cellular content at 260 nm increases progressively as a function of concentration. This release was accompanied by an increase in the mortality rate. However, the bactericidal effect of NPV was more pronounced than that of enramycin. NPV caused a rapid release at 260 nm of absorbing material at a low concentration of 0.062 mg mL^{-1} with total cell destruction at a concentration of 0.25 mg mL^{-1}. The 260 nm release absorbing material remained in a stationary phase for the higher concentrations.

The results obtained with enramycin at increasing concentrations on the release of 260 nm absorbing material show complete cell destruction with 1 mg mL^{-1} concentrations (a concentration twice as large as its MIC = 0.5 mg mL^{-1}). Moreover, the maximal release of material absorbing at 260 nm by *C. perfringens* treated with NPV was more substantial than that obtained after treatment with enramycin.

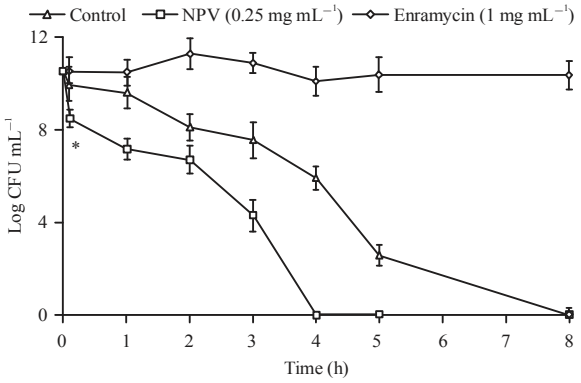

Fig. 2: Time course of *Clostridium perfringens* cell viability after treatment with NPV or enramycin

Figure 2 shows the time course of NPV and enramycin after treatment with an inoculums of 10^{10} CFU mL^{-1} of *C. perfringens* with bactericidal doses. The results show that treatment with NPV at a bactericidal concentration of 0.25 mg mL^{-1} dropped the CFU number rapidly and significantly from 10^{10} to 10^{8} CFU mL^{-1} (2 factor unit of reduction = 99%) in the first 10 min. Then, NPV progressively destroyed the cells during the following 4 h. The treatment with enramycin caused no significant decrease in the first 10 min compared to the control and required 2 h for a reduction of two log factors. Enramycin exerts a total destruction of cells only after eight hours of treatment.

DISCUSSION

Results of the current study showed that oregano and clove EOs have MICs of 1 mg mL^{-1}, while all the other EOs have MICs equal to or greater than 2 mg mL^{-1}. Other studies have shown the anticlostridial activity of various essential oils *in vitro*. Timbermont *et al.*[17] reported that the eucalyptus EO completely inhibited the growth of *C. perfringens* at a concentration of 5.33 mg mL^{-1}. Another *in vitro* study of *C. perfringens* conducted by Radaelli *et al.*[20] showed that the rosemary and peppermint Eos have an MIC of 10 mg mL^{-1} and that the oregano and basil EOs have an MIC of 5 mg mL^{-1}. The MICs obtained in our study are lower than those reported in the studies mentioned above. This could be explained by the fact that the dispersion of EOs using dimethyl sulfoxide (DMSO) or Tween 80 could reduce their antimicrobial activity. Indeed, our laboratory has previously demonstrated that detergents such as Triton-X100 and Tween 80 or solvents such as ethanol decrease the antimicrobial effect of EOs or MCs[21]. The use of agar at 0.2%

as a dispersing agent in this study explains the lower MICs obtained. To optimize the action of the EOs tested, we decided to study the anti clostridial action of their phenolic MCs (thymol, carvacrol and eugenol) and non phenolic MCs (cinnamaldehyde, isopulegol, β-ionone, carvone and cineol). Our data show that EOs are less effective than their MCs as anti clostridial agents. Indeed, we obtained the lowest MICs for thymol and carvacrol (0.25 mg mL^{-1}). The partial inhibition percentages for thymol, for example, were 73 and 93% at 0.06 and 0.125 mg $mL^{-1,}$ respectively. The anti clostridial effect of thymol and carvacrol has already been demonstrated by Timbermont *et al.*[17], who reported that 0.24 mg mL^{-1} of thymol and 0.33 mg mL^{-1} of carvacrol completely inhibited the growth of *C. perfringens*. Another study published by Du *et al.*[2] has shown that thymol and carvacrol have an MIC of 0.375 mg mL^{-1}.

For the antibiotics (ATBs) tested, the results obtained also show that only enramycin and florfenicol have an MIC less than 1 mg mL^{-1}, whereas all the other ATBs have an MIC value greater than 2 mg mL^{-1}. The strong antibacterial effect of enramycin has been documented, especially against *C. perfringens*. Redondo *et al.*[26] showed that among seven antimicrobial agents commonly used in poultry commercial farms as growth promoters, enramycin is the most effective against *C. perfringens* with MICs lower than 8 mg L^{-1}. Moreover, Gharaibeh *et al.*[27] also showed the superiority of florfenicol against *C. perfringens* with an MIC_{50} of 8 mg L^{-1} and the lowest MIC_{90} (32 mg L^{-1}) among thirteen antimicrobials tested. The differences in MIC values in this study can be explained by the sensitivity of the isolates to the agents tested. Furthermore, in this study, we chose the most resistant strain of *C. perfringens* to antimicrobial agents.

All these results show that pure phenolic MCs have the most important anticlostridial efficacy at doses of at least four times lower than those of the oregano and clove EOs and two times lower than those of enramycin and florfenicol. The MCs of EOs should represent an alternative to ATBs for preventing or treating necrotic enteritis caused by *C. perfringens*. On the basis of all these results, our laboratory has developed a patented composition named NPV. The effectiveness of this composition was compared with that of enramycin, which showed the best anticlostridial activity among the ATBs tested. Indeed, NPV has an MIC of 0.25 mg mL^{-1} with a growth inhibition of 97.5% at half concentration (0.125 mg mL^{-1}). These values are much lower than those of enramycin (MIC is 0.5 mg mL^{-1} with a growth inhibition of less than 70% at 0.125 mg mL^{-1}). Based on these results, it can be said that the dose response of NPV is more effective than that of enramycin on the *C. perfringens* isolate used in this study.

In an attempt to explain the cause of cell mortality, we evaluated the lysis of *C. perfringens* cells treated with a range of concentrations of NPV or enramycin by measuring the release of cellular substance absorbing material at 260 nm. The results obtained led us to suggest that the mortality was a consequence of cell lysis for the two tested products. NPV causes 100% mortality of *C. perfringens* at a concentration of 0.25 mg mL^{-1}, while enramycin requires a concentration four times higher to reach 100% mortality. This once again confirms the superiority of NPV to enramycin. The NPV-treated bacteria released a large amount of material absorbing at 260 nm (on the order of 2) from the 0.25 mg mL^{-1} concentration, while enramycin only allowed the release of half of this amount (OD = 1) at a concentration of 1 mg mL^{-1}. In terms of kinetics, 10 minutes of treatment with NPV was sufficient to produce a significant reduction by 2 CFU log units (99%), whereas the same result was obtained with enramycin only after 2 h. Similar results were reported by Matsumoto *et al.*[28], who indicated that enramycin releases a large amount of material absorbing at 260 nm in two hours with an important reduction of viable cells at a concentration higher than 0.78 mg L^{-1}.

CONCLUSION

In conclusion, we consider NPV to be a very good alternative to anticlostridial antibiotics that can decrease the potential cross-resistance to other antibiotics and consequently minimize the risk of spreading antibiotic-resistant bacteria. *In vivo* tests on chicks experimentally infected with *C. perfringens* AM18 and treated with NPV or enramycin showed the superiority of NPV in zootechnical performance (mortality, body weight, bodyweight gain and consumption index) and showed good antibacterial activity against aerobic bacteria (*E. coli* and *Salmonella* sp.) compared with enramycin (results not shown).

SIGNIFICANCE STATEMENT

This study discovered that essential oils (EOs) and their major components (MCs) showed different minimal inhibitory concentration (MIC) values against *C. perfringens*. Phenolic MCs are more effective than EOs as anticlostridial agents. Even

the low concentrations of phenolic MCs inhibited clostridial growth. This natural product can be an efficient alternative to antibiotics and protect poultry against clostridial disease. Moreover, using phenolic MCs can be beneficial by minimizing the risk of antibiotic resistance in livestock due to the misuse of antibiotics in animal feed and can help protect the consumer and the environment.

ACKNOWLEDGMENTS

This work is a partial fulfillment of Aouatef Mzabi's PhD thesis. The authors are deeply grateful to Miss Imane Remmal and Mrs Hélène Mock for their assistance in checking the English of the manuscript.

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