دانشگاه شیراز
Shiraz University

IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 26 No. 4 Ser. No. 93 2025

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

In vitro evaluation of antibacterial and antifungal effects of selected acidifiers and antibacterial effects of essential oils used in poultry feed industry

Omidi, A.[1*]; Rahimi-Kakolaki, M.[2] and Mosleh, H.[3]

[1]Department of Animal Health Management, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Graduated from School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Ph.D. Student in Feed Hygiene, Department of Animal Health Management, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *A. Omidi, Department of Animal Health Management, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: aomidi@shirazu.ac.ir*

10.22099/ijvr.2025.50380.7435

(Received 10 Jul 2024; revised version 11 Aug 2025; accepted 24 Sept 2025)

Abstract

Background: Essential oils and organic acidifiers are used as natural antimicrobial substitutes for chemical additives in poultry feed. Pathogens such as *Salmonella* and *Escherichia coli* pose significant risks. While essential oils possess strong antibacterial and antifungal properties, commercial acidifiers (blends of formic, propionic, and lactic acids) are also commonly applied to reduce microbial contamination in feed. However, comparative data on their efficacy against key poultry pathogens remain limited. **Aims:** The present study investigates the antibacterial and antifungal effects of selected commonly used commercial acidifiers in poultry feed and also compares them with antibacterial effects of essential oils. **Methods:** Antibacterial effects against *Salmonella typhimurium* and *E. coli* were evaluated for both five commercial acidifiers and essential oils (cinnamon, thyme, clove). Antifungal effects against *Fusarium oxysporum* were evaluated for acidifiers only. Microplate and agar-well diffusion methods were used. Acidifiers were tested at 1×, 2×, and 5× the manufacturer's recommended dose; essential oils at 2% and 5% (v/v). Sampling time points for feed pH and microbial counts were days 0, 7, and 21. All tests were performed in triplicate and repeated twice. The impact of acidifiers on feed pH was also evaluated. **Results:** Acidifiers exhibited negligible inhibitory effects against target pathogens even at concentrations five times higher than recommended levels. In contrast, essential oils demonstrated significant inhibitory effects. Furthermore, the addition of acidifiers did not affect the pH of the feed. **Conclusions:** The study underscores the potent antibacterial properties of cinnamon, thyme, and clove essential oils against common poultry pathogens. Comparatively, commercial acidifiers showed limited efficacy. Incorporating essential oils into poultry feed can enhance animal health and safety as effective substitutes for chemical additives. Further research is needed to optimize their use and maximize their antimicrobial benefits.

Key words: Animal health, Feed additives, Feed safety, Pathogen control

Introduction

Livestock and poultry feed industry play a pivotal role in providing balanced and nutritious diets essential for the optimal growth, health, and overall performance of animals. In recent years, efforts for enhancing hygiene requirements and increasing productivity in this sector have intensified. Growing concerns about the toxicity and adverse effects of chemical additives such as formaldehyde (causing respiratory irritation, dermal toxicity, and potential carcinogenicity upon prolonged exposure), have raised interests in finding safer and more sustainable alternatives. Acidifiers and plant essential oils are increasingly recognized as reliable and effective components in modern animal nutrition strategies (Stevanović *et al.*, 2018; Bernardini *et al.*, 2022; Waghmare *et al.*, 2025).

Acidifiers are used as effective antibacterial and antifungal agents in animal feed. Conversely, there is an increasing interest in utilizing plant essential oils as natural alternatives due to their wide-ranging antimicrobial properties and health advantages. These substances can satisfy the rising demand for natural and safe feed additives that work alongside conventional chemical entities (Mucha and Witkowska, 2021; Abd El-Hack *et al.*, 2022; Aguiar Campolina *et al.*, 2023; Sharafi *et al.*, 2023).

In addition to positive health effects, the use of acidifiers reduces dependence on costly antibiotics and synthetic growth promoters. This complies with the increasing trend toward sustainable and economical production in the livestock and poultry sectors. The inclusion of essential oils in animal diets has also contributed to increased feed consumption, enhanced growth, and lower production costs by improving gastrointestinal function and decreasing the feed conversion ratio. These factors are reliable economic indicators that support the replacement of synthetic growth promoters with essential oils and acidifiers (Polycarpo *et al*., 2017; Stevanović *et al*., 2018; Irawan *et al*., 2021; Nehme *et al*., 2021; Vinolya *et al*., 2021; Mincheva *et al*., 2025; Waghmare *et al*., 2025).

Organic acidifiers, particularly their salts are used in livestock and poultry nutrition for promoting intestinal health and enhancing production efficiency. These compounds have diverse benefits, including modulation of the gut microbiota, suppression of pathogenic bacteria, and stimulation of host immune responses (Aliverdi-Nasab *et al*., 2023). Furthermore, acidifiers improve nutrient digestibility, enhance mineral bioavailability, and contribute to superior meat quality (Pearlin *et al*., 2020). Their functional efficacy depends on the chemical nature of the organic acid and its dissociation constant (pKa) (Tugnoli *et al*., 2020). In poultry production systems, acidifiers have demonstrated additional benefits such as increased egg yield, improved feed conversion efficiency, and maintenance of optimal gastrointestinal pH levels. The antimicrobial activity of acidifiers primarily derives from their capacity to lower gut pH, creating an unfavorable environment for pathogenic microorganisms and inhibiting their proliferation (Pearlin *et al*., 2020).

Given the importance of feed additives in enhancing poultry health and productivity, evaluating their effectiveness against major pathogens is essential. Key pathogens that threat poultry health include *Salmonella* spp. (e.g., *S. enteritidis*, *S. typhimurium*, *S. pullorum*, and *S. gallinarum*), which cause gastrointestinal salmonellosis, and *Escherichia coli* (*E. coli*), responsible for colibacillosis, respiratory infections, and septicemia, particularly under stress conditions (Shaji *et al*., 2023; Van *et al*., 2023).

The primary objective of this study is to investigate the *in vitro* antibacterial and antifungal properties of selected commercial acidifiers commonly employed in the livestock and poultry feed industry. Additionally, essential oils derived from cinnamon, thyme, and clove were also evaluated in this regard.

Materials and Methods

Commercial products

Five commercial acidifiers were used: Acidifier 1: cinnamon essential oil (*Cinnamomum cassia*), formic acid, propionic acid, acetic acid, and citric acid (Iran); Acidifier 2: formic acid and propionic acid (France); Acidifier 3: formic acid, propionic acid, phytochemical compounds, and copper sulfate (Iran); Acidifier 4: composition not disclosed by manufacturer (Netherlands); Acidifier 5: formic acid, propionic acid, benzoic acid, and diatomaceous earth (Germany). Due to confidentiality agreements, brand names are not disclosed. Three commercial essential oils were used: cinnamon (*Cinnamomum cassia*), thyme (*Thymus vulgaris*), and clove (*Syzygium aromaticum*). The herbarium number for thyme was DR06763; other plants were purchased from reputable stores and verified by a botanist.

Measurement of acidity (feed pH)

Feed pH was measured at 0, 24, and 48 h after addition of acidifiers (at manufacturer's recommended dose) using a calibrated digital pH meter (AOAC, 2005). Each measurement was performed in triplicate.

Microbial strains

Bacterial strains

Salmonella typhimurium ATCC-14028 and *Escherichia coli* ATCC-10698 were obtained from the microorganisms collection of the food microbiology laboratory, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. They were prepared and activated according to the provided instructions.

Fungal strain

Fusarium oxysporum PTCC-2112 was obtained from the Iranian Research Organisation for Science and Technology (IROST) and activated according to the provided instructions.

Feed experiments

Acidifiers only; essential oils were not tested in feed.

Antimicrobial effects in feed

Total bacterial count and mold count:

Acidifiers were added to poultry feed at the manufacturer's recommended dose. Feed samples were taken on days 7 and 21 (as shown in Tables 1 and 3).

Total bacterial count (CFU/g)

Determined using standard plate count agar.

Mold count (CFU/g)

Determined using potato dextrose agar.

Negative control

Feed without acidifier.

Positive control

Feed with formalin (37%). All tests were performed in triplicate and repeated twice.

Culture media experiments

Preparation of test compounds:

Acidifiers

Tested at 1× (manufacturer's recommended dose), 2×, and 5× that dose. Diluted in sterile distilled water and filtered (0.22 µm).

Essential oils (cinnamon, thyme, clove)

Tested at 2% and 5% (v/v) diluted in Tryptic Soy Broth (TSB) with 5% DMSO (final concentration). Essential oils were tested only for antibacterial activity (not antifungal).

Table 1: Total bacterial count (CFU/g; mean±SD) in feed at days 7 and 21

Sampling time (days)	Negative control (No acidifier)	Positive control (with formalin)	Acidifier 1	Acidifier 2	Acidifier 3	Acidifier 4	Acidifier 5
7	76.1±5.11 [c]	1.6±0.10 [a]	66.6±6.59 [b]	88.5±5.90 [d]	85.5±5.05 [d]	60.8±4.55 [b]	75.7±4.62 [c]
21	230.5±5.85 [e]	195.0±8.0 [d]	155.1±6.10 [b]	180.6±7.51 [c]	130.7±4.23 [a]	225.1±5.75 [e]	205.6±7.51 [d]

Negative control: no additive. Positive control: formalin (37%). Different superscript letters in each row indicate significant differences (P≤0.05). Dissimilar letters in each row indicate significant differences among groups (P≤0.05)

Table 2: pH values (mean±SD) of acidifier solutions (1:10 dilution in distilled water) and poultry feed after addition of each acidifier (manufacturer's recommended dose)

pH	Control	Acidifier 1	Acidifier 2	Acidifier 3	Acidifier 4	Acidifier 5
Acidifier solution	7.03±0.057 [a]	4.57±0.057 [b]	5.57±0.057 [b]	5.47±0.057 [b]	7.0±0.1 [a]	4.47±0.057 [b]
Feed after acidifier addition	6.17±0.057 [ns]	6.17±0.057 [ns]	6.13±0.057 [ns]	6.13±0.057 [ns]	6.13±0.057 [ns]	6.13±0.057 [ns]

Different superscript letters in the same row indicate significant differences (P≤0.05). ns= non-significant difference compared to control (P>0.05). The control value for acidifier solution (7.03±0.057) is the pH of distilled water (1:10 dilution, as per method)

Table 3: Mold count (CFU/g; mean±SD) in feed at days 7 and 21

Sampling time (days)	Negative control (no acidifier)	Positive control (with formalin)	Acidifier 1	Acidifier 2	Acidifier 3	Acidifier 4	Acidifier 5
7	8.6±1.40 [a]	10.0±0.05 [ab]	8.4±1.44 [b]	10.1±0.17 [a]	8.9±0.85 [ab]	9.9±0.23 [ab]	10.0±0.05 [a]
21	260.0±45.82 [c]	10.7±1.15 [a]	135.0±45.0 [b]	250.3±46.37 [c]	175.0±18.02 [b]	253.3±41.63 [c]	259.3±35.23 [c]

Negative control: no additive. Positive control: formalin (37%). Dissimilar letters in each row represent differences between groups (P≤0.05)

Antibacterial activity (for both acidifiers and essential oils)

Microplate method

96-well plates contained 100 µL double-strength TSB + 100 µL test compound + 10 µL bacterial suspension (10^8 CFU/ml); Incubation at 37°C for 24 h; optical density (OD_{600}) measured every hour (microplate reader, shaking 10s every 60min); Positive control: formalin (37%). Negative control: sterile distilled water. DMSO control for essential oils; All tests in triplicate, repeated twice.

Agar well diffusion method

Mueller-Hinton agar plates inoculated with 10^8 CFU/ml bacteria. Wells (5 mm diameter) filled with 50 µL test compound (acidifiers at 1×; essential oils at 2% and 5%); Incubation at 37°C for 24 h; inhibition zone diameters measured; Positive control: nalidixic acid (40 ppm). Negative control: sterile distilled water; All tests in triplicate, repeated twice.

Antifungal activity

For acidifiers only, essential oils were not tested.

Microplate method

Fusarium oxysporum spore suspension (2×10^6 spores/ml) prepared using sterile normal saline (NaCl 0.9%) on five-day-old Potato Dextrose Agar (PDA); 96-well plates contained double-strength Sabouraud Dextrose Broth (SDB) + acidifier (1×, 2×, 5×) + 10 µL spore suspension; Incubation at 25-28°C for 5-7 days; fungal growth assessed visually; Positive control: formalin (37%). Negative control: sterile distilled water; All tests in triplicate, repeated twice.

Agar well diffusion method

PDA plates inoculated with 2×10^6 spores/ml. Wells (5 mm diameter) filled with 50 µL acidifier (1× recommended concentration); Incubation at 25-28°C for 3 days; inhibition zone diameters measured; Positive control: formalin (37%). Negative control: sterile distilled water; All tests in triplicate, repeated twice.

Gas chromatography, mass spectrometry (GC-MS) analysis of essential oils

Chemical components were identified using an Agilent 7890B GC system coupled with a 5977A Series GC/MSD mass spectrometer (70 eV ionization energy, scan range 40-550 amu). A BPX5 capillary column (30 m × 0.25 mm; 0.25 µm film thickness) was used with temperature program: 60°C for 1 min, ramped at 5°C/min to 220°C, then at 3°C/min to 280°C, hold 3 min. Helium carrier gas at 1 ml/min, split ratio 30:1. Injection volume 0.2 µL of diluted samples (1:10 in hexane). Compound identification used the GC-MS library.

Statistical analysis

After checking normality, one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used (SPSS v16, P≤0.05). All data presented as mean±SD. Graphs prepared using Microsoft Office Excel 2016 and GraphPad Prism 8.

Results

The results, depicted in Figs. 1-5 and Tables 1-4, delineate the efficacy of the tested acidifiers (Figs. 1-3, Tables 1-4) and essential oils (Figs. 4-5).

Comparative assessment of acidifiers' anti-

microbial effects in feed and the pH

Among the acidifiers examined, acidifiers 1 and 4 exhibited significant antibacterial effects over a seven-day period, while acidifier 3, demonstrated delayed efficacy only on day 21 (Table 1).

Our study revealed variations in pH levels among the acidifiers. Acidifier 5 displayed the lowest pH (4.47±0.057), whereas acidifier 4 showed the highest (7.0±0.1). Surprisingly, the addition of acidifiers at recommended doses had no significant impact on feed pH (P>0.05) (Table 2).

Comparative evaluation of acidifiers' antimicrobial effects in culture medium

In the liquid medium, Acidifiers 1, 3, and 5 demonstrated inhibitory effects against *Salmonella typhimurium* that were comparable to those of the positive control (nalidixic acid), lasting for

Table 4: Inhibition zone diameters (mm; mean±SD) of acidifiers (agar well diffusion method, 1× concentration)

	Acidifier	*S. typhimurium*	*E. coli*	*F. oxysporum*
	1	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
	2	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
	3	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
	4	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
	5	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]
Positive control	Nalidixic acid (40 ppm) for bacteria	2.3±0.05 [b]	2.5±0.00 [b]	-
	Formalin (37%) for fungi (2%)	2.6±0.05 [b]	1.9±0.00 [b]	1.6±0.11 [b]

Positive controls: nalidixic acid (bacteria) and formalin (fungus). Different superscript letters in each column indicate significant differences (P≤0.05)

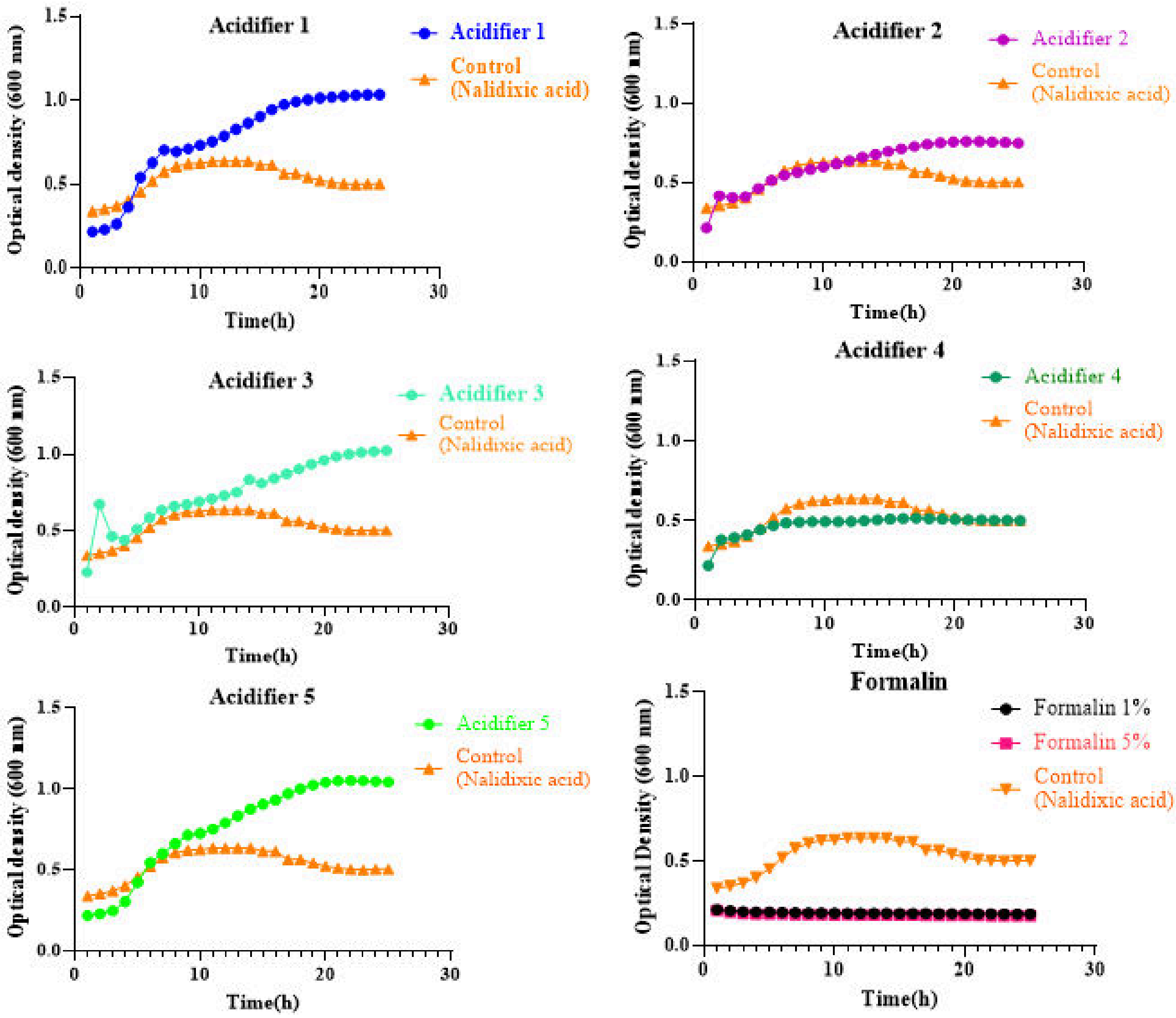

Fig. 1: Antibacterial activity of acidifiers (1×, 2×, 5×) against *S. typhimurium* (OD_{600}, 37°C, 24 h). Positive control: formalin. Negative control: distilled water. Triplicate, representative data

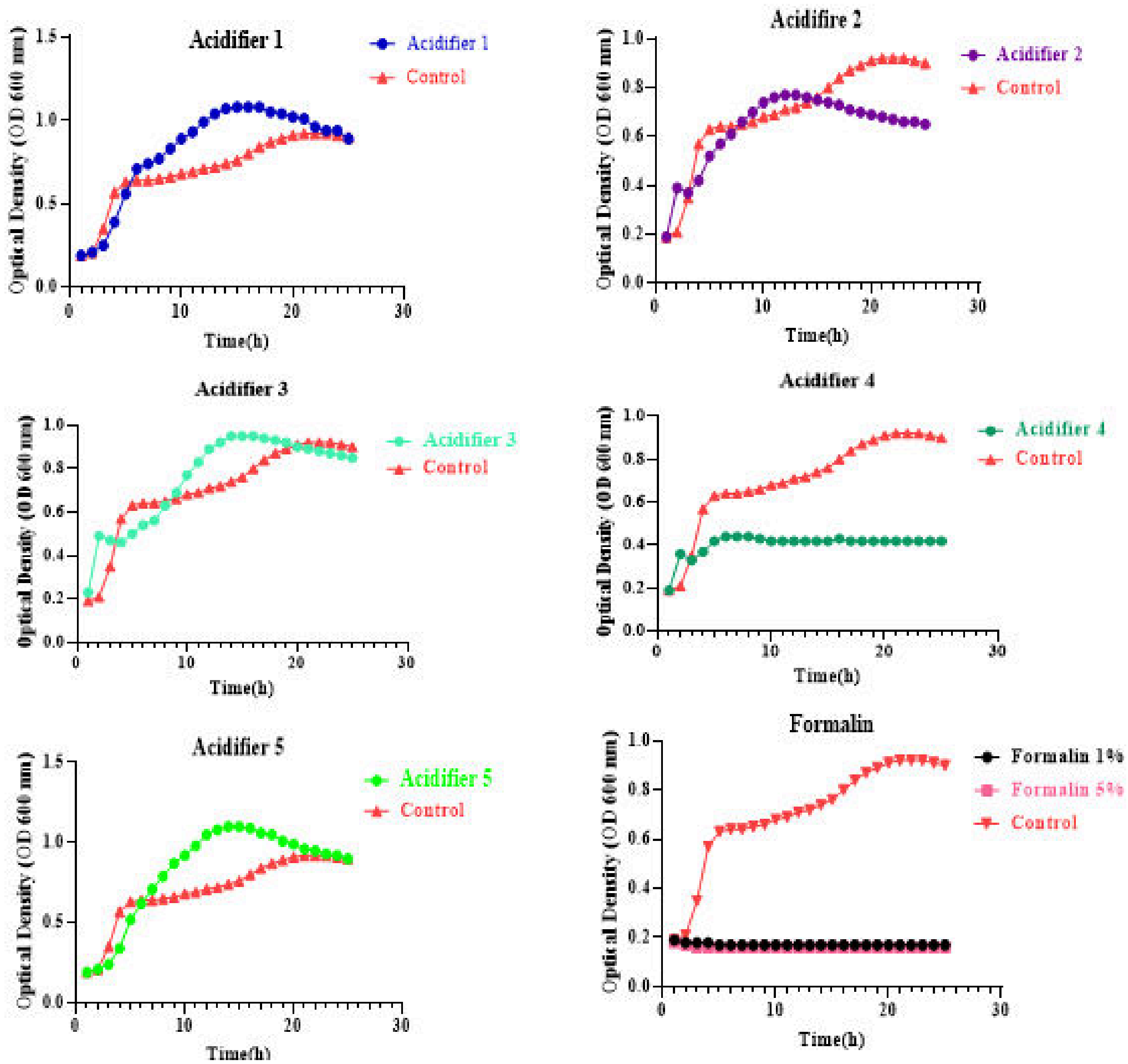

Fig. 2: Antibacterial activity of acidifiers (1×, 2×, 5×) against *E. coli* (OD_{600}, 37°C, 24 h). Positive control: formalin. Negative control: distilled water. Triplicate, representative data

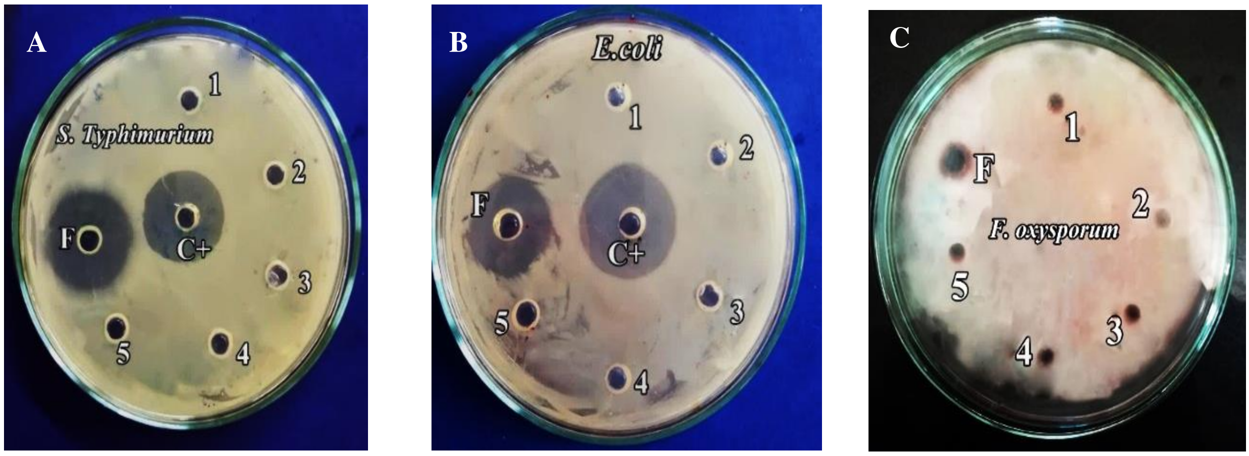

Fig. 3: Comparison of acidifiers' effects on: (**A**) *S. typhimurium*, (**B**) *E. coli* (10^6 CFU/ml), and (**C**) *F. oxysporum* (2×10^6 spores/ml) using agar-well diffusion method. No. 1-5 represent different acidifiers, 'F' denotes formalin, and 'C+' indicates the positive control

(Nalidixic acid)

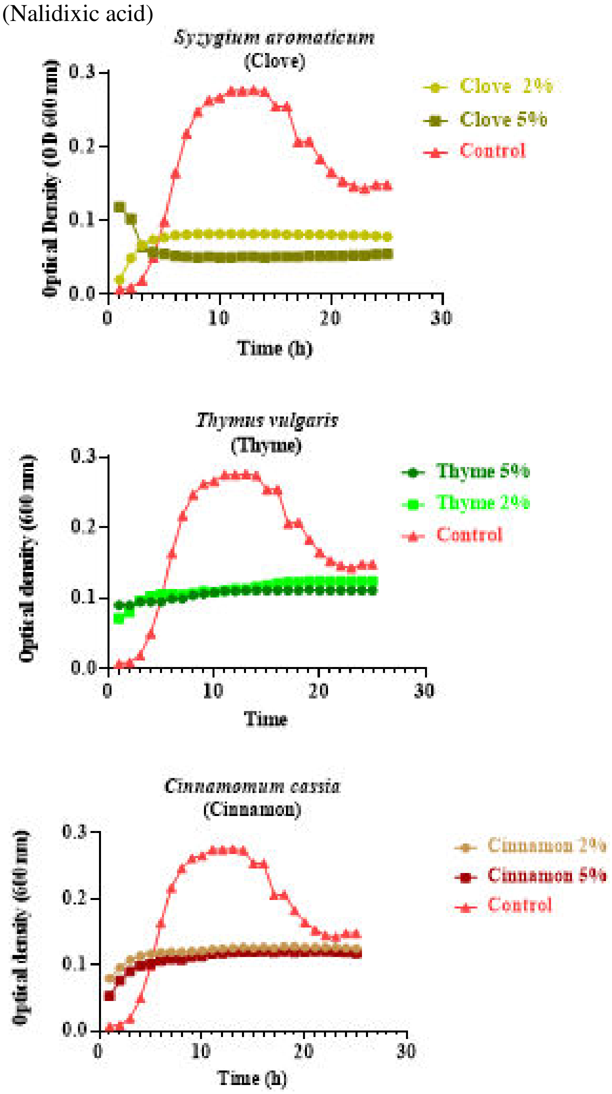

Fig. 4: Antibacterial activity of essential oils (cinnamon, thyme, clove; 2% and 5% v/v) against *S. typhimurium* (OD_{600}, 37°C, 24 h). Positive control: formalin. Negative control: DMSO. Triplicate, representative data. Higher initial OD at 5% due to oil color

approximately 10 h (Fig. 1). Acidifier 2 demonstrated slightly prolonged inhibition (up to 15 h), while acidifier 4 displayed pronounced inhibition throughout the 24 h period, even surpassing the positive control.

Figure 2 depicts the effects of acidifiers on *E. coli*, showing similar inhibitory trends across treatments compared to *S. typhimurium*.

Acidifiers 1, 2, 3, and 5 displayed diminished activity, while acidifier 4 exhibited sustained inhibition for 24 h.

Despite their antibacterial effects, none of the acidifiers demonstrated antifungal properties against *Fusarium oxysporum* in either the feed or the culture medium (Tables 3 and 4, Fig. 3C).

Table 4 and Fig. 3 provide a comprehensive comparison of the acidifiers' efficacy against *S. typhimurium*, *E. coli*, and *F. oxysporum* using the agar well diffusion method. Surprisingly, none of the acidifiers displayed inhibitory effects in this assay.

Comparative analysis with formaldehyde

Formaldehyde exhibited superior efficacy compared to the tested commercial acidifiers against the studied microorganisms (Figs. 1-3, Tables 3 and 4).

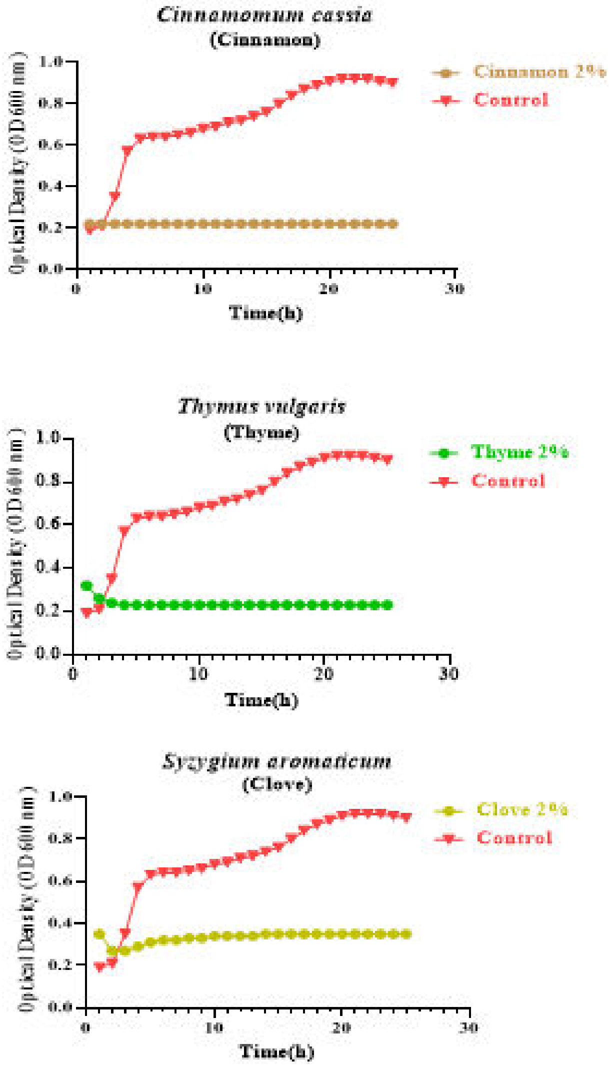

Fig. 5: Antibacterial activity of essential oils (cinnamon, thyme, clove; 2%, 5%) against *E. coli* (OD_{600}, 37°C, 24 h). Positive control: formalin. Negative control: DMSO. Triplicate

Comparative evaluation of natural compounds' antimicrobial effects

The essential oils from the three selected plants were evaluated at concentrations of 2% and 5%.

Both concentrations exhibited comparable, statistically similar antimicrobial effects. The higher optical density (OD) values observed during the initial hours of incubation for thyme and clove essential oils are likely due to their darker color, which is more intense at the 5% concentration than at 2% (Fig. 4). Compared to commercial acidifiers, all three essential oils demonstrated more stable and higher antimicrobial activity at the 2% concentration. Intriguingly, even when administered at five times the recommended dosage, acidifiers only displayed initial-hour inhibitory effects, followed by diminished efficacy and bacterial regrowth.

GC-MS analysis of essential oils

Relative amounts of components were calculated based on GC peak areas. *Cinnamomum cassia* (cinnamon) essential oil contained 32 identified components, with major constituents including 54.38% α-copaene, 8.96% α-muurolene, 6.95% naphthalene, 5.65% (1S, cis)-calamenene, and 4.54% 2-propenal, 3-phenyl (cinnamaldehyde). *Thymus vulgaris* (thyme) essential oil comprised 28 components, with significant concentrations of thymol (34.35%), carvacrol (26.18%), and benzene (13.40%). *Syzygium aromaticum* (clove) essential oil included 19 components, with m-Eugenol (51.04%), eugenyl acetate (23.91%), β-caryophyllene (11.39%), and thymol (3.28%) as major constituents.

Discussion

Acidification emerges as a promising strategy to minimize the use of antibiotics in both human and veterinary medicine (Mantzios *et al*., 2023). Organic acids in the diet, also referred to as acidifiers, have enjoyed widespread use globally for many years due to their antibacterial, antifungal, and antiviral properties (Pearlin *et al*., 2020; Loan *et al*., 2023). Our study sheds light on the efficacy of several commercially available organic acidifiers commonly used in poultry nutrition. Despite their widespread use and purported antimicrobial properties, our results did not demonstrate significant inhibitory effects against targeted pathogens. This outcome challenges the prevailing notion regarding the effectiveness of these acidifiers and underscores the need for a critical evaluation of their performance in real-world settings.

In our laboratory screening, we evaluated the efficacy of five commonly used commercial acidifiers in inhibiting *S. typhimurium*, *E. coli*, and *F. oxysporum*, significant pathogens in human health and poultry. The observed inhibitory effect of acidifiers on the growth of pathogenic bacteria is attributed to their ability to reduce the pH level in both the feed and the digestive system of the animal (Pearlin *et al*., 2020). This pH reduction creates an unfavorable environment for the proliferation of pH-sensitive pathogens like *E. coli* and *Salmonella* spp., while restraining microbial competition for host nutrients without endangering beneficial bacteria, thereby enhancing animal health (Pearlin *et al*., 2020; Aliverdi-Nasab *et al*., 2023; Loan *et al*., 2023).

However, the addition of acidifiers to feed at recommended doses had no effect on feed pH. This lack of change may be attributed to the type of organic acid, dosage, and feed formulation, highlighting the need for optimizing dosage (Nguyen *et al*., 2020; Tugnoli *et al*., 2020). The inhibitory effects observed in this study were not as significant and remarkable as those claimed in other studies for these additives (Vertiprakhov and Grozina, 2019; liverdi-Nasab *et al*., 2023). This limited effectiveness may be attributed to the absence of pH reduction in the feed and environment by the acidifiers under investigation.

It is important to acknowledge that the acidifiers tested in our study represent only a subset of the extensive array of commercial products available in the market. Given the wide range of acidifier formulations and the variability in their chemical compositions, it is conceivable that the effectiveness asserted by manufacturers may not be acheived in all cases (Mantzios *et al*., 2023). Additionally, factors such as the type of organic acid, dosage, administration method, and interaction with other feed components can significantly influence the performance of acidifiers in final formulations (Nguyen *et al*., 2020; Tugnoli *et al*., 2020). The lack of the antifungal effect of acidifiers in feed and culture medium which was observed in our study can also be attributed to these factors. Concerns have also been raised regarding the reactivity of acidifiers with metallic components in feed production facilities and neutralization by bicarbonate secretion in the gut, underscoring the need for non-reactive and targeted delivery of acidifiers for optimal performance (Vertiprakhov and Grozina, 2019; Pearlin *et al*., 2020).

In our study, we observed a difference in the antimicrobial effect of acidifiers between liquid and solid culture media, emphasizing the potential influence of the type of culture media on microbial growth behavior (Brown *et al*., 1980). The high efficacy of formaldehyde as observed in our study is consistent with existing research (Choroszy-Król *et al*., 2023; Omidi *et al*., 2024) and aligns with the findings of Gosling *et al*. (2021), who reported that products based on organic acids currently do not replicate the antibacterial effectiveness seen in formaldehyde-containing products. However, concerns about the specific hazards associated with formaldehyde underscore the necessity to explore safer alternatives, such as natural products.

Our findings highlight the significant antibacterial activity of essential oils as promising alternatives to formaldehyde in feed. Specifically, thyme, cinnamon, and clove essential oils, showed notable inhibitory effects against tested pathogens, indicating their potential as alternatives or complementary strategies for conventional acidifiers.

Initially, the OD values of the essential oil samples were higher than the control due to their darker color, especially at the 5% concentration. However, while the OD of the control gradually increased with bacterial growth, the OD of the essential oil samples remained almost constant, indicating a stable and lasting inhibitory effect of the oils on bacterial proliferation.The antibacterial effects of essential oils are primarily related to their ability to disrupt bacterial cell membranes and inhibit key enzymes (Falleh *et al*., 2025). Major active compounds such as thymol, menthol, and carvacrol (monoterpenes), as well as eugenol and cinnamaldehyde (phenylpropanoids), have shown broad-spectrum efficacy with low toxicity (Burt, 2004; Friedman, 2017). These compounds act through multiple mechanisms, and their activity is influenced by physicochemical properties such as lipophilicity (ability to dissolve in fats), partition coefficient (distribution between aqueous and lipid phases), and hydrogen bonding potential (Khwaza and Aderibigbe, 2025). Moreover, chemical modifications and derivative synthesis of these natural compounds have enhanced their antibacterial potency and broadened their spectrum of activity. Such derivatives often exhibit improved stability, solubility, and bioavailability, making them attractive candidates for future development of antibacterial additives (Friedman, 2017; Liu *et al*., 2021; Khwaza and Aderibigbe, 2025).

In this study, the chemical compositions of cinnamon, thyme, and clove essential oils were investigated. Cinnamon oil contained 32 components, with α-copaene (54.38%) and cinnamaldehyde (4.54%) as the major constituents. Alpha-copaene exhibits antimicrobial activity, particularly against Gram-positive bacteria, while cinnamaldehyde is known for its broad-spectrum effects against bacteria, fungi, and certain viruses (Gupta *et al*., 2008). Their coexistence supports the traditional use of cinnamon oil in infection control and food preservation.

Thyme essential oil comprised 28 compounds, with thymol (34.35%) and carvacrol (26.18%) as the major constituents. Both compounds have exhibited strong antimicrobial and antifungal effects. Thymol disrupts microbial membranes, while carvacrol interferes with cell metabolism and inhibits microbial growth (Burt, 2004).

Clove essential oil included 19 identified constituents, mainly m-eugenol (51.04%) and eugenyl acetate (23.91%). M-eugenol disrupts cell walls and inhibits biofilm formation, whereas β-caryophyllene (11.39%) enhances antimicrobial efficacy by increasing membrane permeability and facilitating antibiotic penetration (Chaieb *et al*., 2007).

The antimicrobial efficacy of cinnamon oil against *Salmonella typhimurium* and *E. coli* has been well documented (Yin *et al*., 2022; Casalino *et al*., 2023). Similar activities have been reported for thyme and clove essential oils (Selles *et al*., 2020; Eslami *et al*., 2022; Morshdy *et al*., 2022). These oils destabilize bacterial membranes, causing cell wall disruption, membrane detachment, and leakage of cytoplasmic contents, leading to cell death (Shen *et al*., 2015; Wydro *et al*., 2017; Andrade-Ochoa *et al*., 2021; Chen *et al*., 2022; Hąc- Yin *et al*., 2022). These effects are primarily attributed to key bioactive compounds such as eugenol, thymol, carvacrol, and cinnamaldehyde (Selles *et al*., 2020; Chen *et al*., 2022; Yin *et al*., 2022).

Essential oils are considered promising alternatives to conventional antibiotics in livestock production. Despite the short-term economic advantages of antibiotics, their long-term use contributes to the development of antimicrobial resistance which increases future treatment costs. Resistance typically develops faster than the discovery of new drugs, limiting therapeutic options and posing a public health risk (Pearlin *et al*., 2020); Evangelista *et al*., 2022). Apart from replacing antibiotic growth promoters, essential oils and their active components have shown synergistic effects when combined with antibiotics. For instance, cinnamaldehyde enhances the susceptibility of erythromycin-resistant *E. coli* and increases the efficacy of tetracycline and novobiocin (Visvalingam *et al*., 2017). Similarly, carvacrol has demonstrated synergism with erythromycin against resistant streptococci and has improved the activity of doxycycline against respiratory pathogens such as *Mannheimia haemolytica* and *Pasteurella multocida* (Magi *et al*., 2015; Kissels *et al*., 2017).

Moreover, supplementation with essential oils has been associated with improvements in animal performance, including increased feed intake and body weight, enhanced intestinal barrier function and nutrient digestibility, improved blood biochemistry, reduced coccidial infections, lower methane emissions in ruminants, and higher egg production in laying hens (Evangelista *et al*., 2022).

Innovative technologies such as nanoencapsulation and microencapsulation have significantly improved the stability, bioavailability, efficacy, and controlled release of active compounds in essential oils by reducing the impact of their volatility and sensitivity to environmental factors like temperature, light, and oxygen in animal feed (Turek and Stintzing, 2013; Stevanović *et al*., 2018; Movahedi *et al*., 2024). These innovations provide a promising path for expanding the industrial use of essential oils as natural, multifunctional additives in sustainable livestock production systems.

Additionally, this study investigated the optimal dosage of acidifiers. It was observed that even at five times the recommended dose, acidifiers only inhibited bacterial growth during the initial hours, after which proliferation resumed. Similar findings were reported by Mantzios *et al*. (2023), who noted that most tested products failed to effectively suppress selected microorganisms at manufacturer-recommended concentrations.

The findings of the present study may serve as a foundation for more extensive and practical investigations in real-world conditions; studies that examine the efficacy of acidifiers and essential oils across diverse environments and against a broader range of microorganisms. Such *in vivo* studies can help in validating and deepenning the existing knowledge and provide practical solutions for optimizing the use of these compounds in the animal feed industry.

This study highlights the critical role of acidifiers and essential oils in enhancing hygiene and productivity within the livestock and poultry feed industry. While commercial acidifiers demonstrated some antibacterial activity, their effectiveness was notably limited when compared to formaldehyde, which exhibited superior performance against key pathogens such as *Salmonella typhimurium* and *E. coli.* In contrast, essential oils derived from cinnamon, thyme, and clove showed satisfactory antibacterial properties, indicating their potential as effective natural alternatives to synthetic additives. The pronounced efficacy of these essential oils not only supports their use in feed formulations but also addresses the growing consumer demand for safe and natural feed ingredients. Given the increasing scrutiny on chemical additives in animal feed and the shift towards sustainable agricultural practices, our findings advocate for further exploration and optimization of both acidifiers and essential oils in feed formulations. This approach can enhance animal health, improve food safety, and contribute to the overall sustainability of the livestock industry. Moreover, the integration of these natural alternatives may reduce reliance on chemical additives, thereby minimizing potential health risks associated with synthetic compounds. Future research should focus on the development of innovative formulations that maximize the antimicrobial benefits of these natural agents while ensuring their stability and efficacy in feed. Continuous efforts are essential to fully harness the potential of essential oils in livestock and poultry nutrition.

Acknowledgements

The authors gratefully acknowledge the School of Veterinary Medicine, Shiraz University, for supporting this study. We thank the laboratory staff of the Department of Animal Health Management for their technical assistance.

Conflict of interest

No conflict of interest to declare.

References

Abd El-Hack, ME; **El-Saadony, MT**; **Saad, AM**; **Salem, HM**; **Ashry, NM**; **Ghanima, MMA**; **Shukry, M**; **Swelum, AA**; **Taha, AE and El-Tahan, AM** (2022). Essential oils and their nanoemulsions as green alternatives to antibiotics in poultry nutrition: a comprehensive review. Poult. Sci., 101: 101584. doi: 10.1016/j.psj.2021.101584.

Aguiar Campolina, G; **D as Graças Cardoso, M**; **Rodrigues-Silva-Caetano, A**; **Lee Nelson, D and Mendes Ramos, E** (2023). Essential oil and plant extracts as preservatives and natural antioxidants applied to meat and meat products: a review. Food Technol. Biotechnol., 61: 212-225. doi: 10.17113/ftb.61.02.23.7883.

Aliverdi-Nasab, K; **Zhandi, M**; **Yousefi, AR**; **Zahedi, V and Rafieian-Naeini, HR** (2023). The effect of acidifier supplementation on egg production performance and intestinal histology of Japanese quail (*Coturnix japonica*). Vet. Med. Sci., 9: 263-271. doi: 10.1002/vms3.1043.

Andrade-Ochoa, S; **Chacón-Vargas, KF**; **Sánchez-Torres, LE**; **Rivera-Chavira, BE**; **Nogueda-Torres, B and Nevárez-Moorillón, GV** (2021). Differential antimicrobial effect of essential oils and their main components: insights based on the cell membrane and external structure. Membranes. 11: 405. doi: 10.3390/membranes11060405.

AOAC (2005). *Official methods of analysis* (18th Edn.), Association of Official Analytical Chemists, Gaithersburg, MD.

Bernardini, L; **Barbosa, E**; **Charão, MF and Brucker, N** (2022). Formaldehyde toxicity reports from in vitro and in vivo studies: a review and updated data. Drug Chem. Toxicol., 45: 972-984. doi: 10.1080/01480545.2020.1795190.

Brown, KJ; **Tannock, GW**; **Elliott, RB andLines, DR** (1980). Comparison of liquid and agar-solidified defined media regarding the physiological mechanism by which β-2-thienylalanine inhibits growth of *Escherichia*, *Shigella*, and *Salmonella* cultures. Microbiol. Immunol., 24: 603-615. doi: 10.1111/j.1348-0421.1980.tb00664.x.

Burt, S (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol., 94: 223-253. doi: 10.1016/j.ijfoodmicro.2004.03.022.

Casalino, G; **Dinardo, FR**; **D'Amico, F**; **Bozzo, G**; **Bove, A**; **Camarda, A**; **Lombardi, R**; **Dimuccio, MM and Circella, E** (2023). Antimicrobial efficacy of cinnamon essential oil against avian pathogenic *Escherichia coli* from poultry. Animals. 13: 2639. doi: 10.3390/ani13162639.

Chaieb, K; **Hajlaoui, H**; **Zmantar, T**; **Kahla-Nakbi, AB**; **Rouabhia, M**; **Mahdouani, K and Bakhrouf, A** (2007). The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. Phytother. Res., 21: 501-506. doi: 10.1002/ptr.2124.

Chen, L; **Zhao, X**; **Li, R and Yang, H** (2022). Integrated metabolomics and transcriptomics reveal the adaptive responses of *Salmonella enterica* serovar Typhimurium to thyme and cinnamon oils. Food Res. Int., 157: 111241. doi: 10.1016/j.foodres.2022.111241.

Choroszy-Król, I; **Futoma-Kołoch, B**; **Kuźnik, K**; **Wojnicz, D**; **Tichaczek-Goska, D**; **Frej-Mądrzak, M**; **Jama-Kmiecik, A and Sarowska, J** (2023). Exposing *Salmonella* Senftenberg and *Escherichia coli* strains isolated from poultry farms to formaldehyde and lingonberry extract at low concentrations. Int. J. Mol. Sci., 24: 14579. doi: 10.3390/ijms241914579.

Eslami, N; **Anzabi, Y and Nourazar, MA** (2022). The role of *Thymus vulgaris* essential oil on the rate of fecal excretion of *Salmonella typhimurium* in broilers infected with bacteria. J. Zoonotic Dis., 6: 157-167. doi: 10.22034/jzd.2022.15683.

Evangelista, AG; **Corrêa, JAF**; **Pinto, ACSM and Luciano, FB** (2022). The impact of essential oils on antibiotic use in animal production regarding antimicrobial resistance – a review. Crit. Rev. Food Sci. Nutr., 62: 5267-5283. doi: 10.1080/10408398.2021.1883548.

Farouk, MM; **El-Molla, A**; **Salib, FA**; **Soliman, YA and Shaalan, M** (2020). The role of silver nanoparticles in a treatment approach for multidrug-resistant *Salmonella* species isolates. Int. J. Nanomed., 15: 6993-7011. doi: 10.2147/IJN.S270204.

Friedman, M (2017). Chemistry, antimicrobial mechanisms, and antibiotic activities of cinnamaldehyde against pathogenic bacteria in animal feeds and human foods. J. Agric. Food Chem., 65: 10406-10423. doi: 10.1021/acs.jafc.7b04344.

Gosling, RJ; **Mawhinney, I**; **Richardson, K**; **Wales, A and Davies, R** (2021). Control of *Salmonella* and pathogenic *E. coli* contamination of animal feed using alternatives to formaldehyde-based treatments. Microorganisms. 9: 263. doi: 10.3390/microorganisms9020263.

Gupta, C; **Garg, AP**; **Uniyal, RC and Kumari, A** (2008). Comparative analysis of the antimicrobial activity of cinnamon oil and cinnamon extract on some food-borne microbes. Afr. J. Microbiol. Res., 2: 247-251.

Hąc-Wydro, K; **Flasiński, M and Romańczuk, K** (2017). Essential oils as food eco-preservatives: model system studies on the effect of temperature on limonene antibacterial activity. Food Chem., 235: 127-135. doi: 10.1016/j.foodchem.2017.05.051.

Irawan, A; **Hidayat, C**; **Jayanegara, A and Ratriyanto, A**

(2021). Essential oils as growth-promoting additives on performance, nutrient digestibility, cecal microbes, and serum metabolites of broiler chickens: a meta-analysis. Anim. Biosci., 34: 1499-1513. doi.: 10.5713/ab.20.0668.

Khwaza, V and Aderibigbe, BA (2025). Antibacterial activity of selected essential oil components and their derivatives: a review. Antibiotics. 14: 68. doi: 10.3390/antibiotics14010068.

Kissels, W; **Wu, X and Santos, R** (2017). Interaction of the isomers carvacrol and thymol with the antibiotics doxycycline and tilmicosin: in vitro effects against pathogenic bacteria commonly found in the respiratory tract of calves. J. Dairy Sci., 100: 970-974. doi: 10.3168/jds.2016-11536.

Liu, T; Kang, J and Liu, L (2021). Thymol as a critical component of *Thymus vulgaris* L. essential oil combats *Pseudomonas aeruginosa* by intercalating DNA and inactivating biofilm. LWT. 136: 110354. doi: 10.1016/j.lwt.2020.110354.

Loan, NVT; **Thong, HT and Duc, HV** (2023). Acidifiers as alternatives for antibiotics reduction and gut health improvement for poultry and swine. In: Babinszky, L; Oliveira, J and Santos, EM (Eds.), *Feed additives - recent trends animal nutrition*. IntechOpen London, UK. PP: 1-22. doi: 10.5772/intechopen.112963.

Magi, G; **Marini, E and Facinelli, B** (2015). Antimicrobial activity of essential oils and carvacrol, and synergy of carvacrol and erythromycin, against clinical, erythromycin-resistant Group A Streptococci. Front. Microbiol., 6: 165. doi: 10.3389/fmicb.2015.00165.

Mantzios, T; **Tsiouris, V**; **Kiskinis, K**; **Economou, V**; **Petridou, E**; **Tsitsos, A**; **Patsias, A**; **Apostolou, I**; **Papadopoulos, GA and Giannenas, I** (2023). In vitro investigation of the antibacterial activity of nine commercial water disinfectants, acidifiers, and glyceride blends against the most important poultry zoonotic bacteria. Pathogens. 12: 381. doi: 10.3390/pathogens12030381.

Mincheva, N; **Oblakova, M**; **Ivanova, I and Hristakieva, P** (2025). Economic evaluation of broiler diets supplemented with either selected herbs or their associated essential oils. Black Sea J. Agric., 8: 3-4. doi: 10.47115/bsagriculture.1564174.

Morshdy, AEM; **El-Tahlawy, AS**; **Qari, SH**; **Qumsani, AT**; **Bay, DH**; **Sami, R**; **Althubaiti, EH**; **Mansour, AM**; **Aljahani, AH and Hafez, AE-SE** (2022). Anti-biofilms' activity of garlic and thyme essential oils against *Salmonella typhimurium*. Molecules. 27: 2182. doi: 10.3390/molecules27072182.

Movahedi, F; **Nirmal, N**; **Wang, P**; **Jin, H**; **Grøndahl, L and Li, L** (2024). Recent advances in essential oils and their nanoformulations for poultry feed. J. Anim. Sci. Biotechnol., 15: 110. doi: 10.1186/s40104-024-01067-8.

Mucha, W and Witkowska, D (2021). The applicability of essential oils in different stages of production of animal-based foods. Molecules. 26: 3798. doi: 10.3390/molecules26133798.

Nehme, R; **Andrés, S**; **Pereira, RB**; **Ben Jemaa, M**; **Bouhallab, S**; **Ceciliani, F**; **López, S**; **Rahali, FZ**; **Ksouri, R and Pereira, DM** (2021). Essential oils in livestock: from health to food quality. Antioxidants. 10: 330. doi: 10.3390/antiox10020330.

Nguyen, DH; **Seok, WJ and Kim, IH** (2020). Organic acids mixture as a dietary additive for pigs—a review. Animals. 10: 952. doi: 10.3390/ani10060952.

Omidi, A; **Mosleh, H and Rahimi-Kakolaki, M** (2024). Comparative evaluation of disinfectants' efficacy in reducing bacterial and fungal contamination in livestock feed production. Iran. J. Vet. Med., 19: 385-396. doi: 10.32598/ijvm.19.2.1005480.

Pearlin, BV; **Muthuvel, S**; **Govidasamy, P**; **Villavan, M**; **Alagawany, M**; **Ragab Farag, M**; **Dhama, K and Gopi, M** (2020). Role of acidifiers in livestock nutrition and health: a review. J. Anim. Physiol. Anim. Nutr., 104: 558-569. doi: 10.1111/jpn.13232.

Polycarpo, GV; **Andretta, I**; **Kipper, M**; **Cruz-Polycarpo, VC**; **Dadalt, JC**; **Rodrigues, PHM and Albuquerque, RD** (2017). Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. Poult. Sci., 96: 3645-3653. doi: 10.3382/ps/pex178.

Selles, SMA; **Kouidri, M**; **Belhamiti, BT and Ait Amrane, A** (2020). Chemical composition, in-vitro antibacterial and antioxidant activities of *Syzygium aromaticum* essential oil. J. Food Meas. Charact., 14: 2352-2358. doi: 10.1007/s11694-020-00482-5.

Shaji, S; **Selvaraj, RK and Shanmugasundaram, R** (2023). *Salmonella* infection in poultry: a review on the pathogen and control strategies. Microorganisms. 11: 2814. doi: 10.3390/microorganisms11112814.

Sharafi, H; **Moradi, M and Sharafi, K** (2023). A systematic review and meta-analysis of the use of plant essential oils and extracts in the development of antimicrobial edible films for dairy application. Vet. Res. Forum. 14: 179-194. doi: 10.30466/vrf.2022.1986122.3730.

Shen, S; **Zhang, T**; **Yuan, Y**; **Lin, S**; **Xu, J and Ye, H** (2015). Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane. Food Control. 47: 196-202. doi: 10.1016/j.foodcont.2014.07.003.

Stevanović, ZD; **Bošnjak-Neumüller, J**; **Pajić-Lijaković, I**; **Raj, J and Vasiljević, M** (2018). Essential oils as feed additives—future perspectives. Molecules. 23: 1717. doi: 10.3390/molecules23071717.

Tugnoli, B; **Giovagnoni, G**; **Piva, A and Grilli, E** (2020). From acidifiers to intestinal health enhancers: How organic acids can improve growth efficiency of pigs. Animals. 10: 134. doi: 10.3390/ani10010134.

Turek, C and Stintzing, FC (2013). Stability of essential oils: a review. Compr. Rev. Food Sci. Food Saf., 12: 40-53. doi: 10.1111/1541-4337.12006.

Van, TTH; **Smooker, PM**; **Wu, S and Wu, Z** (2023). Bacterial diseases in poultry: Biology, virulence and prevention in the age of reduced antibiotic use. Front. Vet. Sci., 10: 1189315. doi: 10.3389/fvets.2023.1189315.

Vertiprakhov, V and Grozina, A (2019). Exocrine pancreatic function in chickens as a result of adding feed acidifiers to their diet. Sib. Herald Agric. Sci., 48: 63-69. doi: 10.26898/0370-8799-2018-6-9.

Vinolya, RE; **Balakrishnan, U**; **Yasir, B and Chandrasekar, S** (2021). Effect of dietary supplementation of acidifiers and essential oils on growth performance and intestinal health of broiler. J. Appl. Poult. Res., 30: 100179. doi: 10.1016/j.japr.2021.100179.

Visvalingam, J; **Palaniappan, K and Holley, RA** (2017). In vitro enhancement of antibiotic susceptibility of drug resistant *Escherichia coli* by cinnamaldehyde. Food Control. 79: 288-291. doi: 10.1016/j.foodcont.2017.04.011.

Waghmare, S; **Gupta, M**; **Bahiram, K**; **Korde, J**; **Bhat, R**; **Datar, Y**; **Rajora, P**; **Kadam, M**; **Kaore, M and Kurkure, N** (2025). Effects of organic acid blends on the growth performance, intestinal morphology, microbiota, and serum lipid parameters of broiler chickens. Poult. Sci., 104: 104546. doi: 10.1016/j.psj.2024.104546.

Yin, L; **Dai, Y**; **Chen, H**; **He, X**; **Ouyang, P**; **Huang, X**; **Sun, X**; **Ai, Y**; **Lai, S and Zhu, L** (2022). Cinnamaldehyde resist *Salmonella typhimurium* adhesion by inhibiting type I fimbriae. Molecules. 27: 7753. doi: 10.3390/molecules

27227753.