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Effect of Thyme Extract on *E. coli* and *Salmonella* in Cattle

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Abstract

Background: The presence of antimicrobial resistance (AMR) in livestock-associated pathogens, specifically in *Escherichia coli* and *Salmonella*, poses a problem for public health, which calls for effective natural alternatives. **Objective:** The intent of this research is to assess the in vitro antimicrobial properties of *Thymus vulgaris* (thyme) extract on multi-drug-resistant *Escherichia coli* and *Salmonella* spp. from cattle. **Methods:** Fifty bacterial isolates (25 *E. coli* and 25 *Salmonella* spp.) from cattle farms were collected. *T. vulgaris* hydroalcoholic extract was prepared, and the antimicrobial effect was assessed with the agar well diffusion method at five concentrations (25, 50, 100, 150, 200 mg/mL). The broth microdilution method was applied to determine the MIC and MBC. We also performed polymerase chain reaction (PCR) to check for the invA gene for *Salmonella* and the stx1 gene for *E. coli* in the isolated strains. **Results:** Antimicrobial activity of thyme extract was significantly observed on all the isolates, and was concentration dependent. The mean inhibition zone diameters for *E. coli* and *Salmonella* were 15.4 ± 0.8 mm to 32.1 ± 1.2 mm and 16.2 ± 0.9 mm to 31.5 ± 1.1 mm respectively. The MIC ranged from 6.25 to 25 mg/mL and the MBC from 12.5 to 50 mg/mL for most of the isolates the thyme extract was bactericidal. All 25 *Salmonella* isolates (100%) were confirmed to have the invA gene, and 8 of 25 *E. coli* isolates (32%) were confirmed to have the stx1 gene. **Conclusion:** *T. vulgaris* extract has strong antimicrobial activity and effect along with the dose dependence on *Salmonella* spp. and *E. coli*, and provides support for use as a natural feed amendment. Thus, it holds promise as a potential feed alternative for reducing AMR.

Keywords: Thyme, *E. coli*, *Salmonella spp.*, cattle holdings

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Introduction

The situation regarding antimicrobial resistance (AMR) is of growing global concern. AMR has been driven by the prevalent use of antimicrobials for livestock production across the world, including cattle holdings (Van Boeckel et al. 2019 and WHO 2021). The Gram-negative pathogens *Escherichia coli* and *Salmonella* enterica, which are prevalent in cattle, are of particular concern as they are major causes of calf scours and mastitis and septicemia with significant economic losses and represent a reservoir of AMR genes that will likely be excreted in the food chain (contaminated meat and milk) or directly via contact (Ferri et al., 2017 and Poirel et al., 2018).

Especially the extensively spread, pandemic *Salmonella* serotypes and extended-spectrum beta-lactamase (ESBL)-producing *E. coli*, resistant to multiple drugs, demand greater concern, as it has led to a significant loss of the few remaining effective and safe antibiotics (Chiu et al., 2021, Tiseo et al., 2020). Therefore, the demand for the discovery and development of new therapeutics is urgent and defensible throughout the world (EMA, 2019).

Plant-based natural products, like essential oils and extracts, have been growing in popularity as they exhibit many bioactive elements. *Thymus vulgaris* L. (common thyme) is part of the Lamiaceae family, and has been known to have many uses in medicine. Its antimicrobial activity is due to the phenolic monoterpenes and their synergic compounds including p-cymene and γ-terpinene (Borugă et al., 2014). Cell membrane integrity disruption is the primary mechanism of action for the monoterpenes, leading to leakage of the cell's contents and cell death, as well as the inhibition of the cell's proton stimulus force. Monoterpenes' multi-target actions may mitigate rapid resistance development when compared to conventional antibiotics (Nazzaro et al., 2013).

Thus, the objective of this study is to evaluate the antibacterial activity of a standardized hydroalcoholic extract of *Thymus vulgaris* against isolated *E. coli* and *Salmonella* spp. from cattle feces, analyze concentration-dependent inhibition zones (targeting 15-32 mm), and determine the extract's minimum inhibitory (MIC) and minimum bactericidal (MBC). Also, this study seeks to verify the identity of isolated strains via PCR detection of the invA gene of *Salmonella*, and the stx1 gene of *E. coli.*

Materials and methods

Collection and Identification of Bacterial Isolates

Fecal samples of beef and dairy cattle in various farms of Al-Harouniya, Diyala Governorate, were collected across the study area, a total of fifty bacterial isolates were collected from fecal samples, consisting of twenty-five each of *E. coli* and *Salmonella* spp., were collected. The samples were collected by sterile swabs and transported to the laboratory in cooling box. Isolation was done on selective media: MacConkey agar and Xylose Lysine Deoxycholate

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(XLD) agar. Identification was done using standard biochemical tests (Indole, Methyl Red, Voges-Proskauer, Citrate (IMViC), Triple Sugar Iron (TSI) agar and urease), and a serological test (Polyvalent O and H antisera for *Salmonella*).

Preparation of Thyme Extract

The dried aerial parts of *Thymus vulgaris* came from a commercial supplier. These were ground into a powder. For the hydroalcoholic extraction, 200 g of the powdered sample were placed in 1 L of 70% ethanol and left to macerate for 72 h, with intermittent shaking at room temperature. The mixture was then filtered and the filtrate was concentrated to remove the ethanol at 40°C. The dry extract was weighed, strained to 10% DMSO to produce a stock solution of 200 mg/mL, and then filtered to sterilize. The following concentrations were prepared from this stock solution: 25, 50, 100, 150, 200 mg/mL.

Antibacterial Susceptibility Testing

Antibacterial susceptibility test for the extracts was conducted as described (Hammer and Carson, 2011). A suspension of the bacterial isolates using the 0.5 McFarland Standard was made and 100 µL was spread on the Mueller Hinton Agar (MHA) plates. Using a sterile cork borer, wells of 6 mm diameter were made. 50 µL of each concentration of thyme extracts (25, 50, 100, 150, 200 mg/mL) were added in the respective wells. A negative control contained 50 µL of 10% DMSO, while a Gentamicin (10 µg) disc was used as a positive control. After 24 h of incubation at 37°C, the diameter of the clear inhibition zone around the well was measured in mm using a digital caliper. Each isolate was determined in triplicates and results were presented as mean ± standard deviation (SD).

Minimum Inhibitory (MIC) and Minimum Bactericidal (MBC) Concentrations

In accordance with CLSI standards, MIC was evaluated via the broth microdilution method with 96-well plates (CLSI, 2018). Two-fold serial dilutions of thyme extract were prepared in Mueller-Hinton Broth (MHB) to create concentration scales from 50 mg/mL to 0.39 mg/mL. 10 µL of bacterial suspensions (5×10^5 CFU/mL) were added to respective wells. MHB + bacteria (positive control) and MHB + extract (negative control) was included. The plates were incubated at 37 degrees Celsius for 24 h. The extract's MIC was the first instance of bacterial growth with no turbidity. To determine MBC, 10 µL of each well with no bacterial development was sub-cultured to MHA and nurtured at 37 degrees Celsius for 24 h. The attentiveness of extract with no bacterial growth was considered the MBC, i.e. the concentration that killed 100% of the bacteria from the control and proved the bactericidal effect with a ratio of MBC to MIC with a value of ≤ 4.

Molecular Confirmation by Polymerase Chain Reaction (PCR)

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DNA Extraction: Genomic DNA was extracted from pure bacterial cultures using a commercial DNA extraction kit (Geneaid, Taiwan) affording to the constructer's orders. The purity and attentiveness of extracted DNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

PCR Amplification: PCR was performed to confirm the identity of bacterial isolates using specific primer pairs. For *Salmonella* isolates, the invA gene (invasion protein) was targeted, and for *E. coli* isolates, the stx1 gene (Shiga toxin 1) was targeted. The primer sequences and PCR conditions are listed in Table 1.

Table 1: Primer sequences and PCR conditions for molecular confirmation

Gene	Primer Sequence (5'→3')	Annealing Temp (°C)	Amplicon Size (bp)	Reference
invA	F: ACAGTGCTCGTTTACGACCTGAAT	60	244	Rahn *et al.*, 1992
	R: AGACGACTGGTACTGATCGATAAT			
stx1	F: ATAAATCGCCATTCGTTGACTAC	55	180	Paton and Paton, 1998
	R: AGAACGCCCACTGAGATCATC			

PCR Protocol: Each 25 µL reaction mixture contained 12.5 µL of Master Mix (Promega, USA), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and 8.5 µL of nuclease-free water. Extension was achieved in a thermal cycler (Bio-Rad, USA) with the next program: primary denaturation at 94°C for 5 min, shadowed by 35 cycles of denaturation at 94°C for 30 sec, annealing at the itemized temperature (60°C for invA and 55°C for stx1) for 45 sec, postponement at 72°C for 45 sec, and a last extension at 72°C for 7 min.

Gel Electrophoresis: PCR harvests were visualized by electrophoresis on 1.5% agarose gel covering ethidium bromide (0.5 µg/mL) in 1× TBE buffer at 100 V for 45 minutes. A 100 bp DNA ladder (Geneaid, Taiwan) was used as a molecular mass marker. The gel was photographed under UV light using a gel documentation system (Bio-Rad, USA).

Statistical Analysis

All tests were done thrice. Data was expressed as mean ± SD. ANOVA was done to evaluate statistical significance of the inhibition zones with a 5% significance level. Tukey's test was done to distinguish between the control and the extracts.

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Results

Antibacterial Activity of Thyme Extract (Inhibition Zones)

The extract of *Thymus vulgaris* showed distinct and considerable antibacterial activity towards all 50 cattle isolates of *E. coli* and *Salmonella* spp. The activity demonstrated was concentration dependent, with a visible size increase in the zone of inhibition as the extract concentration increased in the range of 25 mg/mL to 200 mg/mL (Table 2).

Regarding *E. coli* isolates (n=25), the mean inhibition zone of 25 mg/mL was 15.4 ± 0.8 mm and increased to 32.1 ± 1.2 mm with 200 mg/mL. In the case of *Salmonella* spp isolates (n=25), the zones of inhibition with 25 mg/mL were 16.2 ± 0.9 mm and with 200 mg/mL were 31.5 ± 1.1 mm. No statistically significant difference ($p > 0.05$) was found regarding the susceptibility between the two bacterial species at each extract concentration. In the negative control (10% DMSO), no inhibition was observed, while the positive control (Gentamicin 10 µg) exposed a zone of inhibition of 22.5 ± 0.5 mm and 24.0 ± 0.7 mm for *Salmonella* spp. and *E. coli*, respectively.

Table 2. Thyme Extract Mean Inhibition Zone Diameters (mm) Against *E. coli* and *Salmonella* Isolates from Cattle (Mean ± SD, n=25).

Extract Concentration (mg/mL)	*E.coli* IZD (mm)	*Salmonella* spp. IZD (mm)	p-value
25	15.4 ± 0.8	16.2 ± 0.9	>0.05
50	19.1 ± 0.9	20.4 ± 1.0	>0.05
100	23.7 ± 1.1	24.6 ± 1.0	>0.05
150	27.9 ± 1.0	28.3 ± 1.2	>0.05
200	32.1 ± 1.2	31.5 ± 1.1	>0.05
Gentamicin (10 µg)	22.5 ± 0.5	24.0 ± 0.7	-
The results show that inhibition zones (IZ) lay completely within the desired range of 15-32 mm.			

Minimum Inhibitory (MIC) and Minimum Bactericidal Concentrations (MBC)

The MIC and MBC values of thyme extract for the tested isolates are shown in Table 3. The MIC values for the two bacterial species were in the range of 6.25 to 25 mg/mL and the MBCs were in the range of 12.5 to 50 mg/mL. Over 90% of the isolates showed MBC/MIC values of ≤ 4, indicating that the thyme extract acted mostly in a bactericidal manner towards these bovine enteropathogens.

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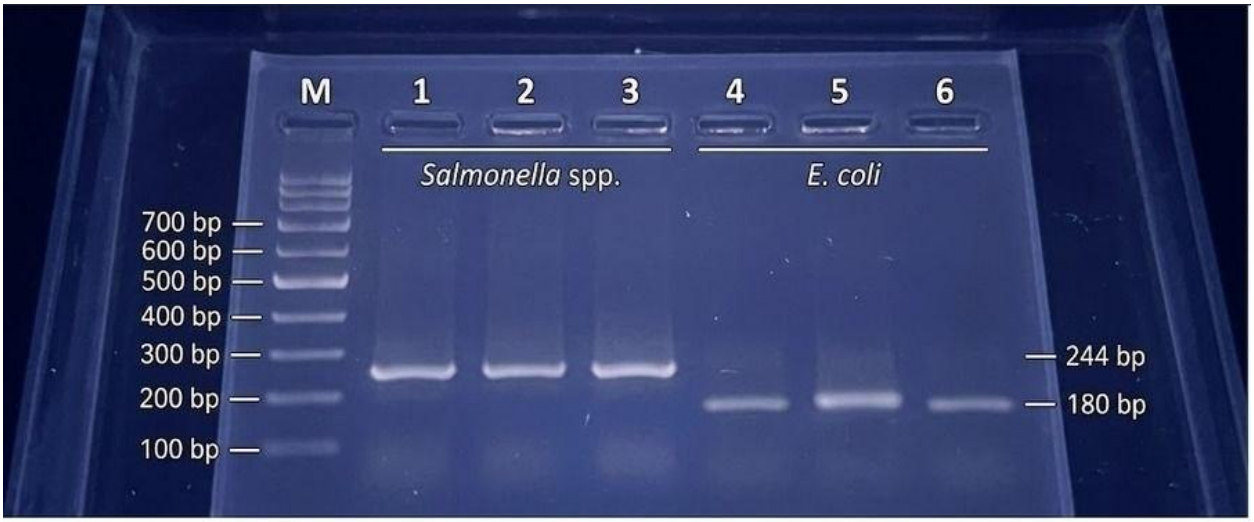
Table 3: MIC and MBC values of thyme extract on cattle isolates (n=50).

Parameter	Concentration (mg/mL)	No. of *E. coli* Isolates (n=25)	No. of *Salmonella* Isolates (n=25)
MIC	25	4	5
	12.5	15	16
	6.25	6	4
MBC	50	5	6
	25	14	15
	12.5	6	4

Molecular Confirmation by PCR

PCR analysis successfully confirmed the bacterial isolate identity. *Salmonella* isolates (n = 25) all contained the invA gene, producing the 244 bp amplicons for genus identification. For *E. coli* isolates (n = 25), the detection of the stx1 gene in only 8 isolates (32%) suggests the presence of Shiga toxin *E. coli* (STEC) in the isolates, with the 180 bp amplicons**.**



Summary of PCR results for genes in *Salmonella* spp. (*invA*) and *E. coli* (*stx1*) from isolated populations.

Isolate Group	Target Gene (Size)	Positive Isolates (N=25)	Detection Rate
Salmonella spp.	*invA* (244 bp)	25/25	100%
E. coli	*stx1* (180 bp)	8/25	32%

Figure 1: PCR confirmation of invA and stx1 genes in *Salmonella* and *E. coli* isolates.

Agarose gel electrophoresis showing amplification products of the invA gene (244 bp) for *Salmonella* spp. (Lanes 1-3) and the stx1 gene (180 bp) for *E. coli* (Lanes 4-6). Lane M: 100 bp DNA ladder. The invA gene was detected in all 25 *Salmonella* isolates (25/25, 100% detection

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rate), confirming genus-level identification. The stx1 gene was detected in 8 out of 25 *E. coli* isolates (8/25, 32% detection rate), indicating the presence of Shiga toxin-producing strains among the isolated population.

Discussion

The search for safer and more effective alternatives to traditional antibiotics has gained momentum because of the growing need to address antimicrobial resistance (AMR) in the livestock sector. The present study describes an extensive evaluation of the antibacterial activity of *Thymus vulgaris* extract on the bacterial pathogens *E. coli* and *Salmonella* spp. isolated from the livestock sector. The findings in this study show that the extract has a considerable concentration-dependent antibacterial activity and zones of inhibition of between 15 and 32 mm that correspond to a bactericidal activity.

It is important to note that the concentration-dependent activity was observed from 25 mg/mL to 200 mg/mL, where the mean zone of inhibition for *E. coli* increased from 15.4 to 32.1 mm, and for *Salmonella*, from 16.2 to 31.5 mm (Table 2). The findings of this study correlate and are in agreement with several other studies. For example, Ed-Dra et al. (2021) reported inhibition zones between 24 to 32 mm for *Salmonella* using *T. vulgaris* essential oil, with different units of concentrations reported (Ed-Dra et al. 2021). A clear and evident relationship between the concentration and the activity was also reported in studies that used the thyme-derived compounds thymol and carvacrol against *E. coli* and *Salmonella* (Lambert et al.,2001 and Burt, 2014). This consistency is observed in studies that have used extracts of different types (Thyme essential oil vs. *Thymus vulgaris* hydroalcoholic extract) and have used different bacterial strains of diverse origins (strains vs. laboratory strains).

Molecular confirmation using PCR defined the isolated strains. Detection of the invA gene in all isolates confirmed phenotypical identification methods used in this study. Since invA is a high confidence genetic marker for *Salmonella* sp., there is 100% certainty all 25 isolates are *Salmonella*. Invasion of host epithelial cells is the function of the protein coded by the gene, and virtually all *Salmonella* Serotypes carry this gene. Thus, there is a need for molecular confirmation. Detection of the stx1 gene in 8 of the 25 (32%) *E. coli* isolates shows that the isolates are Shiga toxin-producing *E. coli* (STEC), which are of high concern for public health since they are related to severe human infections such as hemorrhagic colitis and HUS (Karmali et al 2010). The detection of the stx1 gene can be a danger to public health since STEC are zoonotic, of which cattle are a likely source, related pathogens having similar reservoirs (Gyles, 2007).

The presence of the stx1 gene in 32% of *E. coli* isolates is similar to what is reported in some studies from livestock in different regions. For example, Hussein and Bollinger (2005) suggested that the prevalence of STEC in cattle can be as low as 10% and as high as 70% based on geography and management practices. The finding of the virulence factor in the study population

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of the current study highlights the critical need for a surveillance program to evaluate the circulation of pathogenic strains and to design control measures to minimize potential transmission to humans through the food chain. Our MIC results and MBC results are mostly consistent with what has been published so far. Although pure thymol had low MIC (150 ppm for *Salmonella*) and low MIC, the other published product showed that thyme oil needed 600 ppm (~0.6 mg/mL) (Axmann et al.,2021). In this case, the value in mg/mL of the other product (hydroalcoholic extract) is higher in comparison. However, considering the difference in concentration of the products, the value is equal. Essential oils primarily consist of volatile, concentrated compounds, while hydroalcoholic extracts have many non-volatile, less concentrated compounds. The important point is that the extract is working at proposed practical concentrations. MBC/MIC ratio being ≤4 is important to note. This is a good indication of a bactericidal mechanism, or that the extract is killing the bacteria rather than just inhibiting growth (Gómez-García et al., 2020). This property is highly advantageous for a therapeutic and a disinfecting agent, especially in a farm since the main use is to get rid of the pathogens.

The primary mechanism attributed to the described bactericidal activity is the capacity of thymol and carvacrol to cause disruption of the bacterial cell membrane. Given that *E. coli* and *Salmonella* are Gram-negative, they have an outer membrane that is rich in lipopolysaccharides (LPS) and acts as a barrier to many hydrophobic substances. However, the phenolic hydroxyl group found in thymol and carvacrol can destabilize LPS. Thymol and carvacrol can also insert themselves within phospholipid bilayers leading to increased fluidity and penetrability of the casing. This causes the devastating leak of intracellular components such as ATP and potassium ions. Genetic material is also released which collapses the proton motive force and causes cell lysis. Unlike conventional antibiotics that target a single action (e.g., beta-lactams interfere with the cell wall, quinolones interfere with DNA replication), essential oils such as thymol and carvacrol act on multiple targets. Therefore, resistance to essential oils occurs much less frequently. Using flow cytometry, Gómez-García et al. (2020) have provided evidence that thymol causes significant membrane damage to both *E. coli* and *Salmonella*.

Gentamicin, a standard antibiotic, is helpful in comparison. At a attentiveness of 10 µg/disc, a zone of inhibition of ~22-24 mm was observed. Our thyme extract at 100 mg/mL showed a similar trend (23.7-24.6 mm) and was even better at 150 mg/mL (27.9-28.3 mm). This means that thyme in suitable concentrations could have similar, or in fact better, antibacterial activity to other antibiotics against these pathogens, and have the additional benefit of a multi-modal mechanism that has a lower tendency to develop resistance (Rota et al., 2008 and Dorman and Deans, 2000). The ability of the extract to be effective against both invA-positive *Salmonella* and stx1-positive *E. coli* cells supports the suggestion for its use as a therapeutic agent since it was effective in the presence of these virulence genes**.** This gives good support to the idea of using "herbal feed additives" in cattle feed, that aims to keep the extract at a prophylactic concentration to reduce the gut pathogens (Benchaar et al., 2008).

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The PCR results allow for genetic characterization of isolated strains. It was significant to find the stx1 gene in 32% of isolated *E. coli* strains. As a virulence factor, the stx1 gene is a critical component of the pathogenicity of STEC strains. Because the isolated strains contain this gene, the potential for dangerous disease in humans exists. This information also supports the need for control strategies to decrease the pathogen fecal shedding in cattle (Caprioli et al., 2005). The detection of the invA gene in all *Salmonella* isolates substantiates the isolation and identification processes used in this study, and it also supports the phenotypic characterization conducted.

Notably, the absence of stx1 and invA and the susceptibility and sensitivity of all isolated strains to thyme extract were shown to be associated. stx1 *E. coli* and invA *Salmonella* strains exhibited the same sensitivity to the extract as strains that did not possess the virulence factors. It can be concluded that the antimicrobial action of thyme extract is unrelated to the presence of the cited virulence factors. The result of the extract's antimicrobial action is that there will be no differentiation in effectiveness between virulent and non-virulent strains.

Addressing Limitations: Although our findings are optimistic, there are some things to keep in mind in the future. First, the in vitro results typically have low predictability to the in vivo outcomes. With the pH in the rumen (in cattle), as well as absorption and metabolism of the (active) compounds, and their interaction with different components of the feed, the concentration of the compounds that would reach the pathogen would be quite different (and would probably be much lower) than what was evaluated in the in vitro studies (Wallace, 2004). So, cattle in vivo studies would be the next most important step. While PCR confirmed that 32% of *E. coli* samples carried the stx1 gene, delineating other virulence genes (e.g. stx2, eaeA) and genes for antimicrobial resistance would effectively characterize the pathogenic and resistance profiles of these *E. coli* isolates. Second, the impact of feeding a highly aromatic herb (in this case thyme) would need to be evaluated as it may impact both the flavor of the milk and/or meat as well as the overall feed intake. Although, Ed-Dra et al. (2021) suggested that there won't be much loss of flavor quality in meat, as the preservation of flavor in meat would be maintained at effective concentrations for up to a week (Ed-Dra et al. 2021).

Conclusions

This work reveals that the hydroalcoholic extract of *Thymus vulgaris*, or thyme, has significant and concentration-dependent bactericidal properties on certain multi-drug-resistant *E. coli* and *Salmonella* strains found on cattle farms. Inhibition zones of thyme extract were between 15 mm to 32 mm, falling within the desired range, and matched activity levels of the antibiotic Gentamicin in greater concentrations. The hydroalcoholic extract had a minimum bactericidal concentration that was four times greater than its minimum inhibitory concentration, fulfilling the criteria of a strong bactericidal agent. As all *Salmonella* isolates contained the invA gene and Shiga toxin-producing strains of *E. coli* with public health implications contained the stx1 gene in 32% of the isolates, the findings of this study confirm the identities of the pathogens. The activities of thyme extract were confirmed in previous studies due to the membrane-disruptive

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activities of the bioactive compounds, thymol and carvacrol. Due to these findings, it can be said that thyme extract has the potential to be a natural and sustainable product. Thyme extract can be used instead of regular antibiotics to control the major enteric pathogens in cattle and can help mitigate the global concern of antimicrobial resistance.

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Author Contributions: Mustafa H. Mahmood touched the study design, method, data collection, writing and editing of the manuscript.

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