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Posted Date: 14 July 2026

doi: 10.20944/preprints202607.1007.v1

Keywords: *Salmonella* typhimurium; carvacrol; tetracycline; antimicrobial resistance; tet genes; multidrug resistance; swine

 doi:10.20944/preprints202607.1007.v1

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Carvacrol Modulates Tetracycline Susceptibility and Resistance Gene Expression in Multidrug-Resistant *Salmonella* Typhimurium Strains Isolated from Swine

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Simple Summary

Salmonella Typhimurium is an important pathogen in pig production and a relevant cause of foodborne disease in humans. The increasing occurrence of antimicrobial resistance, particularly multidrug-resistant strains, has encouraged the search for alternative or complementary control strategies. Carvacrol, a natural compound present in oregano (*Origanum vulgare*) essential oil, has recognized antimicrobial properties. In this study, the activity of carvacrol was evaluated against 73 swine-derived *S.* Typhimurium isolates by determining minimum inhibitory and bactericidal concentrations. In addition, the combined effect of carvacrol and tetracycline was assessed in 14 tetracycline-resistant strains. Gene expression analyses were also performed in selected isolates to determine the effect of carvacrol on tetracycline resistance-associated genes. Carvacrol inhibited bacterial growth in most isolates and, when combined with tetracycline, reduced the effective antibiotic concentration in some strains. In several isolates, the combined treatment also reduced the expression of tetracycline resistance genes. These findings suggest that carvacrol could be a useful natural adjuvant to improve antimicrobial efficacy and support resistance control strategies in pig production.

Abstract

Salmonella Typhimurium is an important pathogen affecting both animal production and public health, with pigs representing a major reservoir. The increasing prevalence of antimicrobial resistance highlights the need for alternative or complementary therapeutic strategies. Carvacrol, a phenolic constituent of *Origanum vulgare* essential oil, has recognized antimicrobial activity and may contribute to the modulation of antimicrobial resistance mechanisms. The present study investigated the antimicrobial activity of carvacrol and tetracycline, individually and in combination, against swine-derived *S.* Typhimurium strains. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by broth microdilution. Interactions between both compounds were assessed by checkerboard assays using the fractional inhibitory concentration index (FICI). Relative expression of tetracycline resistance genes was analysed by quantitative real-time PCR in selected isolates exposed to tetracycline alone or combined with carvacrol. Carvacrol MIC values ranged from 0.5 to 16 mg/mL, with MIC_{50} and MIC_{90} values of 2 and 4 mg/mL, respectively. MBC and MIC values were identical in 84.9% of the isolates, indicating a strong bactericidal effect. Tetracycline MIC_{50} and MIC_{90} values were 256 and 512 μg/mL, respectively. Checkerboard assays showed synergistic interactions in 14.3% of strains, additive effects in 50.0%, indifferent responses in 28.6%, and antagonism in 1%. Combined exposure reduced *tetA* and *tetB*

 doi:10.20944/preprints202607.1007.v1

expression in most tested isolates compared with tetracycline alone, although responses were strain dependent. These findings demonstrate that carvacrol exhibits antimicrobial activity against swine-derived multidrug-resistant *S.* Typhimurium and may enhance tetracycline activity while modulating resistance-associated gene expression. Carvacrol could represent a promising natural adjuvant for antimicrobial control strategies in swine production.

Keywords: *Salmonella* typhimurium; carvacrol; tetracycline; antimicrobial resistance; tet genes; multidrug resistance; swine

1. Introduction

Salmonella spp., particularly *S.* Typhimurium, is one of the main gastrointestinal pathogens affecting the swine production sector. Moreover, it represents a major zoonotic and foodborne public health concern, as pigs constitute an important reservoir and may contribute to transmission through contaminated animal-derived products [1,2]. The spread of antimicrobial resistance (AMR) represents one of the major threats to global public and animal health. According to data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), more than 35,000 deaths annually in Europe are attributed to infections caused by antimicrobial-resistant microorganisms [3]. Several factors contribute to the dissemination of resistance in *Salmonella* spp., including selective pressure derived from excessive antimicrobial use in humans and animals, horizontal gene transfer mediated by plasmids, and the emergence of multidrug-resistant (MDR) strains [4,5]. Recent analysis of caecal samples from fattening pigs at slaughterhouses in 2023 revealed that 56.9% of *S.* Typhimurium isolates and 81.1% of its monophasic variant were MDR. Notably, high resistance levels have been reported against ampicillin, sulfamethoxazole, tetracycline, and chloramphenicol, corresponding to the characteristic MDR resistance profile in *Salmonella* [4].

Tetracyclines are broad-spectrum antibacterial agents with relatively low toxicity that have been widely used in both human and veterinary medicine, as well as historically as growth promoters in animal production. This extensive use has contributed to the emergence and dissemination of resistance [6,7]. Tetracycline resistance is mainly mediated by *tet* genes encoding efflux pumps that reduce intracellular antibiotic accumulation. Several *tet* genes, including *tetA*, *tetB*, *tetC*, *tetD* and *tetG*, have been identified in different *Salmonella* serotypes, with *tetA* and *tetB* being the most prevalent [7,8].

As conventional antibiotics lose efficacy, plant-derived secondary metabolites, particularly essential oils (EOs), have emerged as promising candidates to mitigate AMR [9]. EOs are complex mixtures of volatile, lipophilic compounds, generally dominated by a few major constituents that can account for 20–70% of their composition. Their biological activity is largely attributed to these major components, which are mainly responsible for their antimicrobial effects [10,11].

Carvacrol ($C_{10}H_{14}O$) is a phenolic monoterpenoid predominantly found in the essential oils of oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) [12]. It exerts broad-spectrum antimicrobial activity against clinically relevant pathogens of public health importance such as *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Listeria monocytogenes* and *Salmonella enterica* [13,14]. In addition, carvacrol has gained increasing attention in the food industry due to its applications as a natural additive, probiotic and in the development of new antimicrobial packaging systems [15].

Previous studies evaluating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have demonstrated that carvacrol inhibits the growth of different *Salmonella* spp. strains, although its efficacy appears to be strain dependent [16–19].

In this context, natural compounds such as carvacrol can be proposed as antibiotic adjuvants capable of enhancing antimicrobial efficacy and counteracting resistance mechanisms, such as efflux pump activity and membrane permeability alterations. Although synergistic interactions between carvacrol and conventional antimicrobials have been reported in several bacterial species, most studies have focused exclusively on growth inhibition assays, whereas the molecular mechanism

 doi:10.20944/preprints202607.1007.v1

underlying these interactions remain poorly understood, particularly in MDR *Salmonella* of swine origin [20,21].

Despite the documented phenotypic synergy of carvacrol, its molecular impact on specific tetracycline resistance determinants (*tet* genes) in MDR swine isolates remains largely unexplored. Therefore, this study aimed to investigate the antimicrobial interaction between carvacrol and tetracycline and to assess the modulatory effect of carvacrol on the expression of tetracycline resistance-associated genes (*tetA* and *tetB*) in swine-derived MDR *S.* Typhimurium.

2. Material and Methods

2.1. Samples and Antimicrobial Products

Seventy-three *S*. Typhimurium isolates from the strain collection of the Animal Health Department (Faculty of Veterinary Medicine, University of Cordoba, Spain) were included in this study. The isolates were collected between 2010 and 2018 from different closed-cycle free-range pig farms. Samples originated from multiple sources, including lymph nodes, faeces, tonsils, pens, transport, cutting and slaughter [8].

Serotyping was performed by slide agglutination according to the Kauffmann–White–Le Minor scheme [22] and phage typing was conducted at the National Reference Laboratory for *Salmonella* and *Shigella* (Majadahonda, Spain) [8]. All isolates were stored at -20 °C in Brain Heart Infusion (BHI) broth (Oxoid™) supplemented with 15% glycerol until use. Carvacrol (≥98% purity) and tetracycline (98-102% purity) were purchased from Sigma-Aldrich Laboratories (USA). Tetracycline was selected as the antibiotic of interest due to the high prevalence of resistance reported in *Salmonella* spp. isolated from swine [4].

2.2. MIC and MBC Determination

All 73 isolates had been previously characterized as tetracycline-resistant [8]. The minimum inhibitory concentration (MIC) of carvacrol and tetracycline against *S*. Typhimurium was determined by broth microdilution following the Clinical and Laboratory Standards Institute (CLSI) guidelines for bacteria isolated from animals [23]. While carvacrol MIC was evaluated for all 73 isolates, tetracycline MIC was re-evaluated in 14 representative strains using an expanded concentration range to determine the exact MIC values required for subsequent checkerboard assays. These 14 strains were selected to represent the phenotypic and genotypic diversity observed within the collection, including different phage types, carvacrol susceptibility profiles, tetracycline resistance phenotypes, and the presence of the *tetA* and *tetB* genes.

Stock solutions were prepared at concentrations of 128 mg/mL for carvacrol and 8,192 µg/mL for tetracycline. Carvacrol was dissolved in sterile distilled water containing 0.1% dimethyl sulfoxide (DMSO), whereas tetracycline was dissolved in sterile distilled water. Twofold serial dilutions were prepared in 96-well microplates to obtain the desired concentration ranges.

Bacterial inocula were prepared from overnight cultures on Tryptic Soy Agar (TSA; Oxoid) and adjusted to an optical density equivalent to approximately 10^8 CFU/mL. Suspensions were further diluted in Mueller–Hinton broth (MHB) to obtain a final inoculum of 10^6 CFU/mL. Following inoculation, plates were incubated at 37 °C for 18–24 h. MIC, MIC_{50}, and MIC_{90} values were determined.

Minimum bactericidal concentration (MBC) was determined only for carvacrol by subculturing 100 µL from the MIC well and the two higher concentrations onto TSA plates, followed by incubation at 37 °C for 24 h.

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2.3. Antimicrobial Interaction Test

The combined effect of carvacrol and tetracycline was evaluated in 14 representative tetracycline-resistant *S.* Typhimurium strains using the checkerboard method as previously described [20].

Briefly, twofold serial dilutions of tetracycline (11 concentrations) and carvacrol (7 concentrations) were prepared in 96-well microplates. Combinations were tested starting from concentrations equivalent to 4×MIC of each compound for the corresponding strain. Each well contained 100 µL of standardized bacterial inoculum and appropriate volumes of both antimicrobial agents. Positive (growth) and negative (sterility) controls were also included, and plates were incubated at 37 °C for 24 h under aerobic conditions.

The Fractional Inhibitory Concentration Index (FICI) was calculated for each antimicrobial combination using Microsoft Excel (Microsoft Corporation, USA) according to the following formula:

$$\mathrm{FICI}_{\mathrm{index\ x.y}} = \mathrm{FIC}_{\mathrm{CA\ x.y}} + \mathrm{FIC}_{\mathrm{TET\ x.y}}$$

$$\mathrm{FIC}_{\mathrm{CA\ x.y}} = \frac{[CA]\ well\ x.y}{MIC_{CA}}$$

$$\mathrm{FIC}_{\mathrm{TET\ x.y}} = \frac{[TET]\ well\ x.y}{MIC_{TET}}$$

where [CA] and [TET] represent the concentrations of carvacrol and tetracycline in combination that inhibited bacterial growth, whereas MIC_{CA} and MIC_{TET} correspond to the MIC values of each compound when used alone. Interpretation of FICI values followed the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [24]: synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1), indifferent (1 < FICI ≤ 4), or antagonistic (FICI > 4). For each strain, the lowest FICI value obtained in the plate was considered the final result.

2.4. Growth Curve Test

Growth curve assays were performed to determine the subinhibitory concentrations of carvacrol, tetracycline, and their combination for subsequent gene expression analysis. Several subinhibitory concentrations derived from checkerboard assays were initially evaluated. Final concentrations selected (Table 1) for RNA extraction and gene expression analysis ranged from 0.015× to 0.062× MIC for both compounds and were chosen based on their ability to permit bacterial growth and progression to the exponential phase without complete growth inhibition. The assay was conducted over 24 h following the methodology described by Giovagnoni et al. [25].

Table 1. Subinhibitory concentrations of carvacrol (CA) and tetracycline (TET) selected for growth curve assays, RNA extraction, and gene expression analysis in *Salmonella* Typhimurium isolates.

Strain	Interaction profile	MIC_{CA} (mg/mL)	MIC_{TET} (µg/mL)	Selected concentration
1	Synergistic	2	512	0.015x MIC_{CA} + 0.015x MIC_{TET}
2	Synergistic	2	256	0.031x MIC_{CA} + 0.031x MIC_{TET}
3	Additive	2	512	0.015x MIC_{CA} + 0.015x MIC_{TET}
4	Additive	4	256	0.015x MIC_{CA} + 0.015x MIC_{TET}
5	Indifferent	2	256	0.031x MIC_{CA} + 0.015x MIC_{TET}
6	Indifferent	1	256	0.062x MIC_{CA} + 0.062x MIC_{TET}

MIC: minimum inhibitory concentration; CA: carvacrol; TET: tetracycline. Concentrations were selected from checkerboard-derived subinhibitory conditions that allowed bacterial growth to progress to the exponential phase without complete growth inhibition.

Six strains were selected to represent the different interaction profiles identified in the checkerboard assay (synergistic, additive, and indifferent), allowing evaluation of gene expression responses across the range of phenotypic interactions observed. Bacterial cultures were prepared

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from overnight growth on tryptic soy agar (TSA, Oxoid) and inoculated into Mueller–Hinton broth (MHB) to an initial optical density (OD_{595}) of 0.05. Cultures were incubated at 37 °C under aerobic conditions in the presence of carvacrol, tetracycline, or their combination at the selected concentrations. A control without antimicrobial agents was included. Bacterial growth was monitored by measuring optical density (OD_{595}) at 1 h intervals until the stationary phase was reached, with a final measurement at 24 h, using a Multiskan FC spectrophotometer (Thermo Scientific).

2.5. RNA Extraction and cDNA Synthesis

Bacterial samples were collected during the mid-exponential growth phase for each experimental condition, as determined by the growth curve assay. Cells were harvested by centrifugation at 4,000 rpm for 20 min at 4 °C, washed three times with phosphate-buffered saline (PBS), and resuspended in PBS. The resulting pellets were stored at −80 °C until further processing. For cell lysis, bacterial pellets were resuspended in TRI Reagent® and mechanically disrupted using a combination of 0.1 mm and 0.5 mm silica beads (Benchmark Scientific®) in a bead-beating system (Genie™, CViral). The disruption protocol consisted of four cycles of 1 min each, with intermediate cooling on ice. Lysates were clarified by centrifugation at 15,000 rpm for 5 min, and supernatants were collected and stored at -20 °C until RNA extraction.

Total RNA was extracted using the Direct-zol™ RNA Miniprep Plus kit (Zymo Research) according to the manufacturer's instructions. RNA was eluted in DNase/RNase-free water and quantified using a NanoDrop Lite Plus spectrophotometer (Thermo Scientific). To remove residual genomic DNA, samples were treated with the DNA-free™ Kit (Invitrogen) following the manufacturer's instructions. Purified RNA preparations were stored in RNase-free tubes at −80 °C. Complementary DNA (cDNA) synthesis was performed using SuperScript™ II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. The resulting cDNA was purified using the ZR-96 ChIP DNA Clean & Concentrator kit (Zymo Research) and stored at −20 °C until further analysis.

2.6. Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed to evaluate the expression of tetracycline resistance genes. Five candidate reference genes (*16S rRNA*, *icdA*, *rpoD*, *gapA*, and *gmk*) were initially assessed for transcript stability, and the most stable genes were selected for normalization. The target genes analysed were *tetA* and *tetB*. Primer sequences and their respective melting temperatures (Tm) are listed in Table 2.

Table 2. Primer sequences, melting temperatures (Tm), and amplification efficiencies of target and candidate reference genes evaluated for qPCR normalization.

Gen	Sequence (5′–3′)	Product size (bp)	Tm	Reference
tetA-F	GCTACATCCTGCTTGCCTTC	115	60°C	[8]
tetA-R	*ACCTGCCTGGACAAAATTGC*			
tetB-F	CCGAAGTAGGGGTTGAGACG	103	60°C	[8]
tetB-R	TGGCCTATCAATTGCGCTGA			
16S rRNA-F	AGGCCTTCGGGTTGTAAAGT	244	55°C	[26]
16S rRNA-R	GACTCAAGCCTGCCAGTTTC			
icdA-F	TGGTATCGGTGTTGATGTCACTC	140	54°C	[27]
icdA-R	CATCCTGGCCGTAAACCTGTGTG			
rpoD-F	GTGAAATGGGCACTGTTGAACTG	131	60°C	[28]
rpoD-R	TTCCAGCAGATAGGTAATGGCTTC			
gapA-F	GGTGTTGACGTAGTGGCTGAA	204	60°C	[29]

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gapA-R	AGCGTTGGAAACGATGTCC			
gmk-F	TTGGCAGGGAGGCGT TT	84	60°C	[30]
gmk-R	GCGCGAAGTGCCGTAGTAAT			

Reactions were carried out using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in a final volume of 10 µL, containing 5 µL of SYBR Green Supermix, 0.5 µL of each primer (10 µM), 2 µL of cDNA (1 ng/µL), and 2 µL nuclease-free water. All qPCR reactions were performed in technical triplicate, and mean Ct values were used for subsequent analyses. Assays were performed using a CFX Duet Real-Time PCR System (Bio-Rad), and data were analysed using CFX Maestro software.

The amplification protocol consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at the specific primer melting temperature (Tm) for 30 s (Table 2). A melting curve analysis (65–95 °C) was performed at the end of each run to confirm amplification specificity.

To ensure the absence of genomic DNA contamination and reagent impurities, negative controls including no-template controls (NTC) and no-reverse transcriptase (no-RT) controls were included in each run.

2.7. Relative Gene Expression Analysis

Relative gene expression was calculated using the Pfaffl method [31], which accounts for differences in amplification efficiency between target and reference genes.

Primer efficiency (E) was determined from standard curves generated using serial tenfold dilutions of *Salmonella* genomic DNA (50 to 0.005 ng/µL) extracted using the QIAamp DNA kit (Qiagen).

Efficiency for each primer pair was calculated from the slope (m) of the standard curve using the equation $E = 10^{-1/m}$. Efficiency was additionally expressed as a percentage: $E\ (\%) = (10^{(-1/m)} - 1) \times 100$. Only primer pairs with efficiencies between 90 and 110% were considered acceptable or ideal.

The stability of candidate reference genes (*16S rRNA, icdA, rpoD, gapA, and gmk*) was evaluated across all experimental conditions. Following geNorm principles [32], a multigene normalization approach was applied using the two most stable genes, *rpoD* and *gmk.*

Relative expression ratios were calculated using a modified Pfaffl model with multiple reference genes, using the geometric mean of the reference genes as follows:

$$Ratio = \frac{E_{gen}^{\Delta Ct\ (\text{control}-\text{sample})}}{\sqrt[2]{E_{ref1}^{\Delta Ct-ref1\ (\text{control}-\text{sample})} \times E_{ref2}^{\Delta Ct-ref2\ (\text{control}-\text{sample})}}}$$

The control condition corresponded to bacteria grown in MHB without antimicrobial agents, whereas treated samples included exposure to carvacrol, tetracycline, or their combination.

To specifically assess the modulatory effect of carvacrol during combined exposure, relative expression of *tetA* and *tetB* was additionally calculated using the tetracycline-only treatment as calibrator (CA+TET/TET). Ratios were $\log_2$-transformed [$\log_2$(CA+TET/TET)] for interpretation, where negative values indicate downregulation and positive values indicate upregulation relative to tetracycline treatment.

2.8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0.1 (GraphPad Software, USA). Due to the small sample size and variability among isolates, gene expression data were primarily analysed descriptively. Where appropriate, non-parametric tests were applied. Paired comparisons between *tetA* and *tetB* expression were evaluated using the Wilcoxon matched-pairs signed-rank test. Differences were considered statistically significant at $p \leq 0.05$.

 doi:10.20944/preprints202607.1007.v1

3. Results

3.1. Antimicrobial Susceptibility of S. typhimurium Isolates to Carvacrol and Tetracycline

For carvacrol, MIC values against the 73 *Salmonella* strains ranged from 0.5 to 16 mg/mL. The most frequent MIC value was 2 mg/mL (61.6%), followed by 4 mg/mL (19.2%) and 1 mg/mL (13.7%) (Figure 1A). The MIC_{50} and MIC_{90} values were 2 mg/mL and 4 mg/mL, respectively. MBC values coincided with their corresponding MICs in 84.9% of the isolates, suggesting a predominantly bactericidal effect. The most frequent MBC value was 2 mg/mL, followed by 4 mg/mL and 1 mg/mL (Figure 1A). The MBC_{50} and MBC_{90} values were 2 mg/mL and 8 mg/mL, respectively.

Regarding tetracycline, more homogeneous MIC values were observed among the 14 selected isolates. Seven isolates (50%) exhibited an MIC of 256 µg/mL, representing the MIC_{50} value, whereas five isolates showed an MIC of 512 µg/mL, corresponding to the MIC_{90} value (Figure 1B).

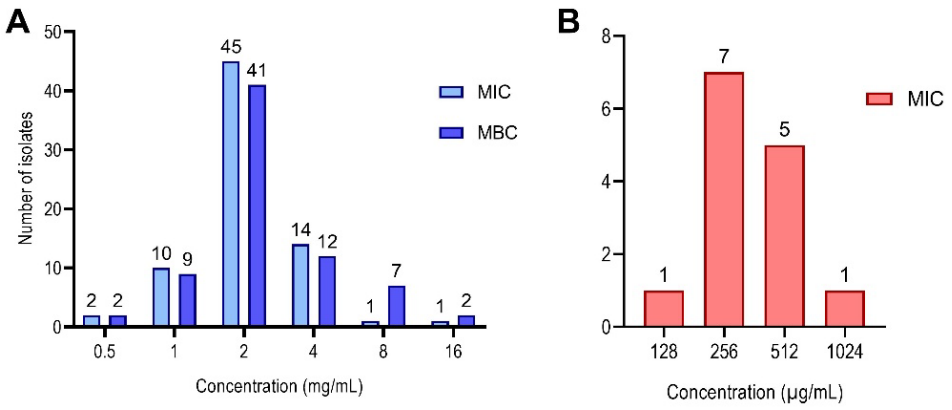

Figure 1. Distribution of antimicrobial susceptibility values for carvacrol and tetracycline. (A) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol against 73 *S.* Typhimurium isolates. (B) MIC values of tetracycline against 14 selected tetracycline-resistant *S.* Typhimurium isolates. Values are expressed in mg/mL for carvacrol and µg/mL for tetracycline.

3.2. Interaction Between Carvacrol and Tetracycline

The interaction between carvacrol and tetracycline varied among isolates, revealing strain-dependent antimicrobial responses in the 14 *S.* Typhimurium isolates tested. Synergistic activity (FICI ≤ 0.5) was observed in 14.3% (n = 2) of the isolates, using combinations of 2 mg/mL carvacrol with 32 or 64 µg/mL tetracycline (Table 3). In these isolates, the inhibitory concentration of carvacrol remained unchanged, whereas the effective concentration of tetracycline was reduced eightfold when used in combination.

Table 3. Summary of checkerboard interaction outcomes between carvacrol and tetracycline against 14 multidrug-resistant *S.* Typhimurium strains.

Interaction Outcome	FICI Range	Number of Strains (n = 14)	Most Effective Combination
Synergistic	≤ 0.5	2 (14.3%)	Carvacrol: 2 mg/mL (1×MIC) Tetracycline: 32–64 µg/mL (0.125×MIC)
Additive	>0.5–1.0	7 (50%)	Carvacrol: 0.25–2 mg/mL (0.25–2×MIC) Tetracycline: 64–256 µg/mL (0.25–0.5×MIC)
Indifferent	>1.0–4.0	4 (28.6%)	Carvacrol: 0.125–4 mg/mL (0.125–4×MIC) Tetracycline: 16–256 µg/mL (0.0625–0.5×MIC)
Antagonistic	>4.0	1 (7.1%)	Carvacrol: 1 mg/mL (0.5×MIC)

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	Tetracycline: 128 μg/mL (0.25×MIC)

Abbreviations: FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration. Interpretation criteria: synergistic (FICI ≤ 0.5), additive (0.5 < FICI ≤ 1), indifferent (1 < FICI ≤ 4), antagonistic (FICI > 4).

Additionally, additive interactions were detected in 50.0% (n = 7) of the isolates, while 28.6% (n = 4) showed indifferent responses. Antagonism was observed in one isolate (7.1%) (Table 3).

To further quantify the impact of the combination on tetracycline activity, MIC reduction was expressed as fold reduction relative to tetracycline used alone. Synergistic isolates showed the highest reduction, with a median of 8-fold (IQR: 8–8). Additive isolates exhibited a moderate reduction (median: 4-fold; IQR: 2–4), whereas indifferent isolates displayed lower reductions (median: 2-fold; IQR: 2–4). These findings are consistent with the interaction patterns observed in the checkerboard assay.

3.3. Effect on Resistance Gene Expression

Growth curve assays confirmed that the selected subinhibitory concentrations caused only moderate reductions in bacterial growth compared with untreated controls and did not prevent progression to the exponential phase. Consequently, samples for gene expression analysis were collected at the mid-exponential phase. Candidate reference genes exhibited variable expression stability across strains and treatment conditions; therefore, *rpoD* and *gmk* were selected as the most stable reference genes for combined normalization of qPCR data.

Under single-compound exposure, gene expression responses varied among isolates. Carvacrol alone generally produced lower or more moderate expression changes in *tetA* and *tetB*, whereas tetracycline treatment more frequently resulted in higher expression levels, particularly for *tetA*. Gene expression profiles across all treatment conditions are summarized in Figure 2.

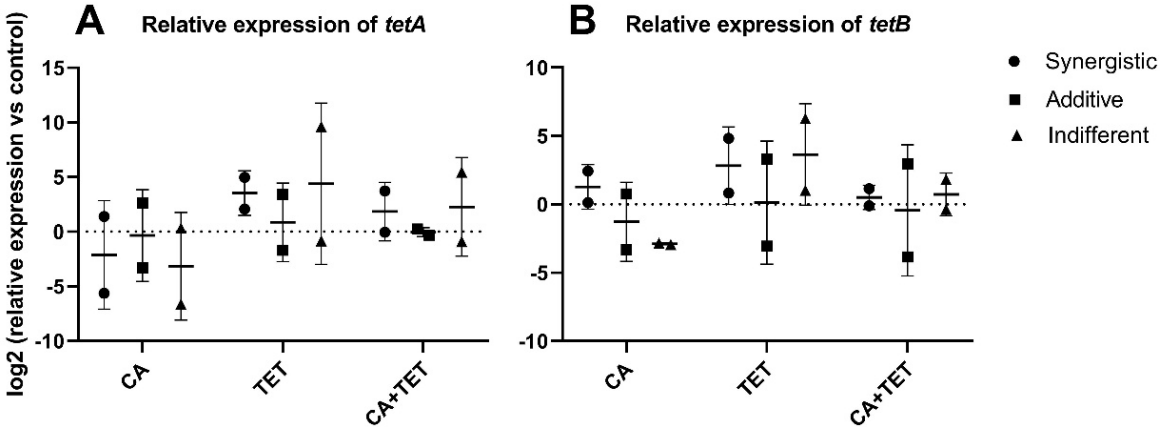

Figure 2. Relative expression of *tetA* (A) and *tetB* (B) in individual *S.* Typhimurium isolates under different treatment conditions. Data are expressed as log2 fold change relative to the untreated control (baseline = 0). Each symbol represents an individual isolate, categorized by its interaction phenotype: synergistic (circles), additive (squares), and indifferent (triangles). Horizontal lines indicate the median values for each group. CA: carvacrol; TET: tetracycline.

To assess the effect of carvacrol on the tetracycline response, gene expression under combined treatment was compared with tetracycline exposure alone (Figure 3). For *tetA*, relative expression under combined treatment was reduced in five of the six evaluated isolates (83.3%), with expression ratios ranging from 0.056- to 0.946-fold. Both isolates previously classified as synergistic showed decreased expression, whereas additive and indifferent isolates displayed more heterogeneous responses, including one isolate with increased expression (2.581-fold).

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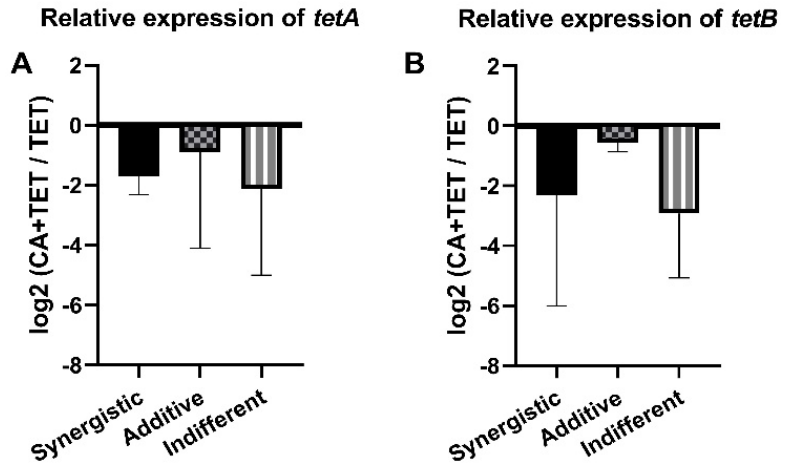

Figure 3. Modulatory effect of carvacrol on tetA (A) and tetB (B) expression during combined treatment. Data are expressed as $\log_2$ fold change of the combination (CA + TET) relative to tetracycline (TET) alone. Bars represent the mean ± SEM of isolates grouped by their interaction phenotype. Negative values indicate that the addition of carvacrol repressed gene expression compared to the antibiotic alone. CA: carvacrol; TET: tetracycline.

Regarding *tetB*, relative expression was also reduced in 83.3% (n = 5) of the isolates, with ratios ranging from 0.033- to 0.781-fold. One synergistic isolate showed marked repression (0.033-fold), whereas the remaining responses varied from moderate reduction to expression levels close to the tetracycline control.

When stratified by interaction phenotype, all synergistic and indifferent isolates exhibited downregulation of *tetA*. In contrast, *tetB* showed more variable responses in the synergistic group but consistent downregulation in additive and indifferent isolates. Overall, downregulation under combined treatment was observed in most of the isolates for both genes.

A paired comparison of gene expression under combined treatment revealed isolate-specific differences between *tetA* and *tetB* (Figure 4). Although both genes generally showed reduced expression relative to tetracycline alone, the magnitude and direction of the response varied between isolates, and no consistent pattern of differential regulation between *tetA* and *tetB* was detected ($p > 0.05$). Taken together, these findings indicate that the combined treatment induced heterogeneous but predominantly repressive effects on tetracycline resistance-associated gene expression.

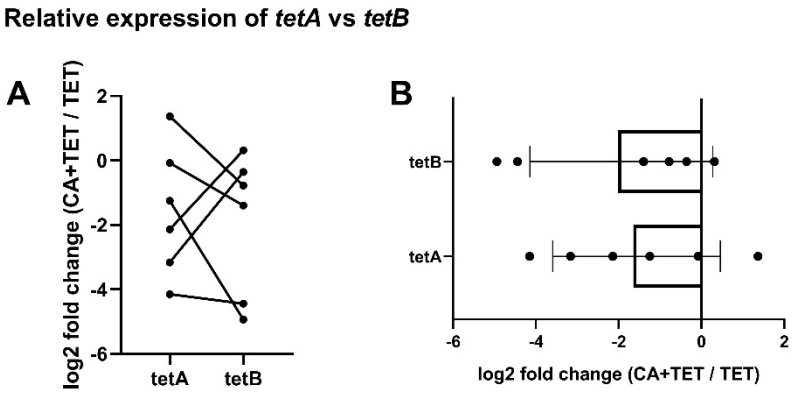

Figure 4. Comparative analysis of *tetA* and *tetB* modulation under combined treatment. Data represents the $\log_2$ fold change of the combination (CA + TET) relative to tetracycline (TET) alone. (A) Paired comparison of gene expression within individual isolates; lines connect *tetA* and *tetB* values for the same isolate to illustrate differential regulation patterns. (B) Distribution of relative expression values across all isolates; each point represents an individual isolate, and box plots indicate the median and interquartile range (IQR). No significant differences were detected between the expression levels of the two genes ($p > 0.05$).

 doi:10.20944/preprints202607.1007.v1

4. Discussion

The increasing dissemination of antimicrobial resistance (AMR), particularly among multidrug-resistant (MDR) *Salmonella* Typhimurium strains of swine origin, represents an important challenge for both public and animal health, as resistance increasingly compromises the efficacy of conventional antimicrobials, including tetracyclines [33,34].

In the present study, the antimicrobial effect of carvacrol was evaluated against 73 swine-derived *S.* Typhimurium strains. MIC values ranged from 0.5 to 16 mg/mL, with variability observed among strains. MBC values coincided with the MIC values in most isolates, suggesting a predominantly bactericidal effect, consistent with the results described by Berdejo et al. [35] and Trevisan et al. [19]. Similar findings have also been reported in recent studies describing the antimicrobial efficacy of carvacrol-rich essential oils against *Salmonella* isolates from the swine food chain [36,37]. However, the concentrations observed in the present study were higher than those commonly reported in previous studies, in which the MIC of carvacrol against *S.* Typhimurium has typically ranged from 0.064 to 0.6 mg/mL [16,19,21,35,38,39]. Comparable carvacrol concentrations have nevertheless been reported against pathogens with high physiological tolerance including *Pseudomonas aeruginosa* and multidrug-resistant *Acinetobacter baumannii* [40,41]. Cho and Lee [42] reported MIC values of 15 and 16 mg/mL against *Staphylococcus aureus* and *Escherichia coli* O157:H7 strains in milk, respectively, likely reflecting reduced carvacrol bioavailability in a lipid-rich food matrix rather than a methodological discrepancy. These findings suggest that higher concentrations of carvacrol may be required in bacterial populations with reduced susceptibility or high antimicrobial pressure.

In addition to strain variability, MIC values may also be influenced by experimental conditions and the physicochemical properties of carvacrol. Factors such as pH, medium composition, and solvent selection may affect its antimicrobial activity, bioavailability, and interaction with bacterial membranes [43,44]. In the present study, 0.1% DMSO was used as a solubilizing agent, consistent with previous studies evaluating hydrophobic compounds such as carvacrol [45–47].

Furthermore, the selected isolates exhibited high tetracycline resistance levels (MIC_{90} = 512 µg/mL), highlighting the need to identify compounds capable of enhancing tetracycline efficacy against resistant *Salmonella* strains.

The combination of carvacrol and tetracycline showed strain-dependent interactions, ranging from synergistic and additive to indifferent and antagonistic responses. In strains where synergistic interactions were observed, up to an eightfold reduction in the effective concentration of tetracycline was achieved. Similar findings have been reported for tetracycline-based combinations. Palaniappan and Holley [48] demonstrated synergistic interactions between carvacrol and tetracycline against *S.* Typhimurium and *Escherichia coli*, whereas Huerta Lorenzo et al. [49] reported predominantly additive effects between *Origanum vulgare* essential oil (containing 63.01% carvacrol) and oxytetracycline in multidrug-resistant *Salmonella enterica* isolates. More broadly, carvacrol has also been shown to enhance the activity of different antibiotic classes [48,50]. Consistent with our findings, Solarte et al. [51] also reported strain-dependent responses in *S.* Typhimurium, supporting the notion that the efficacy of antimicrobial combinations may vary according to both the isolate and the antimicrobial used.

The synergistic and additive interactions observed in the present study may plausibly be explained by the ability of phenolic compounds such as carvacrol and thymol to alter membrane integrity and interfere with efflux pump activity, thereby increasing intracellular antibiotic accumulation and enhancing antimicrobial efficacy [50,52]. However, the interaction between carvacrol and tetracycline varied among isolates, ranging from synergistic to indifferent and antagonistic responses. Similar isolate-dependent variability was reported by Cirino et al. [53] in clinical isolates of *Acinetobacter baumannii*, where differential responses to carvacrol and thymol combinations were associated with distinct resistance profiles identified through genomic analysis. These findings underscore the importance of strain-specific factors when evaluating the efficacy of antimicrobial combinations against MDR pathogens.

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Growth curve assays using subinhibitory concentrations of carvacrol, tetracycline, and their combination allowed moderate growth inhibition while maintaining progression to the exponential phase. Conditions causing complete growth inhibition or insufficient bacterial growth were excluded, as these may substantially alter transcriptional responses and compromise downstream gene expression analyses [54,55]. Under these conditions, gene expression analyses revealed differential responses depending on the antimicrobial treatment applied.

Exposure to tetracycline alone resulted in increased expression of both *tetA* and *tetB*, particularly the former. This finding is consistent with the role of tetracycline as a inducer of tetracycline resistance determinants, including efflux-associated *tet* genes. Among these determinants, *tetA* is one of the most frequently detected tetracycline resistance genes in *Salmonella* spp. [7].

The combination of tetracycline and carvacrol resulted in a decrease in the relative expression of *tetA* and *tetB* compared with tetracycline-only treatment in five of the six isolates. This effect may be related to the mechanism of action of carvacrol. Due to its lipophilic nature, carvacrol can insert into the bacterial lipid bilayer, increasing membrane permeability, depolarizing the membrane, and dissipating the proton motive force (PMF) [56]. Since tetracycline efflux systems such as TetA and TetB depend on the transmembrane proton gradient for antibiotic extrusion, disruption of PMF may interfere with efflux activity and promote increased intracellular accumulation of tetracycline [52,57,58]. Consequently, the combined effect of carvacrol and tetracycline may enhance intracellular antibiotic retention and potentiate ribosomal inhibition [6], which may contribute to the reduced expression of resistance-associated genes observed under combined exposure. Overall, reduced expression of *tetA* and *tetB* was observed in 83.3% of the isolates under combined exposure, with repression ratios as low as 0.056-fold in some strains. Although previous studies have mainly focused on the modulation of non-specific efflux systems such as AcrAB-TolC [59,60], the present findings suggest that carvacrol may also influence the expression of tetracycline-specific resistance determinants. Notably, the consistent downregulation observed for both genes suggests a coordinated transcriptional response rather than a gene-specific effect, potentially reflecting indirect modulation of broader stress-response pathways involved in antimicrobial adaptation and efflux regulation [61,62].

The results of the present study are consistent with previous reports describing resistance gene modulation following exposure to carvacrol or related phenolic compounds. Shen et al. [63] demonstrated that carvacrol combined with streptomycin enhanced susceptibility in antibiotic-resistant strains through the downregulation of resistance-associated genes. Similarly, Bonetti et al. [64] reported reduced expression of resistance determinants following combined treatment with thymol and antibiotics in field strains of *Escherichia coli*. Beyond antimicrobial resistance, carvacrol has also been associated with altered expression of genes involved in quorum sensing [65,66], motility [63], virulence [25], and biofilm formation [67,68], supporting its broader modulatory effect on bacterial physiology.

However, resistance gene expression varied among strains, particularly in isolates exhibiting additive or indifferent interactions. Such heterogeneity is common in *Salmonella* populations and may reflect genomic plasticity, variability in resistance-associated regulatory networks, and adaptive responses to antimicrobial stress [62,69]. In *Salmonella*, resistance determinants such as *tetA* and *tetB* are frequently associated with plasmids, transposons, and diverse regulatory elements, which may influence both basal and inducible expression levels [70]. Moreover, Tet efflux systems are regulated through complex mechanisms involving TetR repressors and proton motive force-dependent transport.

Because tetracycline resistance determinants are embedded within broader regulatory networks, the observed transcriptional changes may also reflect indirect modulation of global stress-response pathways involved in membrane homeostasis and efflux regulation. Consequently, differences in membrane physiology, regulatory sequences, or intracellular tetracycline accumulation may substantially influence transcriptional responses among strains [33,70,71]. Previous exposure to antibiotics, disinfectants, or environmental stressors in animal production systems may further

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contribute to the selection of strains with distinct metabolic and stress-adaptation strategies, resulting in isolate-dependent responses under combined antimicrobial exposure [35,39].

Although subinhibitory concentrations varied among isolates, no consistent relationship between concentration level and gene expression changes was observed, suggesting that isolate-specific regulatory responses may play a more important role than concentration alone. Interestingly, isolates previously classified as synergistic exhibited more consistent downregulation patterns, whereas additive and indifferent isolates showed greater variability, including one isolate with increased *tetA* expression. These findings suggest that modulation of *tet* gene expression may partially contribute to the observed interaction phenotype between carvacrol and tetracycline, although antimicrobial activity is likely multifactorial and also involves membrane destabilization and other stress-associated mechanisms.

Overall, the present findings support the potential use of carvacrol as an adjuvant to improve tetracycline activity against multidrug-resistant *Salmonella* strains. Carvacrol exhibited intrinsic antimicrobial activity and, in combination with tetracycline, was associated with reduced expression of key resistance determinants in most isolates, potentially contributing to improved antimicrobial efficacy. Nevertheless, the variability observed among strains highlights the importance of considering isolate-dependent responses when evaluating antimicrobial combinations. These findings support the future evaluation of carvacrol-based strategies to improve antimicrobial efficacy in swine-associated *Salmonella* populations. Further studies integrating transcriptomic and proteomic approaches are warranted to better understand the molecular mechanisms underlying the interaction between carvacrol and tetracycline.

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