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Effect of combined piperine and amoxicillin as an efflux pump inhibitor for metal-tolerant and antibiotic – resistant *E. coli* in broilers

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Abstract

Background: The co-existence of heavy metal and antibiotic resistance in *Escherichia coli* in poultry farms has emphasized the need for improved therapeutic approaches. This study investigates the combination of piperine as an efflux pump inhibitor, with amoxicillin for enhancing antibacterial efficacy, focusing on pharmacokinetics, residue depletion, and mechanistic assay. Twelve confirmed *E. coli* isolates were serotyped and examined for antibiotic and heavy metal resistance by disc-diffusion and PCR. Amoxicillin and piperine were tested for minimal inhibitory concentration (MIC) and fractional inhibitory concentration index (FIC) against O125 isolate containing *bla*CTX, *pocR*, *cadA*, and *zntA* genes and resistant to ampicillin, amoxicillin, amoxicillin + clavulanic acid, cefoxitin, cefotaxime, oxytetracycline, and doxycycline isolate. Toxicity and oxidative markers for piperine were determined. Twenty-two-day-old chicks were divided into five groups: negative, positive controls, and treatment groups receiving amoxicillin, piperine, and their combination orally post-challenge for five days. Drug concentrations in serum and tissues were quantified using a validated HPLC method followed by pharmacokinetic and residue depletion analyses. Amoxicillin followed a two-compartment model; piperine showed one-compartment kinetics. *E. coli* counts, gene expression, and heavy metal concentrations were measured at different time points.

Results: Amoxicillin and piperine displayed MICs of 32 and 5 µg/mL, respectively; their combinations had FIC of 0.2518. Piperine is well-tolerated and improved antioxidant status at 100 mg/kg feed. In vivo co-administration with amoxicillin resulted in increased amoxicillin C_{max} and AUC, decreased clearance, and enhanced amoxicillin exposure and tissue persistence, with residues remaining compliant with established MRLs by Day 32 (5 days post-cessation), alongside reduced efflux-associated resistance gene expression and lower tissue concentrations of zinc, copper, and cadmium.

Conclusions: Piperine demonstrated a dual functional role as a pharmacokinetic enhancer and resistance modulator in *E. coli*-infected broiler chickens. Integrating phyto-genic bioenhancers into antimicrobial stewardship programs may offer a promising approach to mitigate resistance development.

Keywords: Antimicrobial resistance, heavy metal, resistance, efflux pump, piperine, *E. coli*.

Background

Avian colibacillosis is a widespread infectious disease affecting poultry, caused by avian pathogenic *Escherichia coli* (APEC *E. coli*) that induces a septicemic condition characterized by air sacculitis, pericarditis, perihepatitis, peritonitis, and high mortality rates [1].

Amoxicillin, a broad-spectrum β lactam antibiotic, is often used to treat different bacterial infections in poultry, particularly those caused by *E. coli* [2]. Identifying pharmacokinetics parameters and tissue distribution of antibiotic are important factors that affect their therapeutic effectiveness and residual depletion in edible tissues. Previous studies on the oral administration of amoxicillin in poultry revealed high absorption, moderate bioavailability, short half-life, and minimal tissue binding with the highest distribution in the kidney, liver, and muscle, respectively [3, 4].

Antimicrobial resistance has emerged in the poultry sector due to extensive use of antibiotics such as β -lactams, tetracyclines, macrolides, aminoglycosides, and fluoroquinolones for prophylactic, therapeutic, and growth-promoting purposes [5, 6]. In parallel, contamination with heavy metals is a growing concern in agricultural and farming ecosystems. Metals such as copper (Cu), zinc (Zn), and cadmium (Cd), chromium (Cr), arsenic (As), and lead (Pb) are widely used as food additives, growth promoters, or immunostimulants in poultry farms [5, 6]. In environments where heavy metals and antibiotics coexist, bacteria can develop resistance to both through a process known as co-selection [5, 6]. Several studies have demonstrated the co-occurrence of heavy metal resistance genes, such as (*zntA*, *arsB*, *merA*, *pcoA*, *pcoC*, *chrA*, *PbRT*, *cadD*, *czc*) with antibiotic resistance genes, including (*sul1*, *sul2*, *sul3*, *tetA*, *tetB*, *tetC*, *blaTEM*, *blaSHV*, *blaCTX*, *blaNDM*, and *mcr-2* [7-9]. Efflux pumps, particularly the resistance-nodulation-division (RND) family such as (AcrAB–TolC, CusCBA, and CzcABC), play a key role in this process. These transporter protein systems actively expel a wide range of antibiotics and toxic heavy metals from bacterial cells, facilitating the co-selection and dissemination of these resistance genes through mobile genetic elements [7, 8]. This dual resistance highlights the urgent need for innovative strategies that improve antibiotic efficacy while minimizing resistance development and residue concerns.

Efflux pump inhibitors (EPIs) have emerged as promising agents for RND-mediated efflux resistance by blocking drug efflux pumps, enhancing antibiotic efficacy, and reducing the dissemination of co-resistance determinants [10]. Piperine, a bioactive alkaloid derived from black pepper, has attracted attention due to its ability to modulate drug metabolism, inhibit efflux transporters, and improve drug bioavailability [11, 12]. Piperine has increased the

bioavailability of many drugs such as cefotaxime sodium, amoxicillin trihydrate, and cephadroxi I monohydrate in rats [13]. The co-administration of piperine with marbofloxacin and gatifloxacin have been shown to significantly improved their pharmacokinetics and safety profile in chickens [14, 15]. To date, limited studies have investigated the combination of piperine, an efflux pump inhibitor, with amoxicillin in broiler chickens. Therefore, this study investigated the effect of piperine on the pharmacokinetics, tissue distribution, and antimicrobial efficacy of amoxicillin in broiler chickens experimentally infected with a multidrug-resistant *Escherichia coli* strain harboring the *blaCTX*, *pocR*, *cadA*, and *zntA* genes, with a focus on overcoming efflux-mediated resistance and metal-associated co-resistance within a One Health framework.

Methods

Identification of *E. coli* isolates

A total of 12 *E. coli* isolates were previously recovered from internal organ samples (liver, heart, cecum, trachea, and lung) collected from 44 different enteric diseased broiler farms (1 to 35 days of age) from six governorates (Alexandria, Behera, Cairo, Dakahlia, Giza, and Menofia). The identification was done using buffered peptone water (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and Mac-Conkey agar plates (Oxoid Limited, Thermo Fisher Scientific Inc., UK) according to Sorour et al. [16] and confirmed biochemically as described by Algammal, El-Sayed [17]. Serotyping of *E. coli* isolates was carried out in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Egypt (RLQP, Egypt) according to Lee, Jang [18] using commercial antiserum (SIFIN, Berlin, Germany).

Antibiotic sensitivity test

The antibiogram of 12 *E. coli* isolates was performed by disc-diffusion test against 9 antimicrobial discs in accordance with [19]. The antibiotic discs used: ampicillin (AMP) (10 µg), amoxicillin (AX) (30 µg), amoxicillin + clavulanic acid (AMC) (30 µg), ceftazidime (CAZ) (30 µg), cefotaxime (CTX) (30 µg), cefoxitin (FOX) (30 µg), cephalexin (CL) (30 µg), oxytetracycline (OT) (30 µg), doxycycline (Do) (30µg).

Detection of antibiotic and heavy metal resistance genes using uniplex PCR

All *E. coli* isolates were examined for antimicrobial and heavy metal resistance genes listed in table 1 as described by [20-23]. DNA was extracted using the QIAamp DNA mini kit (Qiagen- Germany- GmbH). PCR was performed in a 25 µL reaction volume containing 5 µL of template DNA, 12.5 µL of ABT 2X Red Mix (Applied Biotechnology, Egypt), 5.5 µL of nuclease-free water, 1 µL of each forward and reverse primer. The reaction was

performed in an applied biosystem 2720 thermal cycler. The PCR products were electrophoresed on 1.5% (w/v) agarose gel, stained by ethidium bromide. The results were documented using a U.V transilluminator.

Table 1. The primers, sequences, amplicon sizes used in the detection of heavy metals and antibiotic-resistant genes using conventional PCR

Type	Heavy metals / antimicrobials	Gene	Primer sequences	Amplicon size	Reference
HRGs	Copper	<i>pcoR</i> -f	CAGGTCGTTACCTGCAGCAG	636	Trajanovska et al [24]
		<i>pcoR</i> -r	CTCTGATCTCCAGGACATATC		
	Cadmium	<i>cadD</i> -f	AATTGCAAGTTGTGGTGCAG	155	Argudin et al. [25]
		<i>cadD</i> -r	CCCACACCAGGAATTCTAGC		
	Zinc	<i>zntA</i> -f	CGA CTC CTG ACA ATC ACGGC	636	Trajanovska et al [24]
		<i>zntA</i> -r	CATTGTCGGCATCGACCACC		
ARG	βeta-lactams	<i>BlaCTX-M</i> -f	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	Archambault et al [27]
		<i>BlaCTX-M</i> -r	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

HRGs: Heavy metal resistant genes and ARGs: antimicrobial resistant genes

Determination of minimum inhibitory concentration of amoxicillin and piperine

The minimum inhibitory concentration of amoxicillin and piperine against *E. coli* isolate containing was assessed using the microbroth dilution method according to Clinical and Laboratory Standards Institute guidelines [24, 25]. Amoxitop 20% was purchased from ATCO pharma, Egypt while piperine 10% ($\geq 98\%$ purity) was obtained from Sigma-Aldrich, USA. Their concentrations were ranged from 64 $\mu\text{g/mL}$ to 0.25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ to 0.02 $\mu\text{g/mL}$, respectively. The selected range to define the *E. coli* against amoxicillin based on the break point (Sensitive ≤ 8 , Intermediate =16, Resistant ≥ 32) [25]. The *E. coli* used was O125 isolate number (7) containing *blaCTX*, *pocR*, *cadA*, and *zntA* genes and resistant to ampicillin, amoxicillin, amoxicillin + clavulinic acid, cefoxitin, cefotaxime, oxytetracycline, and doxycycline. *E. coli* inoculum (10^5 CFU/ml) was prepared as described by [25]. Briefly, 0.1 mL of sterile Muller Hinton broth (Oxoid Limited, Thermo Fisher Scientific Inc., UK) was inoculated in rows A and B a 96-well plate in wells 1–10. After that, 0.1 mL of amoxicillin (64 $\mu\text{g/mL}$) and piperine (50 $\mu\text{g/mL}$) were two-fold serially diluted across wells 1–10, followed by the addition of 0.01 mL of *E. coli* inoculum (10^5 CFU/ml) in wells 1–11. Controls included in wells 11-12 as: negative control (0.1 mL of sterile Muller Hinton broth) and positive control (0.01 mL of *E. coli* inoculum in 0.1 mL of sterile Muller Hinton broth). Rows C and D inoculate with serial dilutions of (0.05 mL of amoxicillin (64 $\mu\text{g/mL}$) and piperine (50 $\mu\text{g/mL}$) in 0.05 mL of sterile

Muller Hinton broth), respectively and served as drug controls. The plates were sealed and incubated at 37 °C and monitored for 24 hours. The assay was performed in triplicates on independent days. The MIC was determined as the lowest concentration of amoxicillin and piperine that produced no visible growth. The MBC was determined after subculturing of samples from clear wells at 37 °C for 24 hours which revealed no colonies on the plate count agar media (Oxoid Limited, Thermo Fisher Scientific Inc., UK).

Determination of synergy between piperine with amoxicillin using checkerboard method

The synergistic interaction between amoxicillin and piperine was tested against *E. coli* O125 isolate no. (7) as described by Jarrar, Abu-Hijleh [26]. *E. coli* O125 isolate (7) was containing *bla*CTX, *pocR*, *cadA*, and *zntA* genes and phenotypically resistant to ampicillin, amoxicillin, amoxicillin + clavulanic acid, cefoxitin, cefotaxime, oxytetracycline, and doxycycline. In rows A and B, amoxicillin at 1/2 MIC was tested with two-fold serially diluted concentrations of piperine starting from 4 MIC and vice versa. The assay was performed in triplicates on independent days. Rows C and D inoculated with dilutions of each drug as previously described in MIC assay and served as drug controls. The fractional inhibitory concentration (FIC) of each amoxicillin and piperine was calculated by dividing the MIC of their combination by the MIC of each one alone. The FIC index = FIC of piperine + FIC of amoxicillin and interpreted as synergistic (FIC < 0.5), indifferent (FIC = 0.5–4), and antagonistic (FIC > 4) [27].

***In Vivo* acute tolerance and subacute safety testing**

A total of 120 one- days old Ross broiler chicks obtained from commercial hatchery in Giza. For each experiment, sixty chicks were randomly assigned to four groups (Control, Low dose, Mid dose, High dose). Each group consisted of three replicates with five chicks per replicate. Lighting and temperature were maintained according to Ross broiler management specifications [28]. All chicks had free access to water and a standard basal starter and grower diet to meet the nutritional standards of the National Research Council (NRC) [29].

For acute tolerance testing, group 1 received corn oil on day 7 and served as negative control group. Chicks in groups 2, 3, and 4 were treated via oral gavage with piperine suspended in corn oil (Merck, Germany) at low dose, mid dose, high dose of 50, 100, and 200 mg/kg body weight (single dose), respectively. Animals were observed for 14 days for mortality and clinical signs.

For subacute safety evaluation, group 1 received standard basal diet for 1-28 days. Chicks in groups 2, 3, and 4 received microencapsulated piperine obtained from (Evonik Nutrition & Care GmbH, Germany) mixed into feed for 1-28 days at 50, 100, and 200 mg/kg, respectively. Animals are observed for 28 days for mortality and clinical signs.

Growth performance metrics, including body weight gain was tested on 0, 7, 14, 21, and 28 days, alongside with feed conversion ratio calculated cumulatively on day 28. Serum biochemistry enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Roche Diagnostics, Germany), and oxidative stress markers such as malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD) (Cayman Chemical, USA) were assessed on day 28.

Effect of piperine, amoxicillin, and their combination on metal-tolerant and antibiotic – resistant *E. coli* in broilers

Bacterial strain

The experimental design used the same *E. coli* O125 isolate (7) used in MIC and FIC assays, which contained the *blaCTX*, *pocR*, *cadA*, and *zntA* genes and was phenotypically resistant to ampicillin, amoxicillin, amoxicillin + clavulanic acid, cefoxitin, cefotaxime, oxytetracycline, and doxycycline.

Sample size

The sample sizes per group were calculated as described by Arifin and Zahiruddin [30] according to Resource Equation method:

$n = \frac{DF}{K} + 1$ (Degree of freedom of the error (DF) are lie between minimum (10) and maximum (20) and K is number of treatments groups (5).

Minimum $n = \frac{10}{5} + 1 = 3$ animals/group and maximum $n = \frac{20}{5} + 1 = 5$ animals/group. However, the number of samples used in the present study per group was ($n=15$). This design allowed for the euthanasia of three birds per replicate across all groups at each time point (5 time points), maintaining consistent group sizes for chronological assessment.

Experimental design

According to [31-33], a total of 225 one day old Ross chicks were purchased from a commercial hatchery in Giza Governorate, Egypt. Ten extra chicks were examined to be free from *E. coli* before starting of the experiment. The birds were raised in a biosecurity level- II room in separated cages and provided with feed and water until the end of the experiment at the 32th day. Lighting and temperature were maintained according to [28] On day 22, chicks were arranged in a randomly blinded allocated design into five groups. Each group consisted of three replicates with twelve chicks per replicate. Groups 1 and 2 were inoculated intraperitoneally (I.P) on day 22 with 0.5 ml of sterile 0.85% saline solution and selected *E. coli* strain (0.5 ml containing 10^8 CFU/ml), respectively and served as negative and positive controls. Chicks in groups 3, 4, and 5 were challenged with the selected *E. coli* strain on day 22 and

treated orally on days 23- 27 with amoxicillin (15 mg/kg body weight), piperine (60 mg/kg body weight), and mix of amoxicillin and piperine (15 and 60 mg/kg body weight), respectively. Mortality and clinical signs were observed throughout the experiment. Scoring of lesions in air sac, heart, and liver in different groups was performed on days 24 and 27 of the experimental study according to Rawiwet and Chansiripornchai [34] as follows: The air sac lesions were scored as follows: 0 no lesions, 1 cloudiness, 2 thickness, 3 accumulations of a cheesy exudate. The pericardial lesions were scored as follows: 0 no lesions, 1 cloudy fluid, 2 extensive fibrination. The hepatic lesions were scored as follows: 0 no lesions, 1—definite fibrination on the surface, 2—extensive fibrination. Birds were subjected for Euthanasia using a gaseous concentration of 45% carbon dioxide to gradually render them unconscious [35].

Evaluation criteria

1- Bacterial analysis

The liver, Kidney, lung and heart of euthanized chickens were removed aseptically and examined on 24 and 27 days for *E. coli* counting on Eosine Methylene Blue (EMB) agar plates [36].

2- Expression of efflux pump associated genes

A reverse transcriptase real-time PCR was used to determine the effect of treatments with amoxicillin, piperine, and their combination on the expression of efflux pump genes (*acrA*, *acrB*, *tolC*) in the selected *E. coli* isolate. The expression levels were normalized to the endogenous control *rpsL* as a housekeeping gene. Liver samples were collected from euthanized chickens on day 27 and preserved in RNA Later (Cat No: AM7021, ThermoFisher Scientific, USA) to protect the mRNA intact at -20 °C until extraction. The RNA extraction has been accomplished with RNeasy Mini Kit (Cat no. / ID. 74104). Then, the extracted RNA was converted into complementary DNA with Xpert cDNA Synthesis Kit (Cat no. GK80.0100). Gene expression testing was performed using Maxima SYBR Green/ROX qPCR Master Mix (Cat. No. K0221) following the kit's instructions. The amplification was performed as described by [37] using specific primers for the *acrA*, *acrB*, *tolC* and *rpsL* genes, at concentration of 300 nM.

Their sequences were outlined as *acrA* F CTCTCAGGCAGCTTAGCCCTAA R TGCAGAGGTTTCAGTTTTGACTGTT, *acrB* F GGTCGATTCCGTTCTCCGTTA R CTACCTGGAAGTAAACGTCATTGGT, and *tolC* F AAGCCGAAAAACGCAACCT R CAGAGTCGGTAAGTGACCATC, and *rpsL* F GCAAAAACGTGGCGTATGTACTC R TTCGAAACCGTTAGTCAGACGAA. The formula of $2^{(-\Delta\Delta C_t)}$ was used to calculate the fold changes in the expression of genes [38].

3) Pharmacokinetic and tissue distribution study

Stock solutions of amoxicillin and piperine were prepared in methanol: water (50:50, v/v). An intermediate solution (100 mg/L) was prepared by serial dilution. Matrix-matched calibration standards were prepared by spiking blank serum, muscle, liver, and kidney samples from untreated birds to final concentrations of 10–1000 µg/L for each analyte. Quality control samples were prepared independently at three levels corresponding to 0.5×, 1×, and 2× the maximum residue limit (MRL) of amoxicillin (50 µg/kg), in accordance with [33, 39] bioanalytical method validation guidelines. All calibration and QC samples were processed using the same extraction and chromatographic procedures as study samples.

For pharmacokinetic study, blood samples were collected from groups 3, 4, and 5 from the wing vein at 0 (pre-dose), 0.5, 1, 2, 4, 8, 12, and 24 h following the amoxicillin administration on day 23. Serum was separated (4,000 × g, 10 min, 4 °C) and stored at –80 °C. Amoxicillin and piperine were extracted and quantified from serum samples as described by Fadel et al. [40] with some modifications in the chromatographic conditions. An Agilent 1200 series HPLC system (Agilent Technologies, Germany) equipped with a reversed-phase C18 column (250 × 4.6 mm, 5 µm, 100 Å) was used for the analysis. This method was conducted under isocratic mobile phase of 0.02 M phosphate buffer adjusted to pH 3.0 and acetonitrile (93:7, v/v) at a flow rate of 1.0 mL/minute. The column temperature was maintained to 35 °C and sample injection volume was 20 µl. Detection of analytes was achieved using a UV-Vis detector set at a wavelength of 230 nm. Sample extraction and preparation were performed according to Fadel et al. [40] using protein precipitation with acetate buffer (pH 5.0) and acetonitrile (3:1, v/v). The method was validated following ICH Q2(R1) guidelines [39], confirming linearity ($r^2 \geq 0.995$), precision (RSD < 5%), accuracy (95–105%), and appropriate LOD/LOQ values. Pharmacokinetic modeling and parameter estimation were conducted in accordance with EMA guidance for target animal species [41].

For residue depletion study, birds were euthanized in groups 3, 4, and 5 on days 28, 30, and 32. Tissue samples from muscle (pectoralis major), liver, and kidney were excised for residue determination. Samples were homogenized in acetonitrile, centrifuged, filtrated using (0.22 µm) pore syringe filters (Corning, NY, Germany), evaporated, and reconstituted [42].

4) Mechanistic assays

To explore piperine's modulatory role, liver, kidney, and lung samples were collected from groups 3, 4, and 5 on day 27 and examined for zinc, copper, and cadmium concentrations using atomic absorption spectrophotometry

(A7942 SensAA Dual, GBC Scientific Equipment, Braeside, Victoria, Australia) following standard procedures for trace metal determination in biological tissues [43]. This analysis was performed to evaluate potential interactions between heavy metals, antimicrobial exposure, and resistance modulation.

Statistical analyses

Two-way ANOVA with Tukey's post hoc test was used to study the difference in the log₁₀ *E. coli* count in different treatments and organs at 24 and 27 days. The log₁₀ *E. coli* counts were recorded as means ± SE. One-way ANOVA with Tukey's post hoc test was used to assess the difference in growth performance, biochemical markers, and oxidative stress parameters, expression for each gene, and concentrations of heavy metals in different treatment groups. Mean lesion scores in air sac, liver and heart were calculated as sum of scores divided by the total number of euthanized chicks. Mann-Whitney test was used to compare mean lesion score at each time point in different treatment groups. The P-values were considered significant at values ≤ of 0.05.

Results

Isolation and identification of *E. coli*

All isolates were confirmed to be *E. coli* and confined to 10 serotypes. The most common serotypes were O125, O111, O86, and O91 in percentages of 26.38, 22.22, 20.83, and 12.5% followed by O78 and O44 with a percentage of 4.16% each. O127 and O55 were recorded in a percentage of (2.77%) each. One strain of O119 and O166 was recorded in a percentage of 1.38% each.

Antimicrobial sensitivity of *E. coli* strains

Twelve isolates were screened for their sensitivity to different β-lactams and tetracyclines. Among penicillin group, the isolates showed highest resistance to ampicillin and amoxicillin in percentages of 91.6% each (Table 2). Among cephalosporins, the highest resistance was recorded against first generation cephalixin in a percentage of 75% followed by second generation cefoxitin and cefotaxime (66.7%) (Table 2). The resistance of examined *E. coli* isolates against tetracyclines (oxytetracycline and doxycycline) were nearly similar (Table 2).

Table 2. Distribution of resistance genes and antimicrobial resistance profile in twelve selected multi-resistant isolates

Strain	Serotype	Resistance genes		Antimicrobial resistance
		ARGs	HRGs	
1	O166	–	<i>zntA</i>	AMP, AX, CL, CTX, AMC, DO, OT
2	O91	–	<i>zntA</i>	CL, FOX, CAZ, AMC, OT
3	O125	–	<i>zntA</i>	AMP, AX, FOX, AMC, DO

4	O55	–	<i>zntA</i>	AMP, AX, CL, FOX, CAZ, CTX, AMC, OT
5	O111	<i>blaCTX</i>	<i>zntA</i>	AMP, AX, CL, FOX, CAZ, CTX, AMC, DO
6	O125	<i>blaCTX</i>	<i>pocR</i> , <i>zntA</i>	AMP, AX, CL, AMP, CL, DO
7	O125	<i>blaCTX</i>	<i>pocR</i> , <i>cadA</i> , <i>zntA</i>	AMP, AX, FOX, CTX, AMC, OT, DO
8	O127	<i>blaCTX</i>	<i>zntA</i>	AMP, AX, FOX, CTX, SXT, OT
9	O86	–	<i>pocR</i> , <i>zntA</i>	AMP, AX, CL, FOX, CAZ, CTX, AMC, DO
10	O44	–	<i>zntA</i>	AMP, AX, CL, OT
11	O119	<i>blaCTX</i>	<i>zntA</i>	AMP, AX, CL, FOX, CAZ, CTX, OT
12	O158	<i>blaCTX</i>	<i>pocR</i> , <i>zntA</i>	AMP, AX, CL, CAZ, CTX, AMC, OT

ARGs (Antibiotic resistance genes), HRGs (Heavy metal resistance genes), Ampicillin (AMP), Amoxicillin (AX), Amoxicillin + Clavulanic acid (AMC), Cefoxitin (FOX), Cefotaxime (CTX), Ceftazidime (CAZ), Cephalexin (CL), Oxytetracycline (OT), Doxycycline. (Do).

PCR detection of antibiotic and heavy metal resistance genes:

The *bla*-CTX gene was identified in 50% of isolates. Among heavy metal resistance genes, *zntA* gene was detected in all isolates, while *pocR* and *cadA* genes were detected in 33.3% and 8.3%, respectively (Table 2).

Determination of minimum inhibitory concentration of amoxicillin and piperine

Our study revealed that amoxicillin and piperine showed antibacterial properties against tested *E. coli* strain at MIC of 32 and 5 μgml^{-1} , respectively.

Determination of synergy between piperine with amoxicillin using checkerboard method

The combination of amoxicillin and piperine has a synergistic effect on the tested *E. coli* strain, as the FIC index was 0.2518 (Table S1).

	MIC μgml^{-1}	FIC	FIC index
Piperine (2-fold diluted) in combination with amoxicillin	1.25	FIC piperine in combination with amoxicillin = MIC piperine in combination with amoxicillin / MIC piperine alone = 1.25 / 5 = 0.25	= FIC piperine in combination with amoxicillin + FIC amoxicillin in combination with piperine = 0.25 + 0.0018 = 0.2518
Piperine alone	5		
Amoxicillin (2-fold diluted) in combination with piperine	0.06	FIC amoxicillin in combination with piperine = MIC amoxicillin in combination with piperine / MIC amoxicillin alone = 0.06 / 32 = 0.0018	
MIC amoxicillin alone	32		

FIC: Fractional inhibitory concentration

Acute and subacute tolerance for safety assessment of piperine

Overall, no mortality or severe adverse signs were observed. However, transient mild hypoactivity occurred at 200 mg/kg in acute tolerance testing and resolved within 6 hours. In subacute tolerance testing, one-way ANOVA with Tukey's post hoc test revealed no significant changes in the serum ALT and AST in different dose groups, while significant changes were recorded in the TAC, SOD, and MDA in the 100 mg/kg feed group compared to control and 50 mg/kg feed groups ($p \leq 0.05$) (Table 3). Furthermore, there is significant improvement in the growth performance in the 100 mg/kg group compared to other groups ($p \leq 0.05$) (Table 3).

Table 3. Growth performance, biochemical markers, and oxidative stress parameters in broiler chickens supplemented with microencapsulated piperine in subacute toxicity testing

Parameter	Control	50 mg/kg feed	100 mg/kg feed	200 mg/kg feed
Body weight (g)				
Day 0	40 ± 2 ^a	40 ± 2 ^a	40 ± 3 ^a	40 ± 2 ^a
Day 7	320 ± 15 ^a	330 ± 1.6 ^{ab}	340 ± 1.7 ^b	335 ± 16 ^b
Day 14	720 ± 25 ^a	740 ± 2.8 ^{ab}	770 ± 3.2 ^b	750 ± 29 ^b
Day 21	1150 ± 31 ^a	1180 ± 32 ^{ab}	1220 ± 34 ^b	1190 ± 33 ^{ab}
Day 29	1600 ± 35 ^a	1650 ± 36 ^{ab}	1700 ± 38 ^b	1660 ± 37 ^{ab}
Feed Conversion Ratio	1.70 ± 0.05 ^a	1.68 ± 0.04 ^a	1.60 ± 0.04 ^b	1.69 ± 0.05 ^a
ALT (U/L)	25 ± 0.3 ^a	26 ± 0.3 ^a	27 ± 0.4 ^a	30 ± 0.4 ^a
AST (U/L)	120 ± 1.02 ^a	122 ± 1.1 ^a	125 ± 1.2 ^a	132 ± 1.2 ^a
MDA (nmol/mL)	5.0 ± 0.4 ^a	4.8 ± 0.3 ^a	4.2 ± 0.3 ^b	4.6 ± 0.3 ^{ab}
TAC (mmol/L)	1.2 ± 0.1 ^a	1.3 ± 0.1 ^{ab}	1.5 ± 0.1 ^b	1.4 ± 0.1 ^{ab}
SOD (U/mL)	80 ± 0.5 ^a	82 ± 0.6 ^a	90 ± 0.6 ^b	85 ± 0.6 ^{ab}

Values are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test versus control ($p < 0.05$ considered significant). "ab" indicates no significant difference between those groups. ALT = alanine aminotransferase; AST = aspartate aminotransferase; MDA = malondialdehyde; TAC = total antioxidant capacity; SOD = superoxide dismutase; FCR = feed conversion ratio.

Effect of piperine, amoxicillin, and their combination on metal-tolerant and antibiotic – resistant *E. coli* in broilers

1. Clinical signs, mortalities and lesion scores

No mortalities were recorded in all treatment groups. The birds in groups 2, 3, 4, and 5 suffered from sneeze, depression, fever, cough, diarrhea and skin lesions at 1st day post challenge. In contrast, no signs were recorded in groups 1. Upon necropsy, no lesions were observed in the negative control group (I). A significant high air sac, hepatic and heart lesions score ranging from 1 to 3 was observed in the groups 2 and 3 (positive control and amoxicillin treated groups) on day 27 (Table 4). Low air sac and liver lesion scores (score = 1) were observed in

groups 4 and 5 treated with piperine and mix, respectively on day 27 (Table 4). Furthermore, no heart lesions were observed in these treated groups (Table 4).

Table 4. Scoring of air sac, heart, and liver lesions in different groups on days 24 and 27 of the experimental study

Air sac										
Group	Score on day 24				Mean lesion score	Score on day 27				Mean lesion score
	0	1	2	3		0	1	2	3	
2	0	0	15	0	2 ^a	0	0	0	15	3 ^a
3	0	0	15	0	2 ^a	0	0	0	15	3 ^a
4	0	0	15	0	2 ^a	0	15	0	0	1 ^b
5	0	4	11	0	1.4 ^b	0	15	0	0	1 ^b

Heart								
Group	Score on day 24			Mean lesion score	Score on day 27			Mean lesion score
	0	1	2		0	1	2	
2	0	15	0	1 ^a	0	0	15	1 ^a
3	0	15	0	1 ^a	0	0	15	1 ^a
4	0	15	0	1 ^a	15	0	0	0 ^b
5	4	11	0	0.7 ^b	15	0	0	0 ^b

Liver								
Group	Score on day 24			Mean lesion score	Score on day 27			Mean lesion score
	0	1	2		0	1	2	
2	0	0	15	2 ^a	0	0	15	2 ^a
3	0	0	15	2 ^a	0	0	15	2 ^a
4	0	0	15	2 ^a	0	15	0	1 ^b
5	0	4	11	1.4 ^b	0	15	0	1 ^b

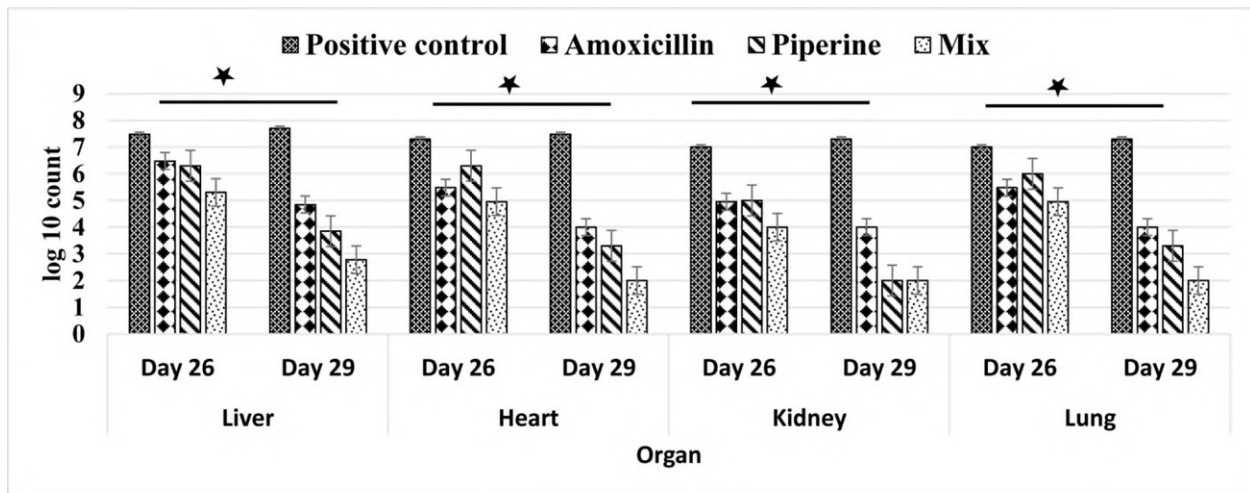
The air sac lesions were scored as follows: 0 no lesions, 1 cloudiness, 2 thickness, 3 accumulations of a cheesy exudate. The pericardial lesions were scored as follows: 0 no lesions, 1 cloudy fluid, 2 extensive fibrination. The hepatic lesions were scored as follows: 0 no lesions, 1—definite fibrination on the surface, 2—extensive fibrination. Values with different superscript letters (a and b) in the mean lesion score column for each time points are significantly different ($p > 0.05$). Number of euthanized birds in each group 15 (5 in each replicate) in each time point

2. *E. coli* counting in chicken organs

Two-way ANOVA with Tukey post hoc test revealed that there were significant differences in the *E. coli* count between all treatments at 24 and 27 days ($P < 0.05$) (Table S2 and Fig 1). In addition, there is no interaction between

type of organs and both time points and treatment type ($P > 0.05$) (Table S2). The results revealed lowest mean $\log_{10}$ *E. coli* count to 4.8 and 2.2 after treatment with mix of amoxicillin and piperine at 24 and 27 days (Fig 1). There is no significant difference in *E. coli* count after treatment with amoxicillin and piperine alone ($P > 0.05$) (Table S2). On the other hand, the highest mean $\log_{10}$ count was recorded in the positive control group (Fig 1). There is no significant difference in the count between different organs except liver and kidney ($P = 0.009$) (Table S2).

Fig 1. Bar chart represented *E. coli* counts in liver, heart, kidney, and lung in positive control and Amoxicillin, piperine, and mix treatment groups on days 24 and 27



Bar chart represents mean of *E. coli* counts ($\log_{10}$ CFU/gram of tissue) $\pm$ standard deviation that indicated by error bars. Asterisks indicated statistically significant differences in the count between organs ($P = 0.017$) using Two-way Anova post hoc test.

Table S2. Two-way Anova post hoc test results of the difference of *E. coli* counting in chicken organs in different treatment groups on days 24 and 27.

Source	P value
Between organs	0.017*
Between treatments	0.000*
Between organ and time points	0.977
Between organ and treatments	0.954
Between treatments and time points	0.000*
Between treatments	
Group 1 and groups 2, 3, 4, 4	0.000*
Groups 2 and 3	0.612
Groups 3 and 4	0.000*
Groups 4 and 5	0.014*

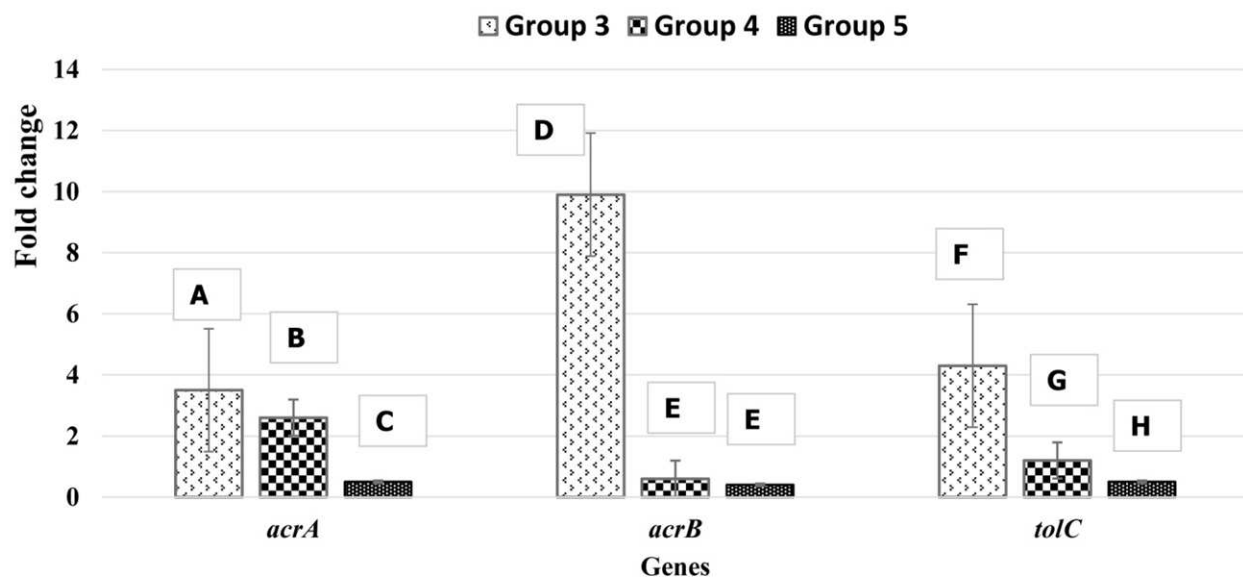
Between organs	
Liver and kidney	0.009*
Liver and heart	0.438
Liver and lung	0.277
Heart and kidney	0.302
Heart and lung	0.991
Kidney and lung	0.469

* Indicated significant differences in the gene expression between groups, groups 1 and 2 were assigned to negative and positive controls, respectively. Groups 3, 4, and 5 were challenged with *E. coli* ad treated with amoxicillin, piperine and mix

3. Expression of efflux pump genes

It was noticed that group 5 treated with piperine and amoxicillin showed the highest down-expression of *acrA*, *acrB*, and *Tol-C* genes with fold change of 0.5, 0.4 and 0.5 respectively (Fig 2). In contrast, groups 3 treated with amoxicillin only showed significant up regulation of the expression of *acrA*, *acrB* and *tolC* genes with fold change of 3.5, 9.9 and 4.3 respectively (Fig 2). Furthermore, group 4 treated with piperine only showed significant up regulation of the expression of *acrA* and *tolC* genes with fold change of 2.6 and 1.2, respectively (Fig 2). One-way Anova was used to assess the difference in the effect of each treatment on the expression of each gene, the results revealed there is no significant difference in the effect of piperine and mix treatments on *acrB* ($P > 0.05$). Furthermore, there is significant difference in the effect of all treatments on *acrA* and *Tol-C* genes ($P < 0.05$).

Fig 2. Expression of *acrA*, *acrB*, and *tolC* in the treatment groups on day 27

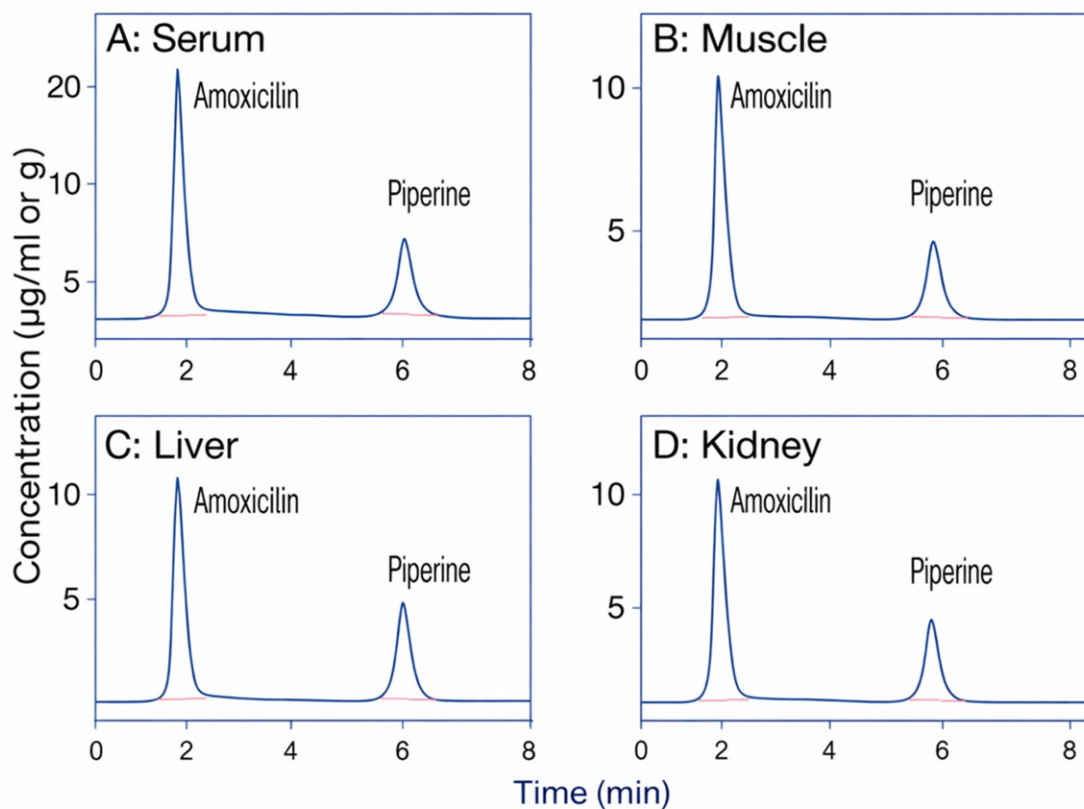


Groups 3, 4, and 4 were treated with amoxicillin, piperine, and mix, respectively. Results of relative expression for each gene in each treatment group were expressed as fold change relative to the control group. Different letters for each gene indicated statistically significant differences in the gene expression between treatments ($P < 0.05$), while gene with the same letter showed no significant difference between treatments ($P > 0.05$).

4. Method validation

The RP-HPLC method achieved clear baseline separation of amoxicillin and piperine in serum and tissue extracts with no endogenous interference (Fig 3). The validated method met international acceptance criteria. Extraction recoveries were 90% for amoxicillin and 95% for piperine across all matrices. The HPLC method showed excellent linearity for both analytes ($r \geq 0.995$) over the tested serum and tissue ranges. LODs were 0.005 $\mu\text{g/mL}$ or $\mu\text{g/g}$ for amoxicillin and 0.003 $\mu\text{g/mL}$ or $\mu\text{g/g}$ for piperine, while LOQs were 0.01 and 0.008 $\mu\text{g/mL}$ or $\mu\text{g/g}$, respectively. Precision was within 5% RSD and accuracy ranged from 95–105%, demonstrating method robustness and reproducibility in accordance with ICH Q2(R1) (2005).

Fig 3. Representative HPLC chromatograms of amoxicillin (20 $\mu\text{g/kg}$) and piperine (10 $\mu\text{g/kg}$) in different broiler matrices.



Chromatograms show retention peaks for amoxicillin (3.2 minutes) and piperine (6.1 minutes) in serum, muscle, liver, and kidney samples, confirming trace-level detection and matrix recovery. Co-administration with piperine significantly reduced apparent clearance and volume of distribution of amoxicillin, resulting in increased systemic exposure.

5. Pharmacokinetics, tissue residues, and modulatory effects of piperine on amoxicillin disposition and antimicrobial resistance in *E. coli* infected broiler chickens

Amoxicillin in group 3 exhibited reduced C_{max} and AUC with delayed T_{max} and a prolonged absorption half-life (t_{1/2}ka) (Table 5). Notably, co-administration of piperine in group 5 significantly increased amoxicillin C_{max} and AUC while reducing apparent clearance and t_{1/2}ka (Table 5). Also, piperine in group 5 showed similarly higher systemic exposure and a prolonged terminal half-life compared with piperine alone (group 4) (Table 5 and Fig 4). Tissue residue analysis demonstrated measurable concentrations of amoxicillin and piperine in muscle, liver, and kidney in amoxicillin treated group (group 2). Group 5 displayed elevated levels of amoxicillin and piperine residues across all tissues; however, their concentrations in all edible tissues fell below the maximum residue limit (MRL) by day 32 (5 days post-cessation of treatment).

Table 5. Pharmacokinetic parameters of amoxicillin and piperine in *E. coli*-infected broiler chickens

Group	t _{1/2} ka(h)	C _{max} (µg/mL)	T _{max} (h)	t _{1/2} β(h)	AUC _{0–24} (µg·h/mL)	CL (L/h/kg)	Vd (L/kg)
Gp3 Amoxicillin	0.63	15.8	1.5	10.9	135	0.45	1.6
Gp4 Piperine	1.15	2.8	2.0	12.3	30	0.80	2.1
Gp5 Amoxicillin	0.43*	20.5*	1.0	8.7	180*	0.35*	1.2*
Gp5 Piperine	0.77*	4.1*	1.5	14.0	52*	0.60*	2.5*

C_{max} = maximum serum concentration; T_{max} = time to reach C_{max}; t_{1/2} = elimination half-life; AUC_{0–24} = area under the concentration–time curve from 0–24 h; CL = apparent clearance; Vd = apparent volume of distribution. *Significantly different from corresponding single-treatment group (p < 0.05). Pharmacokinetic parameters were estimated using two compartmental analyses.

Table 6. Depletion of amoxicillin and piperine residues in edible tissues of *E. coli*-infected broiler chickens following cessation of treatment

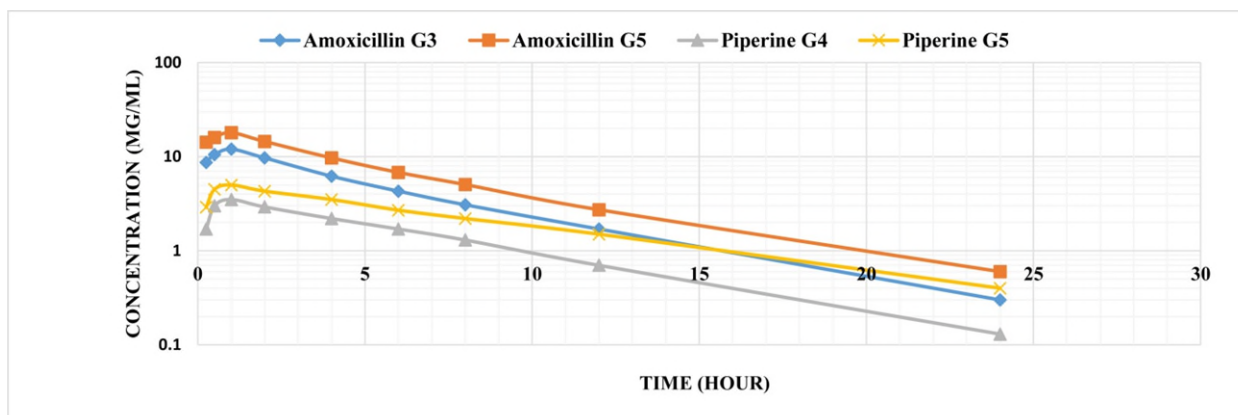
Tissue	G3 (Amoxicillin) (µg/kg)	G4 (Piperine) (µg/kg)	G5 (Amoxicillin) (µg/kg)	G5 (Piperine) (µg/kg)	Amoxicillin MRL (µg/kg)
Day 28 (1day post-cessation)					
Muscle	42 ± 0.6	15 ± 0.3	55 ± 0.7*	25 ± 0.4*	50
Liver	76 ± 0.9	30 ± 0.5	102 ± 1.01*	40 ± 0.6*	50
Kidney	90 ± 1.1	35 ± 0.6	120 ± 1.2*	48 ± 0.7*	50

Day 30 (3 days post-cessation)					
Muscle	18 ± 0.4	9 ± 0.2	28 ± 0.5*	14 ± 0.3*	50
Liver	32 ± 0.6	18 ± 0.4	48 ± 0.7*	22 ± 0.4*	50
Kidney	40 ± 0.7	20 ± 0.4	65 ± 0.8*	30 ± 0.5*	50
Day 32 (5 days post-cessation)					
Muscle	8 ± 0.2	ND	14 ± 0.3*	6 ± 0.2	50
Liver	12 ± 0.3	ND	20 ± 0.4*	8 ± 0.2	50
Kidney	15 ± 0.4	ND	25 ± 0.5*	10 ± 0.3	50

ND = Not detected (below LOQ) *Significantly higher than Group 3 at the same time point (p < 0.05).

Concentrations expressed as mean ± SD

Fig 4. Semilogarithmic serum concentration–time profiles of amoxicillin and piperine following oral administration in *Escherichia coli*–infected broiler chickens.



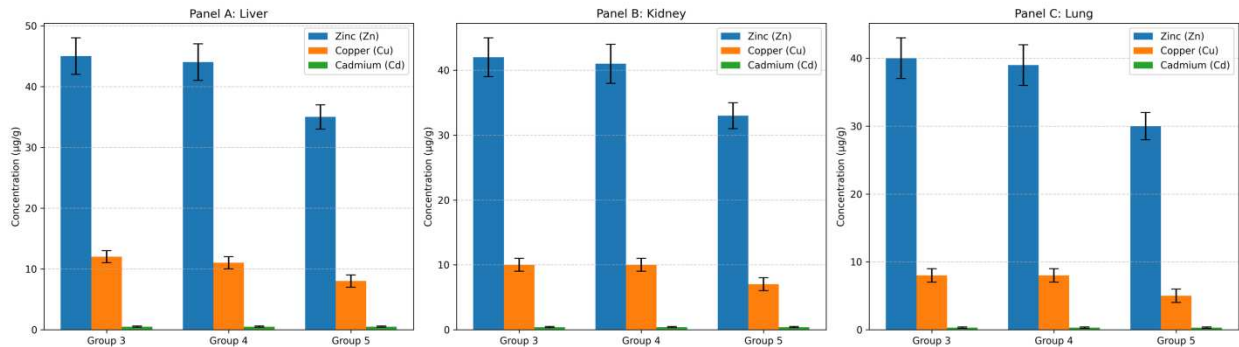
Concentration data are plotted on a logarithmic scale to illustrate elimination kinetics. Co-administration with piperine in group 5 increased systemic exposure of amoxicillin compared with amoxicillin alone (Group 3), while piperine concentrations were also elevated in the combined treatment group relative to piperine alone (Group 4).

6. Heavy-metal analysis

Zinc and copper were detected in all tissues at baseline levels needed in poultry in groups 3 and 4, but significant reductions were observed in group 5 ($p < 0.05$). Cadmium levels remained negligible in all tissues and treatments (Fig 5).

Fig 5. Organ-specific concentrations of zinc (Zn), copper (Cu), and cadmium (Cd) in liver, kidney, and lung of *E. coli*-infected broiler chickens treated with amoxicillin (group 3), piperine (group 4), and the amoxicillin–piperine combination (group 5).

Figure X. Organ-specific concentrations of zinc (Zn), copper (Cu), and cadmium (Cd) in broiler chickens



Zinc and copper were detected at background levels in all tissues in groups 3 and 4, but significant reductions were recorded in group 5 ($p < 0.05$). Cadmium levels remained negligible across all tissues and treatments. Data were expressed as mean $\pm$ SD.

Discussion

In this study, the *E. coli* serotypes used in this study were similar to those obtained in previous studies recovered from chickens [44-46]. In contrast, other serogroups have been reported to be predominant as O78, O2, and O53 [47]. In particular, the difference of the geographical region affects the antigenic diversity of APEC [47].

The antibiogram profiling of the isolates was similar to those obtained in previous findings in Egypt and Iran [48, 49] and higher than reported by Fatoba, Amoako [50] in South Africa. The findings revealed that the resistance to penicillin's was relatively higher than cephalosporins and amoxicillin clavulanic acid. This may be related to hydrolysis of ampicillin and amoxicillin by β -lactamases, which are produced by many bacteria compared to cephalosporins that is not effectively hydrolyzed by them. Therefore, amoxicillin clavulanic acid is frequently used as and β -lactamase inhibitor [51]. On the other hand, amoxicillin-clavulanic acid resistance was higher than tetracycline resistance may be related to widespread plasmid-mediated resistance mechanisms and extensive empirical, besides tetracycline resistance necessitates particular resistance mechanisms and subjected to less selective pressure [52, 53].

The *E. coli* isolates were further screened for ARG (*bla*CTX) and HMR (*pco*R, *cad*D, and *znt*A) genes using conventional PCR. β -lactam resistance is the common resistance encountered in *E. coli* which is caused by extended spectrum β -lactamase (ESBL) genes [54]. β -lactamase gene *bla*CTX was detected among 50% of the isolates that is similar to that recorded in previous studies (Ibrahim et al. [48]) and lower than reported in Sweden

and Pakistan [55, 56]. This argue might be related to that CTX-M genes are the most common ESBL and have a higher spread rate than other β -Lactamases [57]. This explains the high prevalence of β -lactams resistant isolates phenotypically.

Our results reported that *zntA* was detected in 100% of isolates, while, *pocR* was found in 33.3% of isolates. One isolate only was positive for *cadA* gene. Yang, Deng [8] recorded that the *zntA* gene was most widespread (77.0%), the frequency of was lowest (6.1%, the frequency of *merA* was lowest (2.5%). The prevalence of *zntA* and *pocR* might be related to their location: *pocR* is commonly found on plasmids, while *zntA* is normally integrated into the bacterial chromosome [58, 59]. Yang, Agouri [60] displayed that the *pocR* gene was associated with *bla*NDM-1 and *bla*CTX-M-15 β -lactamase resistance in Enterobacteriaceae. The high percentage of HMRs and ARGs in this study might be related to the using of metal-based feed additives (such as Cu and Zn) and the regular administration of antibiotics make intensive livestock production a significant source of HMRs and ARGs [61].

The efficacy of the combined amoxicillin and piperine was assessed *invito* using MIC and FIC tests. The results revealed enhanced antibacterial activity against selected *E. coli* isolate, reducing the MIC up to 16-fold compared to amoxicillin alone. This indicated by the synergistic interaction between both that confirmed by the FIC index. Similarly, Haroun and Al-Kayali [62] displayed that β -lactam antibiotics were among the most antimicrobial classes associated with a positive synergistic potential.

The efficacy of piperine, amoxicillin, and their combination against metal-tolerant and antibiotic-resistant *E. coli* was assessed in broilers. The determination of acute and subacute toxicity is a fundamental step in determining the safety and biological limits of piperine before using in poultry. Piperine is acute and subacute tolerated safe in broiler chickens up to 200 mg/kg feed, with optimal antioxidant and performance benefits at 100 mg/kg which is ethically compliant design. These findings align with previous reports of piperine supplementation improving oxidative status and productivity in poultry [63]. The challenged birds in the positive control group (group2) revealed necropsy findings such as pericarditis, airsacculitis, cellulitis, perihepatitis and this is consistent with previous findings of Glombowsky et al [31]. Amoxicillin treated group (group 3) displayed the failure of amoxicillin the to lower the *E. coli* count. This failure of treatment was related to the type of strain used in this study as the strain used was phenotypically and genotypically β -lactam resistant O125 strain. Groups 5 had the best growth performance. The use of piperine in group 4 significantly reduced the pathogenic bacterial counts; this might be related to the antibacterial effect of piperine as piperine could suppress the motility and cellular adhesion potential

of *E. coli* in particular [64, 65]. There are limited available data on the effect of piperine as an efflux pump inhibitor against *E. coli* in chickens. The transcriptional effect of piperine as efflux pump inhibitor on resistant *E. coli* isolates was assessed. Our results demonstrated that treatment with amoxicillin in group 3 revealed a marked upregulation of these genes, particularly *acrB*, which showed nearly a 10-fold increase in expression relative to the control. Previous studies have reported the relation between the elevated expression of efflux pump and resistance to multiple classes of antibiotics, including fluoroquinolones, β -lactams, aminoglycosides, tetracyclines, and macrolides [10, 66-68]. Acriflavine resistance B (*AcrB*) was identified as the catalytic core of the AcrAB-TolC efflux pump and energy transduction center for the drug/proton antiport process [69-71]. The expression of *acrB* in group 3 was higher than *acrA* and *tolC* that suggested an improvement in the substrate recognition and initiation of transport and reducing intracellular drug accumulation [72]. In contrast, treatment with piperine in group 4 significantly downregulated *acrB* expression, while *acrA* and *tolC* near baseline levels. This indicates that piperine may exert an inhibitory effect on efflux pump activity, particularly targeting the AcrAB-TolC system, which plays a major role in multidrug resistance in Gram-negative bacteria. Notably, the combination treatment in group 5 produced the strongest suppressive effect on all three genes, with significant downregulation of *acrA* and *acrB*, and *tolC* expression. These findings suggest a synergistic interaction between amoxicillin and piperine, thereby potentially restoring or enhancing antibiotic susceptibility and this is consistent with the obtained FIC index value. Studying pharmacokinetics and tissue distribution is critical for optimizing dosing regimens by determining systemic exposure and assuring that the drug reaches its therapeutic target. The present study demonstrates that piperine significantly modulates the pharmacokinetics and tissue distribution of amoxicillin in *E. coli*-infected broiler chickens. The selected amoxicillin dose is consistent with published poultry pharmacokinetic and therapeutic studies and lies within the dose range evaluated by the EMA-CVMP for amoxicillin use in food-producing animals, where 10–20 mg/kg has been shown to achieve effective plasma concentrations against susceptible pathogens [4, 73]. The extraction and analytical methods employed were robust and suitable for simultaneous quantification of a hydrophilic β -lactam antibiotic and a lipophilic phytochemical in complex biological matrices. Amoxicillin treated group (group 3) displayed extended the absorption phase which consequently lowered peak and overall drug exposure, indicating infection-associated impairment of intestinal absorption and distribution and this is consistent with the increment of *E. coli* count findings. In contrast, the observed enhancement of amoxicillin exposure in the presence of piperine in group 5 is consistent with the downregulation findings of efflux-associated resistance genes.

This confirms that piperine modulates efflux-associated resistance pathways, thereby increasing intracellular antibiotic exposure and partially reversing phenotypic resistance [74]. Similarly, previous reports displayed that piperine enhances drug bioavailability by inhibiting metabolizing enzymes and efflux transporters. [75-77]. Furthermore, Hiwale et al., [13] reported the ability of piperine to enhance the bioavailability of amoxicillin. Tissue residue analysis demonstrated that amoxicillin was preferentially distributed to kidney, followed by liver and muscle, consistent with its hydrophilic character and predominant renal elimination in poultry species [4, 73]. Co-administration with piperine in group 5 significantly increased amoxicillin concentrations across all examined tissues, indicating that piperine modifies drug disposition beyond plasma pharmacokinetics and enhances tissue persistence, through inhibition of drug-metabolizing enzymes and efflux transporters [18, 75]. The detection of piperine residues in edible tissues further confirms its systemic absorption and distribution, this is consistent with previous pharmacokinetic reports on its bioavailability and tissue penetration [78, 79]. From a regulatory perspective, the persistence of amoxicillin residues above the established maximum residue limit (MRL) in liver and kidney of the combination group indicated the delayed depletion kinetics. Previous studies displayed that amoxicillin residues in the monotherapy group fell below MRL thresholds within the anticipated withdrawal period (80, 81). Such prolongation of tissue residues under co-administration conditions may necessitate extension of withdrawal periods to ensure compliance with residue regulations following co-administration and minimize its risk in poultry products [79]. Co-administration of amoxicillin and enrofloxacin yielded comparable results, displaying altered clearance pattern and increased tissue binding [40].

In parallel, reduced tissue concentrations of zinc, copper, and cadmium were observed in the combination-treated birds. zinc and copper are recognized co-selective factors for antimicrobial resistance and may influence bacterial stress responses and efflux-associated resistance mechanisms [82-83]. As direct metal-binding assays were not performed, these findings interpreted as evidence of altered metal disposition rather than definitive proof of metal chelation. Trace metals are recognized inducers of efflux systems and co-resistance via mobile genetic elements, thereby promoting multidrug resistance even in the absence of antibiotic exposure [84].

Conclusions

Piperine co-administration significantly altered the pharmacokinetic disposition of amoxicillin in *E. coli*-infected broiler chickens, increasing systemic exposure and enhancing tissue persistence while downregulating efflux

511 resistance genes and lowering tissue heavy metal levels. Notably, amoxicillin and piperine residues fall below
 512 regulatory MRL limits by day 32 (5 days post-treatment), ensuring food safety compliance.

513 **Study limitations**

514 Some gene determinants of heavy metal resistance were present in all twelve tested isolates, so the absence of
 515 negative cases made statistical analysis of association between antibiotic and heavy metal resistance not feasible.
 516 Therefore, the results only support co-occurrence, but no correlation can be established. Further research is needed
 517 on the molecular mechanisms behind lowering levels of heavy metals, metal-binding or chelation assays
 518 formulation-related effects on drug disposition and commercial application.

519 **List of Abbreviations**

MIC	minimal inhibitory concentration
FIC	fractional inhibitory concentration index
C max	Peak serum or plasma concentration
AUC	Area Under the Curve
MRLs	maximum residue levels
APEC <i>E. coli</i>	avian pathogenic <i>Escherichia coli</i>
MFS	major facilitator superfamily
SMR	multidrug resistance family
MATE	multidrug and toxic compound extrusion
RND	resistance nodulation cell division family
ABC	ATP-binding cassette superfamily
EPIs	Efflux pump inhibitors
CLSI	Clinical and Laboratory Standards Institute guidelines
ALT	alanine aminotransferase
AST	aspartate aminotransferase
SOD	superoxide dismutase
MDA	as malondialdehyde
TAC	total antioxidant capacity
I.P	intraperitoneally
EMB	Eosine Methylene Blue
MRL	maximum residue limit
HMRs	Heavy metal resistant genes
ARGs	antimicrobial resistant genes
FCR	feed conversion ratio

520 **Declarations**

521 **Ethics approval**

522 Acute oral toxicity and subacute oral toxicity studies were carried out in accordance with ARRIVE 2.0 guidelines
523 and current animal welfare recommendations outlined in [85]. Acute oral toxicity was evaluated in accordance with
524 the Organization for Economic Co-operation and Development Test Guideline 223 [86].

525 The experimental study was approved by then Regulations of the Institutional Animal Care and Use Committee
526 (ARC-IACUC) (Agricultural Research Center) under registration number (ARC AHRI, 6725).

527 **Consent to participate**

528 Not applicable

529 **Consent for publication**

530 Non to declare

531 **Availability of data and material**

532 All data generated or analyzed during this study are included in this published article and its supplementary
533 information files. The authors confirm that the data supporting the findings of this study are available within the
534 article and its supplementary information. Preprint is available in research square

535 **Competing interests**

536 The authors declare that they have no competing interests.

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540 **Authors' contributions**

541 Reham A. Hosny, Amara F. Ebrahim, and Mai F. Abdelaty performed isolation, identification of *E. coli* and assess
542 their antibiotic susceptibility. Also, performed MIC and FIC testing for amoxicillin, piperine, and their combination
543 against *E. coli*. All researchers designed and performed the workflow of the experiment. Reham A. Hosny
544 performed all statistical analyses throughout the manuscript. Amany Adel and Zeinab A. El-badia studied the
545 presence of antibiotic resistance and heavy metal resistance genes and efficacy of amoxicillin, piperine, and their
546 combinations on the expression of efflux pump genes of *E. coli*. Mai A. Fadel conducted acute and subacute toxicity
547 studies of piperine; developed and applied an HPLC-based analytical method for quantification of amoxicillin and
548 piperine; performed pharmacokinetic, tissue residue, and mechanistic analyses; and conducted statistical analysis

and data interpretation. All researchers studied performed the experiment, wrote, revised and approved the manuscript.

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Non to declare

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Supporting information

Table S1. Fractional inhibitory index of the combined treatment of amoxicillin with piperine

Table S2. Two-way Anova post hoc test results of the difference of *E. coli* counting in chicken organs in different treatment groups on days 24 and 27

Figure legends

Fig 1. Bar chart represented *E. coli* counts in liver, heart, kidney, and lung in positive control and Amoxicillin, piperine, and mix treatment groups on days 24 and 27

Bar chart represents mean of *E. coli* counts ($\log_{10}$ CFU/gram of tissue) $\pm$ standard deviation that indicated by error bars. Asterisks indicated statistically significant differences in the count between organs ($P = 0.017$) using Two-way Anova post hoc test.

Fig 2. Expression of *acrA*, *acrB*, and *tolC* in the treatment groups on day 27

Groups 3, 4, and 4 were treated with amoxicillin, piperine, and mix, respectively. Results of relative expression for each gene in each treatment group were expressed as fold change relative to the control group. Different letters for each gene indicated statistically significant differences in the gene expression between treatments ($P < 0.05$), while gene with the same letter showed no significant difference between treatments ($P > 0.05$).

Fig 3. Representative HPLC chromatograms of amoxicillin (20 μ g/kg) and piperine (10 μ g/kg) in different broiler matrices.

Chromatograms show retention peaks for amoxicillin (3.2 minutes.) and piperine (6.1 minutes.) in serum, muscle, liver, and kidney samples, confirming trace-level detection and matrix recovery. Co-administration with piperine significantly reduced apparent clearance and volume of distribution of amoxicillin, resulting in increased systemic exposure.

Fig 4. Semilogarithmic serum concentration–time profiles of amoxicillin and piperine following oral administration in *Escherichia coli*–infected broiler chickens.

Concentration data are plotted on a logarithmic scale to illustrate elimination kinetics. Co-administration with piperine (group 5) increased systemic exposure of amoxicillin compared with amoxicillin alone (group 3), while piperine concentrations were also elevated in the combined treatment group relative to piperine alone (group 4).

Fig 5. Organ-specific concentrations of zinc (Zn), copper (Cu), and cadmium (Cd) in liver, kidney, and lung of *E. coli*-infected broiler chickens treated with amoxicillin (group 3), piperine (group 4), and the amoxicillin–piperine combination (group 5)

Zinc and copper were detected at background levels consistent with commercial poultry production, with modest but significant reductions in group 5 compared with group 3 ($p<0.05$). Cadmium levels remained negligible across all tissues and treatments. Data were expressed as mean $\pm$ SD.

Table legends

- Table 1. The primers, sequences, amplicon sizes used in the detection of heavy metals and antibiotic-resistant genes using conventional PCR.
- Table 2. Distribution of resistance genes and antimicrobial resistance profile in twelve selected multi-resistant isolates.
- Table 3. Growth performance, biochemical markers, and oxidative stress parameters in broiler chickens supplemented with microencapsulated piperine in subacute toxicity testing.
- Table 4. Scoring of air sac, heart, and liver lesions in different groups on days 24 and 27 of the experimental study
- Table 5. Pharmacokinetic parameters of amoxicillin and piperine in *E. coli*–infected broiler chickens.
- Table 6. Depletion of amoxicillin and piperine residues ($\mu\text{g/kg}$, mean $\pm$ SD) in edible tissues of *E. coli*–infected broiler chickens following cessation of treatment.