Poultry Science

Multidrug resistant and multivirulent avian bacterial pathogens: tackling experimental leg disorders using phytobiotics and antibiotics alone or in combination

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ABSTRACT Locomotor disorders caused by multidrug-resistant (**MDR**) bacterial pathogens denote one of the most detrimental issues that collectively threaten the poultry industry leading to pronounced economic losses across the world. Hence, searching for effective alternatives, especially those extracted from plant origins became of great priority targeting a partial or complete replacement of chemical antimicrobials to tackle their developing resistance. Therefore, we aimed to determine the prevalence and antimicrobial resistance of *Staphylococcus aureus* (***S. aureus***), *Salmonella* species, *Mycoplasma synoviae* (***M. synoviae***), and *Escherichia coli* (***E. coli***) recovered from 500 broilers and ducks (250 each) with locomotor disorders in various farms in Dakahlia and Sharkia Governorates, Egypt. Additionally, we assessed, for the first time, the *in vitro* antimicrobial effectiveness of marjoram, garlic, ginger and cinnamon essential oils (**EOs**) against MDR and multivirulent bacterial isolates as well as the *in vivo* efficiency of the most effective antibiotics and EOs either separately or in combination in the treatment of experimentally induced poultry leg disorders. The overall prevalence rates of *S. aureus, E. coli, Salmonella* species, and *M. synoviae* were 54, 48, 36, and 2%, respectively. *Salmonella* species and *S. aureus* prevailed among ducks and broilers (36 and 76%, respectively). Notably, MDR was observed in 100, 91.7, 81.1, and 78.5% of *M. synoviae, E. coli, Salmonella*, and *S. aureus* isolates, respectively. Our *in vitro* results displayed that marjoram was the most forceful EO against MDR and multivirulent chicken vancomycin-resistant *S. aureus* (**VRSA**) and duck *S.* Typhimurium isolates. The current *in vivo* results declared that marjoram in combination with florfenicol or amoxicillin/clavulanic acid succeeded in relieving the induced duck and chicken leg disorders caused by *S.* Typhimurium and VRSA, respectively. This was evidenced by improvement in the clinical and histopathological pictures with a reduction of bacterial loads in the

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experimental birds. Our encountered successful *in vitro* and *in vivo* synergistic effectiveness of marjoram combined with florfenicol or amoxicillin/clavulanic acid recommends their therapeutic application for leg disorders and offers opportunities for reducing the antibiotics usage in the poultry industry.

Key words: poultry, *Salmonella*, *S. aureus*, leg disorder, marjoram

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INTRODUCTION

The locomotive disorders represent eminent challenges to the poultry industry causing great economic problems worldwide. These disorders usually occur at 14 to 70 days-old, but mostly at 35 days-old and they are implicated in tenosynovitis, arthritis, synovitis, osteomyelitis, femoral head necrosis, and bumble foot in broiler chickens (Itakura et al., 1976). Bacterial arthritis frequently occurs after localized infection to the joints or septicemic infections and it is associated with several bacterial pathogens including *Staphylococcus aureus* (***S. aureus***), *Salmonella* species, *Mycoplasma synoviae* (***M. synoviae***), and *Escherichia coli* (***E. coli***), which have direct associations with leg pathologies in poultry (Abd El-Hamid et al., 2019a). This disorder is mostly caused by *S. aureus*, which is a common bacterial pathogen of chickens and ducks causing up to 15% mortality rate and reduction in the production performance with a consequence of significant economic losses (Shiozawa et al., 1980). Moreover, *E. coli* has been reported as one of the major bacterial agents associated with musculoskeletal infections in poultry (Chansiripornchai, 2009). *Salmonella* species and *M. synoviae* had been also proposed to be prominent causes of arthritis and they have been implicated in joint lesions in poultry (Abd El-Hamid et al., 2019a). *M. synoviae* infection is mostly subclinical, but in cases in combination with secondary bacterial infections, it becomes systemic and results in acute and chronic cases of infectious synovitis (Landman and Feberwee, 2012). The success of any bacterial species as a pathogen is affected by its extraordinary aptitude to express an enormous repertoire of specialized virulence attributes, which aid in the occurrence of diseases with a consequence of detrimental toxic effects (Ahmed et al., 2019; Awad et al., 2019; Bendary et al., 2022c).

The identification of bacterial agents incriminated in the locomotor disorders simplifies control and preventive measures in poultry flocks, which consequently decreases the resulted economic losses (Costa et al., 2016). Owing to the lack of efficient vaccines for bacterial diseases, antibiotic agents are widely recommended for controlling these diseases in poultry flocks. The nonjudicious utilization of antibiotics causes increased resistance rates to the frequently used ones in the poultry field (Ahmed et al., 2021; Ammar et al., 2021b; Aljazzar et al., 2022). Currently, this problem is intensified by the rising emergence of multidrug-resistant (**MDR**) bacterial species, which is becoming an increasing concern in the poultry sector (Ammar et al., 2021a).

From this point, it is crucial to search for alternative therapies to fight the virulent and MDR bacterial strains (El-Sheikh et al., 2021; Abd El-Hamid et al., 2022a). There is an intensifying demand for new natural antimicrobials to improve the control of bacterial pathogens incriminated in leg affections in the poultry sector. In this direction, thousands of phytochemicals such as essential oils (**EOs**) have been used as effective and safe promising natural alternative agents and their potential antimicrobial effects against a broad range of Gram-positive and Gram-negative bacterial species are under comprehensive investigations (Aljazzar et al., 2022; Hashem et al., 2022). The benefits of EOs over antibiotics are their great antibacterial potency without inducing any bacterial resistance. This potency has been presented by various EOs like marjoram, garlic, ginger and cinnamon during previous *in vitro* studies against avian bacterial pathogens (Abd El-Hamid et al., 2019a; Elmowalid et al., 2019). Moreover, the combination of EOs with antibiotics offers an optimistic solution for combating MDR bacteria (Langeveld et al., 2014). Yet, the *in vivo* efficacy of the above-mentioned EOs either alone or in combination with antibiotics against bacterial pathogens incriminated in avian leg disorders needs further inquiry.

Insight of the above-mentioned threats, this study was undertaken to determine the occurrence and antimicrobial susceptibility patterns of some bacterial pathogens recovered from broiler chickens and ducks presenting locomotor disorders in Sharkia and Dakahlia Governorates, Egypt. Additionally, the most recovered MDR chicken and duck bacterial isolates were further characterized via virulence genes profiling. Moreover, we sought to explore the *in vitro* antimicrobial activities of marjoram, garlic, ginger and cinnamon EOs against MDR and multivirulent bacterial isolates. Finally, we targeted to evaluate the antibacterial efficiency of the most effective antibiotics and EOs either separately or in combination in broiler chicken and duck experimental models of leg disorders.

MATERIALS AND METHODS

Ethical Statement

All the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Zagazig University with the reference number of ZU-IACUC/2/F/386/2022.

Clinical Examination and Sampling

Five hundred diseased broiler chickens aged 20 to 35 d and ducks aged 20 to 60 d (250 each); 300 from 60 farms in Dakahlia Governorate (150 birds/30 farms for each species) and 200 from 40 farms in Sharkia Governorate (100 birds/20 farms for each species) were included in this study. The investigated birds were clinically examined and clinical signs and postmortem (PM) lesions of freshly dead and sacrificed birds were recorded. Descriptive data of the investigated birds in Sharkia and Dakahlia Governorates are listed in Supplementary Tables 1 to 4. Swabs from synovial fluid and joint lesions were collected, labeled, and transported aseptically in an ice box to the Reference laboratory for Veterinary Quality Control on Poultry Production, Sharkia, Egypt for further bacteriological examination.

Microbiological Characterization of Avian Bacterial Isolates

Isolation and identification of *Salmonella* species were carried out via standard bacteriological techniques adopting the International Organization for Standardization (**ISO**) 6579-1 (ISO 6579-1, 2017). In brief, the collected samples were transferred to sterile buffered peptone water before being inoculated into Rappaport-Vassiliadis broth and incubated at 42°C for 24 h. Subsequently, a loopful of Rappaport-Vassiliadis broth was streaked onto MacConkey's and xylose lysine desoxycholate agar plates and the plates were incubated at 37°C for 24 h. For *E. coli* isolation, all collected samples were suspended in buffered peptone water, then the enrichment broth was streaked onto MacConkey's and eosin methylene blue agar media. Subsequently, the suspected *E. coli* and *Salmonella* cultures were identified using standard conventional phenotypic techniques (Cruickshank et al., 1975; Ammar et al., 2015; Bendary et al., 2022a,b; Elfaky et al., 2022). Furthermore, presumptive isolates were confirmed via API20E identification system (BioMérieux, Marcy l'Etoile, France) following the manufacturer's directions. The preliminarily identified *Salmonella* isolates were serotyped according to Kauffman-white scheme (Kauffman, 1974) using polyvalent and monovalent O and H antisera (Denka-Seiken, Tokyo, Japan). Moreover, serotyping of *E. coli* isolates was conducted via slide agglutination test using commercial polyvalent and monovalent O antisera (Test Sera Enteroclon, Berlin, Germany). Isolation of *S. aureus* was firstly performed onto mannitol salt and Paired Parker agar media. Primary phenotypic identification of *S. aureus* was carried out through standard bacteriological methods (ISO 6888-1, 2003) based on mannitol fermentation, β hemolysis, Gram-positive grape-like cocci appearance and biochemical reactions including catalase and coagulase tests (Abd El-Hamid and Bendary, 2015; Ammar et al., 2016a; Abd El−Hamid et al., 2019b; Abd El-Hamid et al., 2022b). Isolation of *M. synoviae* was conducted using Frey's broth and agar media under microaerophilic humid conditions (10% CO_2) at 37°C. Preliminary identification of *M. synoviae* was achieved using digitonin and traditional biochemical tests and definitive characterization was performed serologically via growth inhibition test employing definite antisera (Ammar et al., 2022; Awad et al., 2022). All media used for the isolation and identification of investigated bacterial isolates were provided by Oxoid (Oxoid, England, UK).

Molecular Identification of Avian Bacterial Isolates

On the subject of molecular characterization of the isolated *Salmonella* species, all the recovered isolates were molecularly screened at the genus level via PCR amplification of *inv*A (invasion A) gene, which is unique for genus *Salmonella* adopting the previously described PCR procedures (Oliveira et al., 2003; Ammar et al., 2016b). Moreover, the identities of *E. coli* isolates were confirmed by PCR assay based on alkaline phosphatase structural gene (***phoA***) specific primer and the conditions detailed previously (Hu et al., 2011). *S. aureus* isolates were molecularly identified through specific PCR amplification of the 23S ribosomal ribonucleic acid (***23S rRNA***) gene following the previously published PCR protocol (Bhati et al., 2016). Finally, all mycoplasma isolates were confirmed to be *M. synoviae* via PCR amplification using one primer set targeting variable lipoprotein hemagglutinin A (***vlhA***) gene according to the procedures previously reported (Jeffery et al., 2007; Abd El-Hamid et al., 2019a).

Antimicrobial Susceptibility Patterns of the Recovered Bacterial Isolates

Antimicrobial susceptibility rates of the recovered *Salmonella, E. coli*, and *S. aureus* isolates were detected by standard Kirby−Bauer disc diffusion approach as stated formerly (Bauer et al., 1966) utilizing commercial drug disks and Mueller Hinton agar medium (Oxoid); meanwhile, the *in vitro* susceptibility of all *M. synoviae* isolates to routine antimicrobials was determined via broth microdilution test (Hannan, 2000). The activities of amoxicillin/clavulanic acid (20/10 μg, **AMC**), ampicillin (10 μg, **AMP**), neomycin (30 μg, **N**), streptomycin (10 μg, **S**), oxytetracycline (30 μg, **OTC**), doxycycline (30 μg, **DO**), erythromycin (15 μg, **E**), trimethoprim/sulfamethoxazole (1.25/23.75 μg, **SXT**), florfenicol (30 μg, **FF**), and colistin sulfate (10 μg, **CT**) antimicrobials were tested against *Salmonella* and *E. coli* isolates. Meanwhile, amoxicillin/clavulanic acid (20/10 μg, **AMC**), ampicillin (10 μg, **AMP**), oxytetracycline (30 μg, **OTC**), oxacillin (1 μg, **OX**), vancomycin (30 μg, **VA**), streptomycin (10 μg, **S**), erythromycin (15 μg, **E**) and trimethoprim/sulfamethoxazole (1.25/23.75 μg, **SXT**) antimicrobials were evaluated against *S. aureus* isolates. The inhibition zone diameters were measured, in duplicate, and the tested isolates were categorized as sensitive or resistant in accordance with the interpretative

criteria recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI), 2020). Based upon CLSI breakpoints, the disk diffusion test is not dependable for assessing vancomycin resistance, so the minimum inhibitory concentration (**MIC**) of vancomycin (Oxoid, England, UK) was further detected via broth microdilution test as endorsed by CLSI (Clinical and Laboratory Standards Institute (CLSI), 2020). Finally, all *M. synoviae* isolates were tested for a panel of 8 antimicrobials including erythromycin, doxycycline, oxytetracycline, spectinomycin, spiramycin, tilmicosin, tylosin, and tiamulin. The isolates being resistant to at least one agent in 3 or more various antimicrobial classes were categorized as MDR.

Molecular Characterization of Virulence Determinants of the Selected Bacterial Isolates

Genomic DNAs from the most isolated MDR *S. aureus* and *S.* Typhimurium from chickens and ducks were extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) adopting the manufacturer's instructions. Extracted DNAs were subsequently used for various PCR assays, in triplicate, to detect some virulence-associated genes; *S. aureus* staphylococcal surface protein A (***spa***), toxic shock syndrome toxin (***tsst***) and collagen adhesin gene (***cna***) and *S.* Typhimurium fimbrial gene (***fimH***), *Salmonella* plasmid virulence C (***spvC***), and *Salmonella* outer protein D2 (***sopD2***) using target primers and cycling protocols detailed elsewhere by several investigators (Mehrotra et al., 2000; Tristan et al., 2003, Huehn et al. 2010; Wada et al., 2010; Hojati et al., 2015 ; Elnady, 2019). The sequences of all oligonucleotide primers and their corresponding genes with PCR amplified products' sizes are illustrated in Table 1. The amplified PCR products were subjected to gel electrophoresis and visualized under UV illumination. For each PCR assay, applicable positive (DNAs from isolates previously affirmed to possess sequences for any of the target virulence genes) and negative (DNase/RNase free water) controls were involved.

Herbal Essential Oils Antibacterial Activities

The antimicrobial properties of an array of marjoram, garlic, ginger and cinnamon EOs (Sigma, Taufkirchen, Germany) against the most common chicken and duck MDR and multivirulent isolates were evaluated using agar well diffusion and standard broth microdilution techniques (Elmowalid et al., 2022). Each assay was carried out in triplicate.

Essential Oils Interaction With Antibiotics via Checkerboard Assay

Evaluation of the interaction between the most effective antibiotics and EOs against the most dominant chicken and duck isolates showing MDR and multivirulence characteristics was achieved using a checkerboard method, in triplicate, following the method previously detailed (Hriouech et al., 2020). Analysis of the interaction between the screened antimicrobial compounds was carried out by calculating the fractional inhibitory concentration index (**FICI**) utilizing the formula described formerly (Hriouech et al., 2020). The FICI values were interpreted as following: FICI $\leq$ 0.5 (synergism), 0.5 $<$ FICI $\leq$ 1 (additivity), 1 $<$ FICI $\leq$ 4 (indifference) and FICI $>$ 4 (antagonism).

In Vivo *Antibacterial Activities*

Experimental Birds and Design Two hundred one-day-old broiler chicks (Ross 308) and Muscovy ducklings (100 each) were obtained from Dakahlia and El-Wafaa Companies, respectively and kept in separate cages at Animal Biosafety Level Two Facility at Animal Health Research Institute, Giza, Egypt. The birds were reared in

Table 1. Sequences of oligonucleotide primers and amplified PCR products of target genes.

Bacterial species	Target gene	Primer sequence (5′-3′)	Amplified product (bp)	Reference
Staphylococcus aureus	*23S rRNA*	F: ACGGAGTTACAAAGGACGAC R: AGCTCAGCCTTAACGAGTAC	1250	Bhati et al., 2016
	spa	F: TCAACAAAGAACAACAAAATGC R: GCTTTCGGTGCTTGAGATTC	226	Wada et al., 2010
	tsst	F: ACCCCTGTTCCCTTATCATC R:TTTTCAGTATTTGTAACGCC	326	Mehrotra et al., 2000
	cna	F: GTCAAGCAGTTATTAACACCAGAC R: AATCAGTAATTGCACTTTGTCCACTG	423	Tristan et al., 2003
Salmonella Typhimurium	*invA*	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284	Oliveira et al., 2003
	fimH	F: GTGCCAATTCCTCTTACCGTT R: TGGAATAATCGTACCGTTGCG	164	Hojati et al., 2015
	spvC	F: ACCAGAGACATTGCCTTC C R: TTCTGATCGCCG CTATTCG	467	Huehn et al., 2010
	sopD2	F: ACCATGCGCTGGAAGTGTTA R: GCGGGACGCATCATCTCATA	430	Elnady, 2019
Escherichia coli	*phoA*	F: CGATTCTGGAAATGGCAAAAG R: CGTGATCAGCGGTGACTATGAC	720	Hu et al., 2011
Mycoplasma synoviae	*vlhA*	F: TACTATTAGCAGCTAGTGC R: AGTAACCGATCCGCTTAAT	350−400	Jeffery et al., 2007

a floor- based system in experimental partitions under optimal conditions of light, humidity and temperature and received antimicrobial-free balanced ration. The broiler chickens diet for starter, grower and finisher periods was formulated adopting Aviagen recommendations (Aviagen, 2018) as listed in Supplementary Table 5. Chemical analyses of feed ingredients were carried out following the standard protocols supplied by the Association of Official Analytical Chemists (**AOAC**) (AOAC, 2012). Moreover, starter and grower diets of Muscovy ducks were formulated in accordance with the guidelines of the National Research Council (National Research Council (NRC), 1994) as shown in Supplementary Table 6. All birds had ad libitum access to feed and water. The experimental chickens were vaccinated with Hitchiner B1 + IB vaccine at 3 days-old, Gumboro vaccine at 10 days-old and LaSota NDV vaccine at 7 and 21 days-old via intraocular route. The experimental ducks were vaccinated with AI vaccine at 3 and 21 days-old and the fowl cholera vaccine at 18 days-old via subcutaneous injection.

Two independent *in vivo* experimental models of leg disorders were carried out. Using standard bacteriological methods, chickens (Groups 1−5; 20 each) and ducklings (Groups 6−10; 20 each) were examined at their arrival and weekly until the time of experimental bacterial infection (21 days-old), to confirm that they were free from any bacterial infection. Birds in groups 1 and 6 were kept as negative controls (noninfected and nontreated). Chickens in groups 2 to 5 and ducks in groups 7 to 10 were experimentally infected with 10^7 colony-forming units (**CFU**)/mL of the most prevalent MDR and multivirulent isolates; vancomycin-resistant *S. aureus* (**VRSA**) via intra articular and *S.* Typhimurium via intra muscular route, respectively (Gu et al., 2013; Guo et al., 2019). The bacterial experimental infections were confirmed through clinical signs, PM findings and re-isolation and identification of the infecting pathogens. Additionally, establishment of the bacterial infection models were affirmed via re-investigating the antimicrobial susceptibility and virulence genes' profiles of both chicken and duck bacterial pathogens. Following the clinical signs appearance, infected birds in groups 2 and 7; 3 and 8; 4 and 9 were treated for 5 consecutive days with the most effective antibiotics, EO and antibiotics/EO in combination, respectively using selected concentrations based on their *in vitro* MIC values.

Evaluation Parameters

Clinical Examination All experimental birds were observed on a daily basis and clinical signs, the number of dead birds and PM lesions on freshly dead birds were recorded from the time of bacterial infection until the end of the experiment.

Bacteriological Examination

After the end of the treatment, the synovial fluids of both chickens and ducks were collected and then subjected to re-isolation and identification of the experimentally infecting bacterial isolates in addition to estimation of the numbers of their respective CFU/mL of synovial fluids.

Histopathological Examination

Specimens from the joints were collected from sacrificed chickens and ducks after the end of the treatment. Histopathological examination of these samples was performed after fixation, decalcification and paraffin embedding. The paraffin tissue sections of 5 to 7 μm thickness were cut by Histotome (Leica RM2135, Heidelberger, Germany) and stained with hematoxylin-eosin (**H&E**) stain (Suvarna et al., 2018).

Statistical Analyses

The chi-square and one-way ANOVA tests were carried out to define the statistically significant differences, which were determined at a P value < 0.05. A heatmap with hierarchical clustering was performed using the "heatmap" package in R software. The correlation between antimicrobial resistance phenotypes and virulence genes and visualization were done using the R packages ggcorrplot (version 4.0.2; https://www.r-project.org). The results of antimicrobial susceptibility testing were graphed as heatmaps through GraphPad Software (version 8.0.1, GraphPad Software Inc., La Jolla, CA).

RESULTS

Clinical Signs and Postmortem Findings

The clinical signs of the investigated naturally infected chickens and ducks were swollen footpads, swelling and inflammation of the hock joint, lameness, inability to stand and gradual emaciation. Some diseased birds showed respiratory signs in the form of sneezing and coughing. PM examination of the freshly dead and sacrificed birds revealed caseous exudates in the swollen joints, cartilage injury and synovial membrane thickening in addition to liver congestion, pneumonia and airsaculitis in some cases (Supplementary Tables 1−4).

Occurrence and Distribution of Avian Bacterial Species

Microbiological and molecular analysis revealed the occurrence of 4 major bacterial pathogens; *Salmonella, E. coli, S. aureus* and *M. synoviae*. Of all examined birds (n = 500), 270 (54%), 240 (48%) and 180 (36%) were positive for *S. aureus, E. coli* and *Salmonella* species, respectively and only 10 (2%) were infected by *M. synoviae*. Notably, *S. aureus* was the most prevalent (76%, 190/250) among the examined chickens, followed by *E. coli* (66%, 165/250) and *Salmonella* (36%, 90/250)

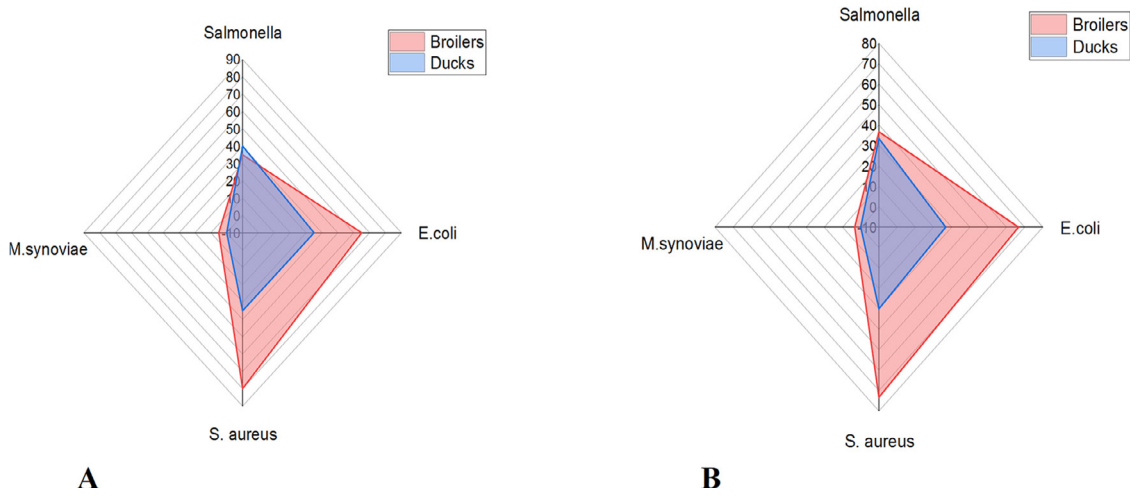

Figure 1. Occurrence rates of *Staphylococcus aureus* (*S. aureus*), *Salmonella* species, *Mycoplasma synoviae* (*M. synoviae*), and *Escherichia coli* (*E. coli*) in diseased broilers and ducks from poultry farms in Sharkia (A) and Dakahlia (B) Governorates. Pink and blue colors denote broiler chickens and ducks, respectively.

isolates. Regarding the investigated ducks, *Salmonella* and *S. aureus* isolates were recovered with high frequencies (36%; 90/250 and 32%; 80/250, respectively). Obviously, *M. synoviae* displayed a low isolation percentage relative to the total number of diseased chickens (10/250, 4%) and it did not be isolated from diseased ducks. Altogether, *S. aureus* was highly distributed among the diseased birds in both Sharkia and Dakahlia Governorates (57.5 and 51.7%, respectively), followed by *E. coli* isolates (50 and 46.7%, respectively). In Sharkia and Dakahlia Governorates, *S. aureus* isolates recorded the highest isolation percentages from diseased chickens (80 and 73.3%, respectively); meanwhile, *Salmonella* species were the predominant bacterial isolates among the examined ducks (40 and 33.3%, respectively) (Figure 1). Notably, there were no statistically significant differences ($P > 0.05$) in the occurrence rates of all recovered chicken and duck isolates between Sharkia and Dakahlia Governorates. Meanwhile, the occurrence rates of recovered chicken and duck isolates exhibited statistically significant variations ($P < 0.0001$) in each investigated Governorate. Moreover, there were statistically significant differences ($P < 0.0001$) in the occurrence rates of the recovered *E. coli* and *S. aureus* isolates between chickens and ducks, but no statistically significant differences ($P > 0.05$) were recorded for the occurrence rates of *Salmonella* species between both avian hosts.

Occurrence Rates of Single and Mixed Bacterial Isolates in Chickens and Ducks

Overall, *S. aureus* had the highest occurrence rate as a single bacterial isolate among the investigated birds with a percentage of 16.2%, while *M. synoviae* did not be recovered singly. Among the identified bacterial isolates, *S. aureus* and *Salmonella* species predominated singly in the investigated chickens and ducks (28.8 and 5.6%, respectively). Totally, the bacterial community profiling of mixed isolates revealed 6 different combinations (Table 2). The predominant mixed bacterial profiles were detected for *E. coli* and *S. aureus* in the diseased chickens (27.2%) and *S. aureus* plus *E. coli* and *Salmonella* species in the total examined birds and ducks (17 and 16%, respectively).

Table 2. Occurrence of single and mixed bacterial infections in chickens and ducks.

Single or mixed bacterial isolate (s)	No. of recovered isolates from avian source (%)		
	Chickens (250)	Ducks (250)	Total (500)
Staphylococcus aureus	72 (28.8)	9 (3.6)	81 (16.2)
E. coli	11 (4.4)	0 (0)	11 (2.2)
Salmonella species	9 (3.6)	14 (5.6)	23 (4.6)
E. coli + *Staphylococcus aureus*	68 (27.2)	15 (6)	83 (16.6)
E. coli + *Salmonella* species	31 (12.4)	20 (8)	51 (10.2)
E. coli + *M. synoviae*	5 (2)	0 (0)	5 (1)
Staphylococcus aureus + *Salmonella* species	0 (0)	16 (6.4)	16 (3.2)
Staphylococcus aureus + *E. coli* + *Salmonella* species	45 (18)	40 (16)	85 (17)
Staphylococcus aureus+ *E. coli* + *Salmonella* species + *M. synoviae*	5 (2)	0 (0)	5 (1)

Occurrence of E. coli and Salmonella Serotypes Among Examined Birds

Serotyping of all recovered *E. coli* isolates revealed 6 different recognized serotypes; O91, O126, O78, O2, O153, and O103. Interestingly, O78 was the prevailing serotype among the typed *E. coli* isolates recovered from total birds, chickens and ducks accounting for 41.7, 39.4, and 46.7%, respectively (Figure 2I). Moreover, O78 was found to be the most predominant serotype identified among *E. coli* isolates recovered from both Sharkia and Dakahlia Governorates (45 and 39.3%, respectively).

Five *Salmonella* serovars were characterized with *S.* Typhimurium, *S.* Enteritidis, and *S.* Kentucky being the most dominant ones among the total examined birds (47.2, 25, and 22.2%, respectively). Other 2 serotypes; *S.*

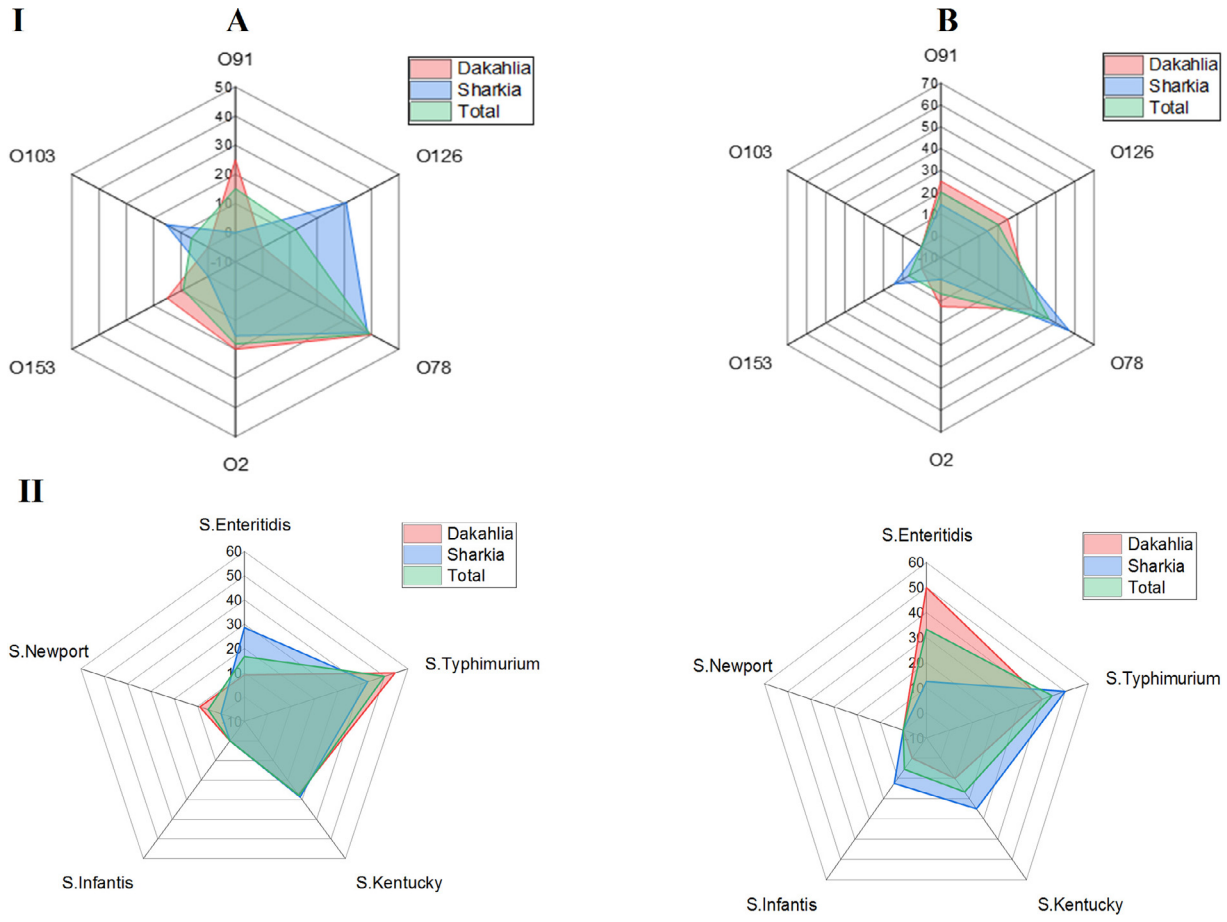

Figure 2. Total occurrence rates of various *Escherichia coli* (I) and *Salmonella* (II) serotypes in diseased broiler chickens (A) and ducks (B) and their distribution schemes in Sharkia and Dakahlia Governorates.

Infantis and *S.* Newport, were recovered from duck and chicken origins, respectively with a total prevalence rate of 2.8% each. Totally, *S.* Typhimurium serotype displayed the highest occurrence rate among recovered isolates from chickens (50%) and ducks (44.4%) in both Sharkia (46.7%) and Dakahlia (47.6%) Governorates. Among chicken isolates, *S.* Typhimurium was the most predominant serotype in Sharkia (42.9%) and Dakahlia (54.6%) Governorates. Among duck isolates, *S.* Typhimurium serotype predominated in Sharkia Governorate (50%); meanwhile, *S.* Enteritidis was the most recovered serotype in Dakahlia Governorate (50%, Figure 2II). Notably, there were no statistically significant differences ($P > 0.05$) in the occurrence rates of *E. coli* and *Salmonella* serotypes between Sharkia and Dakahlia Governorates. Meanwhile, the occurrence rates of recovered *E. coli* and *Salmonella* serotypes exhibited statistically significant variations ($P < 0.0001$) in each studied Governorate. Moreover, there were statistically significant differences ($P < 0.05$) in the occurrence rates of all *E. coli* serotypes, *S.* Enteritidis *S.* Infantis and *S.* Newport between chickens and ducks, but no statistically significant differences ($P > 0.05$) were recorded for the occurrence rates of *S.* Typhimurium and *S.* Kentucky between both avian hosts.

Antimicrobial Susceptibility Patterns of the Recovered Avian Bacterial Isolates

All *E. coli* and *Salmonella* isolates recovered from chickens and ducks were examined for their susceptibilities against a spectrum of 10 selected antimicrobials of various classes as summarized in Table 3 and Supplementary Figures 1 to 4. The analyzed data demonstrated that all recovered *E. coli* isolates from chickens showed higher resistance rates towards the majority of the tested antimicrobials with absolute resistance (100%) to doxycycline, neomycin and oxyetracycline. On the other side, colistin sulfate was the most effective antibiotic against those isolates (100%). Antibiogram results of the recovered *E. coli* isolates from ducks revealed that the highest resistance levels were observed against amoxicillin/clavulanic acid, ampicillin and oxyetracycline (100% each). Meanwhile, streptomycin and colistin sulfate reported the maximum sensitivity rates against those isolates (86.7 and 80%, respectively).

On the topic of antimicrobial susceptibility profiles of avian *Salmonella* isolates, the results revealed noticable high resistance rates of chicken isolates. The more precise interpretation of resistance data displayed that all chicken isolates were resistant to amoxicillin/clavulanic

Table 3. Antimicrobial susceptibility patterns of *E. coli* and *Salmonella* isolates recovered from chickens and ducks.

Antimicrobial agent	No. of avian *E. coli* isolates (%) showing antimicrobial susceptibility patterns				No. of avian *Salmonella* species (%) showing antimicrobial susceptibility patterns			
	Chickens (165)		Ducks (75)		Chickens (90)		Ducks (90)	
	Sensitivity	Resistance	Sensitivity	Resistance	Sensitivity	Resistance	Sensitivity	Resistance
Doxycycline	0 (0)	165 (100)	15 (20)	60 (80)	22 (24.4)	68 (75.6)	73 (81.1)	17 (18.9)
Amoxicillin/clavulanic acid	27 (16.4)	138 (83.6)	0 (0)	75 (100)	0 (0)	90 (100)	22 (24.4)	68 (75.6)
Ampicillin	29 (17.6)	136 (82.4)	0 (0)	75 (100)	0 (0)	90 (100)	24 (26.7)	66 (73.3)
Streptomycin	27 (16.4)	138 (83.6)	65 (86.7)	10 (13.3)	45 (50)	45 (50)	73 (81.1)	17 (18.9)
Neomycin	0 (0)	165 (100)	30 (40)	45 (60)	20 (22.2)	70 (77.8)	69 (76.7)	21 (23.3)
Oxytetracycline	0 (0)	165 (100)	0 (0)	75 (100)	10 (11.1)	80 (88.9)	60 (66.7)	30 (33.3)
Erythromycin	80 (48.5)	85 (51.5)	45 (60)	30 (40)	22 (24.4)	68 (75.6)	30 (33.3)	60 (66.7)
Colistin sulfate	165 (100)	0 (0)	60 (80)	15 (20)	0 (0)	90 (100)	35 (38.9)	55 (61.1)
Trimethoprim/sulfamethoxazole	108 (65.5)	57 (34.5)	45 (60)	30 (40)	90 (100)	0 (0)	90 (100)	0 (0)
Florfenicol	80 (48.5)	85 (51.5)	45 (60)	30 (40)	45 (50)	45 (50)	90 (100)	0 (0)

acid, ampicillin, and colistin sulfate (100% each) and a high proportion of the isolates was resistant to oxyetracycline 88.9%. However, trimethoprim/sulfamethoxazole was the most effective antimicrobial against these isolates (100%). The antimicrobial susceptibility profiles of duck *Salmonella* isolates demonstrated that high levels of resistance were documented for amoxicillin/clavulanic acid (75.6%). From the therapeutic viewpoint, trimethoprim/sulfamethoxazole, and florfenicol showed absolute sensitivity against those isolates (100%).

The *in vitro* antimicrobial susceptibility patterns of avian *S. aureus* isolates against antimicrobial agents of various classes are illustrated in Table 4 and Supplementary Figures 5 and 6. Chicken *S. aureus* isolates demonstrated high rates of susceptibility to amoxicillin/clavulanic acid (63.2%) and vancomycin (60.5%); however, they displayed high resistance rates against trimethoprim/sulfamethoxazole (84.2%) and oxyetracycline (73.7%). Among duck *S. aureus* isolates, the highest resistance rates were observed for oxacillin (87.5%), followed by trimethoprim/sulfamethoxazole (50%); meanwhile, they were highly susceptible to streptomycin, vancomycin and erythromycin (85, 80 and 80%, respectively). Notably, 75 chicken *S. aureus* isolates (39.5%) and 16 duck *S. aureus* isolates (20%) were resistant to vancomycin with MIC values ranging from 64 to 1024 μg/mL being classified as VRSA in agreement with CLSI breakpoints.

Another striking feature was that all *M. synoviae* isolates from chickens were resistant to erythromycin, doxycycline, oxytetracycline, tilmicosin and tylosin and sensitive to spectinomycin, spiramycin and tiamulin (Supplementary Figure 7).

Regarding the resistance patterns of various chicken *E. coli* serotypes, high levels of resistance to amoxicillin/clavulinc acid were displayed by O91, O126, O2, and O78 serotypes (92, 90, 86.7, and 83.1%, respectively). Moreover, O103 and O153 serotypes recorded high resistance rates to streptomycin (80 and 73.3%, respectively). Antibiogram of duck *E. coli* O126, O78, and O91 serotypes demonstrated high rates of resistance to doxycycline (86.7, 82.9, and 73.3%, respectively). Moreover, *E. coli* O153, and O2 serotypes exhibited high resistance levels to doxycycline (80 and 60%, respectively) and neomycin (80 and 60%, respectively).

Concerning the resistance profiles of investigated chicken *Salmonella* serovars, it was found that *S.* Newport displayed full resistance to erythromycin and oxyetracycline (100% each). Moreover, *S.* Typhimurium and *S.* Kentucky demonstrated high resistance rates to oxyetracycline (93.3 and 88%, respectively) and neomycin (82.2 and 92%, respectively). Additionally, *S.* Enteritidis showed high resistance percentages to erythromycin and doxycycline (80% each). More detailed interpretation of antimicrobial resistance patterns of duck *Salmonella* serovars revealed that the highest levels of resistance of *S.* Typhimurium and *S.* Enteritidis were obtained against amoxicillin/clavulinc acid (77.5 and 73.3%, respectively). Furthermore, a high proportion of *S.*

Table 4. Antimicrobial susceptibility patterns of *S. aureus* isolates recovered from chickens and ducks.

	No. of avian *S. aureus* isolates (%) showing antimicrobial susceptibility patterns			
	Chickens (190)		Ducks (80)	
Antimicrobial agent	Sensitivity	Resistance	Sensitivity	Resistance
Amoxicillin/clavulanic acid	120 (63.2)	70 (36.8)	45 (56.3)	35 (43.8)
Ampicillin	110 (57.9)	80 (42.1)	41 (51.3)	39 (48.8)
Oxyetracycline	50 (26.3)	140 (73.7)	45 (56.3)	35 (43.8)
Oxacillin	90 (47.4)	100 (52.6)	10 (12.5)	70 (87.5)
Vancomycin	115 (60.5)	75 (39.5)	64 (80)	16 (20)
Streptomycin	85 (44.7)	105 (55.3)	68 (85)	12 (15)
Erythromycin	90 (47.4)	100 (52.6)	64 (80)	16 (20)
Trimethoprim/sulfamethoxazole	30 (15.8)	160 (84.2)	40 (50)	40 (50)

Kentucky and *S.* Infantis serovars was resistant to erythromycin (80% each).

On overall, MDR was notably observed in 91.7, 81.1, and 78.5% of *E. coli, Salmonella*, and *S. aureus* isolates, respectively. Moreover, the MDR patterns of the recovered isolates of chicken and duck origins were highly variable. *Salmonella* and *S. aureus* isolates of chicken origin exhibited high MDR levels (89.9 and 91.1%, respectively) as compared to those recovered from duck origin (63.3 and 48.8%, respectively). Additionally, duck and chicken *E. coli* isolates displayed high MDR percentages (96 and 89.7%, respectively). It is worth noting that all chicken *E. coli* O2 and duck O91 and O153 serotypes were MDR. Moreover, all chicken *E. coli* serotypes O2, O126 and O91 and duck *E. coli* serotypes O126 and O91 were resistant to at least 5 of the 10 examined antimicrobials (Figures 3 and 4). There were great variations in the antimicrobial resistance patterns among different *Salmonella* serovars from duck and chicken origins with high levels of MDR displayed by chicken *Salmonella* serovars, unlike the duck ones. Analysis of the results revealed that all chicken *S.* Enteritidis, *S.* Kentucky and

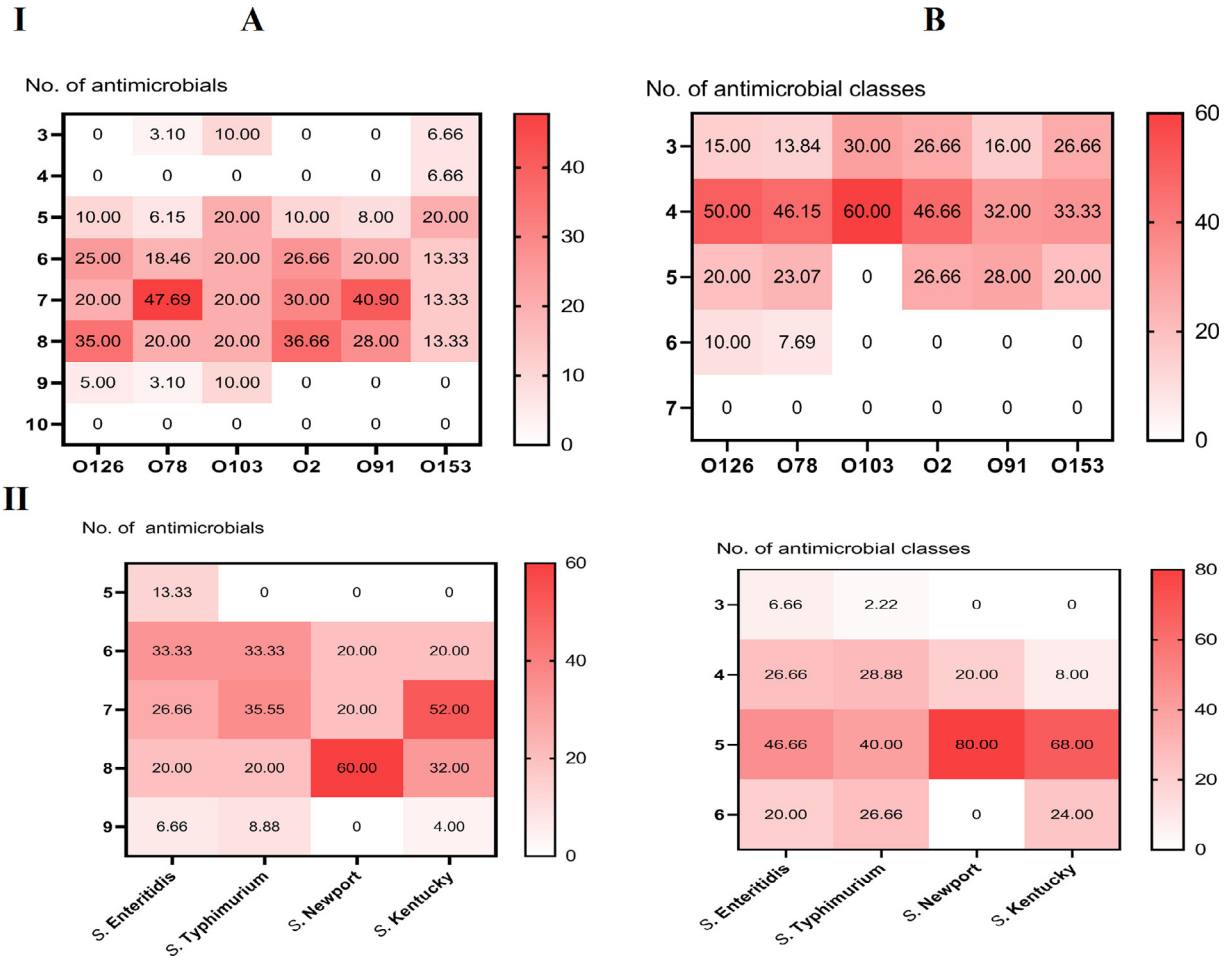

Figure 3. *In vitro* antimicrobials resistance profiling of chicken isolates considering the percentages of *Escherichia coli* (I) and *Salmonella* (II) serotypes resistant to various antimicrobials (A) and antimicrobial classes (B).

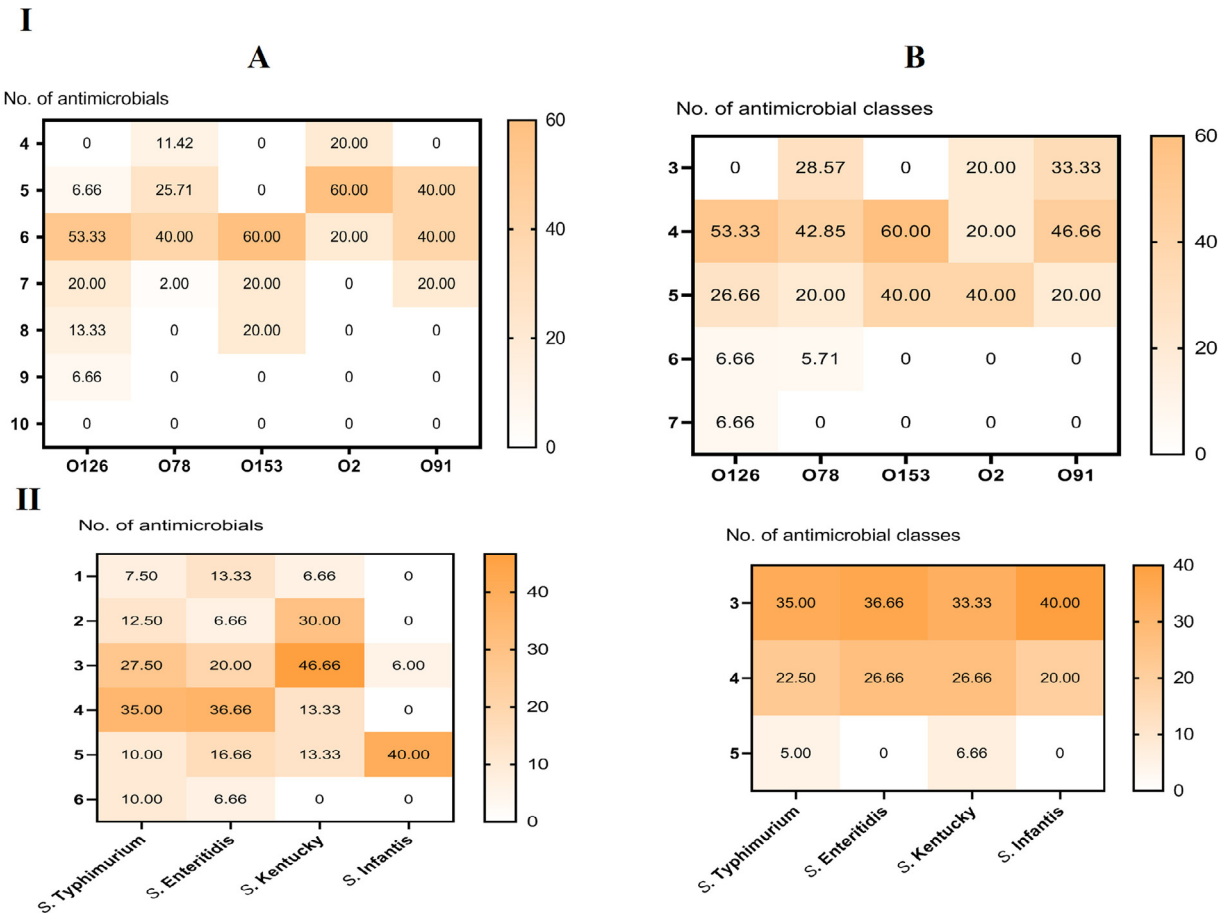

Figure 4. *In vitro* antimicrobials resistance profiling of duck isolates considering the percentages of *Escherichia coli* (I) and *Salmonella* (II) serotypes resistant to various antimicrobials (A) and antimicrobial classes (B).

S. Newport were MDR. Among duck *Salmonella* serovars, *S.* Kentucky and *S.* Enteritidis exhibited higher MDR patterns with percentages of 66.67 and 63.3%, respectively. Regarding *S. aureus* antimicrobial resistance patterns, it was found that 91.1% of chicken isolates were MDR with 65.8% of them having phenotypic resistance to at least 4 different antimicrobial classes. Furthermore, 48.8% of duck *S. aureus* isolates expressed MDR with no isolates presented resistance to more than 6 antimicrobials (Figure 5). Notably, all chicken *M. synoviae* isolates (100%) had phenotypic resistance to 5 antimicrobial drugs of 3 different classes being MDR.

Antibiotyping, Genotyping, and Virulence Gene Profiling of the Selected Avian Isolates

Basing on antimicrobial resistance data, 37 *S. aureus* and 22 *S.* Typhimurium isolates that were resistant to at least 6 and 4 antimicrobial drugs, respectively were selected for further investigations. Overall, 24 and 15 different antimicrobial resistance patterns of the investigated *S. aureus* (Figure 6) and *S.* Typhimurium (Figure 7) isolates were recognized. The most common pattern displayed by 5 *S. aureus* isolates (13.5%) included resistance to AMP, OTC, OX, S, E, and SXT antimicrobials and that demonstrated by 4 *S.* Typhimurium isolates (18.2%) comprised resistance to AMC, AMP, OTC, E, and CT antibiotics.

The distribution of examined virulence genes among the selected isolates revealed that all examined chicken *S. aureus* isolates (100%) harbored *spa* and *cna* genes and only 59.5% of them (22/37) were positive for *tsst* gene. Moreover, all duck *S.* Typhimurium isolates (100%) possessed *fimH* and *spvC* genes, while *sopD2* gene was detected in only 77.3% (17/22) of these isolates (Figure 8). Various combinations of 3 tested virulence genes among the investigated *S. aureus* and *S.* Typhimurium isolates demonstrated that each of them exhibited 2 virulence genes' profiles. It is of interest to reveal that the most dominant profile was that of *S. aureus* and *S.* Typhimurium isolates possessing all examined virulence genes (59.5 and 77.3%, respectively) being multivirulent isolates. Interestingly, out of 22 chicken multivirulent *S. aureus* isolates, 16 (72.7%) were confirmed to be VRSA.

Correlation Analyses Between Phenotypic Antimicrobial Resistances and Virulence Genes and Among Resistances to Antimicrobials

As illustrated in Figure 9A, *S.* Typhimurium *sopD2* gene was positively correlated with resistances to erythromycin ($r = 0.22$) and oxytetracycline ($r = 0.06$), while it was negatively correlated with the

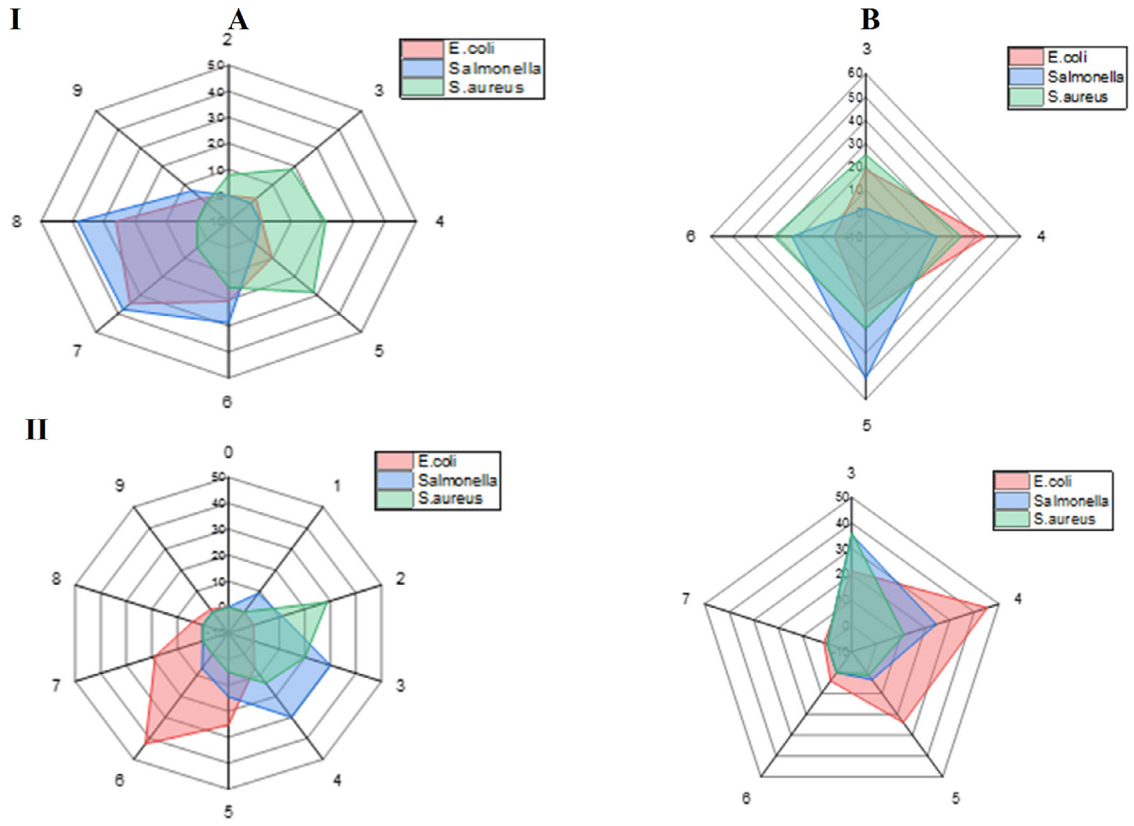

Figure 5. *In vitro* antimicrobials resistance profiling of chicken (I) and duck (II) isolates considering the percentages of *Escherichia coli* (*E. coli*), *Salmonella* species and *Staphylococcus aureus* (*S. aureus*) resistant to various antimicrobials (A) and antimicrobial classes (B).

other examined antibiotics with r values ranging from - 0.03 to - 0.21. Moreover, we noted positive correlations between resistances to colistin sulfate and both streptomycin ($r = 0.22$) and oxytetracycline ($r = 0.06$), erythromycin and both doxycycline ($r = 0.26$) and neomycin ($r = 0.01$), neomycin and

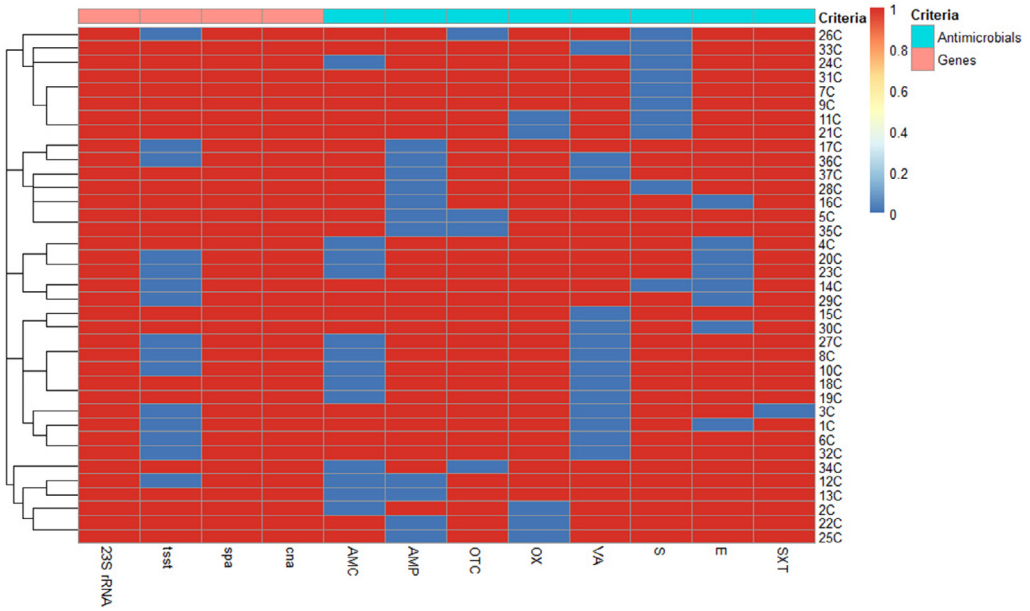

Figure 6. Heat map and hierarchical clustering of the examined 37 chicken *Staphylococcus aureus* isolates based on the occurrence of antimicrobial resistance and target genes. In the heat map, red and blue colors denote the resistance/sensitivity to an antimicrobial agent and the presence/absence of the target genes, respectively. The code numbers on the right of the heat map infer the chicken (C) isolates. Abbreviations: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; *cna*, collagen adhesin gene; E, erythromycin; OTC, oxytetracycline; OX, oxacillin; S, streptomycin; *spa*, staphylococcal surface protein A; SXT, trimethoprim/sulfamethoxazole; *tsst*, toxic shock syndrome toxin and *23S rRNA*: 23S ribosomal ribonucleic acid; VA, vancomycin.

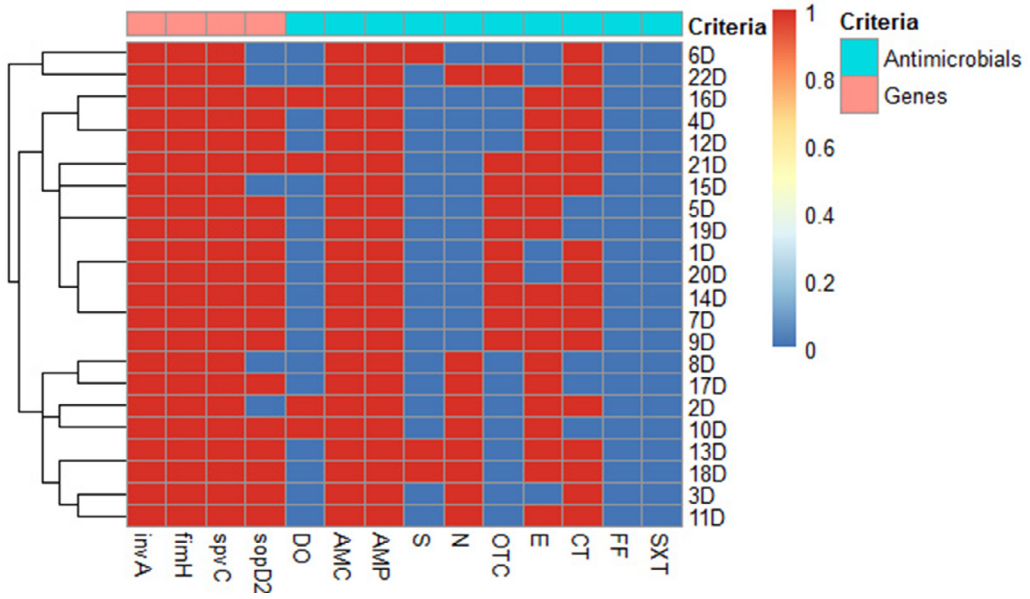

Figure 7. Heat map and hierarchical clustering of the examined 22 duck *Salmonella* Typhimurium isolates based on the occurrence of antimicrobial resistance and target genes. In the heat map, red and blue colors denote the resistance/sensitivity to an antimicrobial agent and the presence/absence of the target genes, respectively. The code numbers on the right of the heat map infer the duck (D) isolates. Abbreviations: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CT, colistin sulfate; DO, doxycycline; E, erythromycin; FF, florfenicol; *fimH*, fimbrial gene; *invA*, invasion A gene; N, neomycin; OTC, oxytetracycline; S, streptomycin; *sopD2*, salmonella outer protein D2; *spvC*, salmonella plasmid virulence C; SXT, trimethoprim/sulfamethoxazole.

both streptomycin ($r = 0.21$) and doxycycline ($r = 0.09$).

According to Figure 9B, *S. aureus tsst* gene was positively correlated with resistance to erythromycin ($r = 0.23$), vancomycin ($r = 0.22$), trimethoprim/sulfamethoxazole ($r = 0.20$) and amoxicillin/clavulanic acid ($r = 0.04$), while it was negatively correlated with the other tested antibiotics with r values ranging from - 0.04 to - 0.33. Furthermore, we recorded positive correlations between resistance to trimethoprim/sulfamethoxazole and vancomycin ($r = 0.20$), streptomycin and both oxacillin ($r = 0.12$) and oxytetracycline ($r = 0.04$), oxacillin and both ampicillin ($r = 0.09$) and amoxicillin/clavulanic acid ($r = 0.02$) and oxytetracycline and ampicillin ($r = 0.02$).

In Vitro *Antibacterial Activities of Herbal Essential Oils*

The antibacterial potentials of 4 herbal EOs against MDR and multivirulent chicken VRSA ($n = 16$) and duck *S.* Typhimurium ($n = 17$) isolates were studied in terms of assessing the bacterial growth inhibition zones and MIC values. All used EOs showed excellent

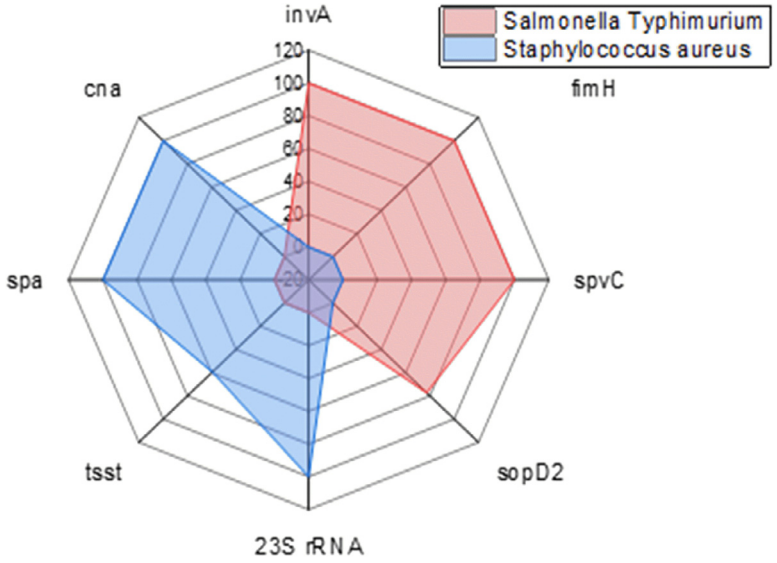

Figure 8. Distribution of target genes among selected 37 chicken *Staphylococcus aureus* and 22 duck *Salmonella* Typhimurium isolates. Abbreviations: *cna*, collagen adhesin gene; *fimH,* fimbrial gene; *invA*, invasion A gene; *spa*, staphylococcal surface protein A; *23S rRNA*, 23S ribosomal ribonucleic acid; *sopD2*, salmonella outer protein D2; *spvC:* salmonella plasmid virulence C; *tsst*, toxic shock syndrome toxin.

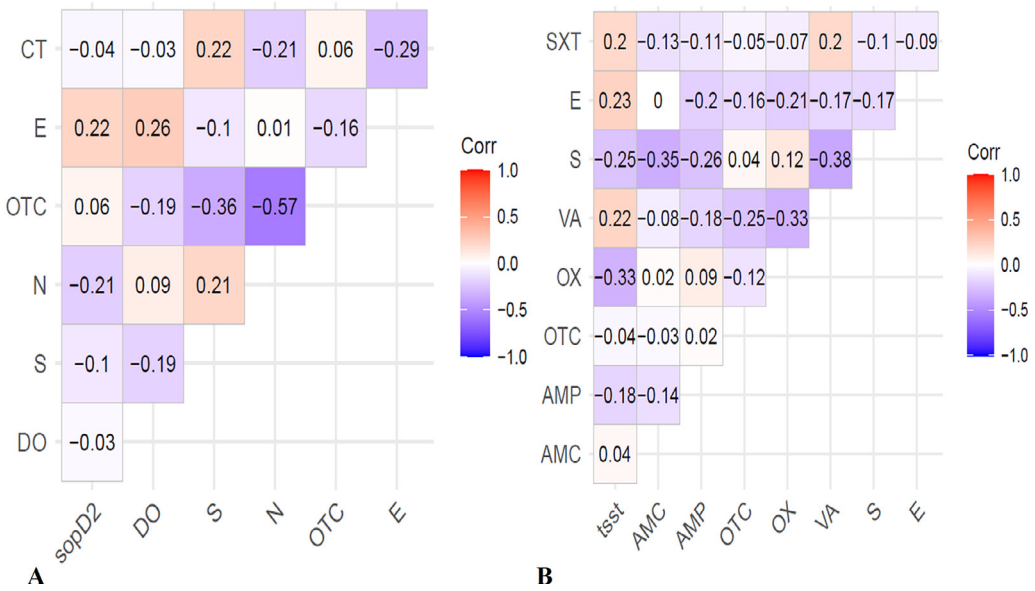

Figure 9. Correlation (r) analyses among resistances to antimicrobials and between phenotypic antimicrobial resistance and virulence genes of duck *Salmonella* Typhimurium (A) and chicken *Staphylococcus aureus* (B) isolates. Red and blue colors indicate positive and negative correlations, respectively. The color key denotes the correlation coefficient (r). Abbreviations: AMC, amoxicillin/clavulanic acid; AMP, ampicillin; CT, colistin sulfate; DO, doxycycline; E, erythromycin; N, neomycin; OTC, oxytetracycline; OX, oxacillin; S, streptomycin; *sop*D2, salmonella outer protein D2; SXT, trimethoprim/sulfamethoxazole; *tsst*, toxic shock syndrome toxin; VA, vancomycin.

antibacterial activities against all investigated bacterial isolates. Concerning the susceptibility of chicken VRSA to the tested EOs, they were all sensitive with relevant inhibition zones of diameters ranging from 16 to 31 mm and resultant MIC concentrations in the range from 64 to 8 ug/mL. The sensitivity to examined EOs was superior for duck *S.* Typhimurium isolates with estimated inhibition zones' diameters and MIC values ranging from 22 to 38 mm and 16 to 4 ug/mL, respectively. Among the 4 tested EOs, marjoram was the most potent one against chicken VRSA and duck *S.* Typhimurium isolates with maximum inhibition zones' diameters (up to 31 and 38 mm, respectively) and this effectiveness was reflected with recording very low MIC values (up to 8 and 4 μg/mL, respectively).

Assessment of Interaction Between Essential Oils and Antibiotics

According to the results of *in vitro* antimicrobial susceptibility patterns alongside the EOs antibacterial activities, the combination between the most effective antibiotics (florfenicol and amoxicillin/clavulanic acid) and EO (marjoram) against the most dominant MDR and multivirulent isolates; chickens VRSA ($n = 16$) and ducks *S.* Typhimurium ($n = 17$) exhibited synergistic interactions with FICI values ≤ 0.5.

In Vivo *Efficacy of Antimicrobials in Chickens and Ducks*

Clinical Signs, Mortalities, and Postmortem Lesions None of chickens or ducks in groups 1 and 6 (negative controls) showed any clinical signs, mortality rates or PM lesions through the entire experimental period. Meanwhile, the experimentally VRSA-infected chickens via intra articular route in group 5 (positive control) showed numerous clinical signs in the form of gradual loss of appetite, weakness, swollen hock and toe joints, arthritis (Figure 10A and B), lameness, reluctance to move and emaciation with a mortality percentage of 20%. The PM examination of freshly dead and sacrificed chickens exhibited that the affected joints and their cavities were surrounded with yellowish-white caseous exudate. Moreover, the experimentally *S.* Typhimurium infected ducks via intra muscular route in group 10 (positive control) displayed anorexia, depression, diarrhea, pasty vent, swollen hock and toe joints, arthritis (Figure 10C and D), inability to stand, lameness and panophthalmitis in some ducks with a mortality percentage of 40%. The PM findings revealed fibrinous exudates in the affected joints, white cecal cores and generalized septicemia in freshly dead and sacrificed ducks. All the treatment regimens succeeded in reliving the clinical signs and PM lesions in all experimentally infected and treated chickens and ducks. Birds treated with amoxicillin/clavulanic acid (intramuscularly) or florfenicol (orally) in combination with marjoram (orally) presented the most pronounced improvement in the clinical pictures of the established experimental bacterial leg infections in chickens and ducks.

Bacteriological Studies After the end of the treatment period, all treatment regimens displayed reductions in the viable bacterial counts in the examined synovial fluids. Interestingly, treatment of chicken VRSA and duck *S.* Typhimurium experimental infections using marjoram and amoxicillin/clavulanic acid or florfenicol in combination demonstrated the lowest bacterial counts (up to 1×10^1 CFU/mL of synovial fluids).

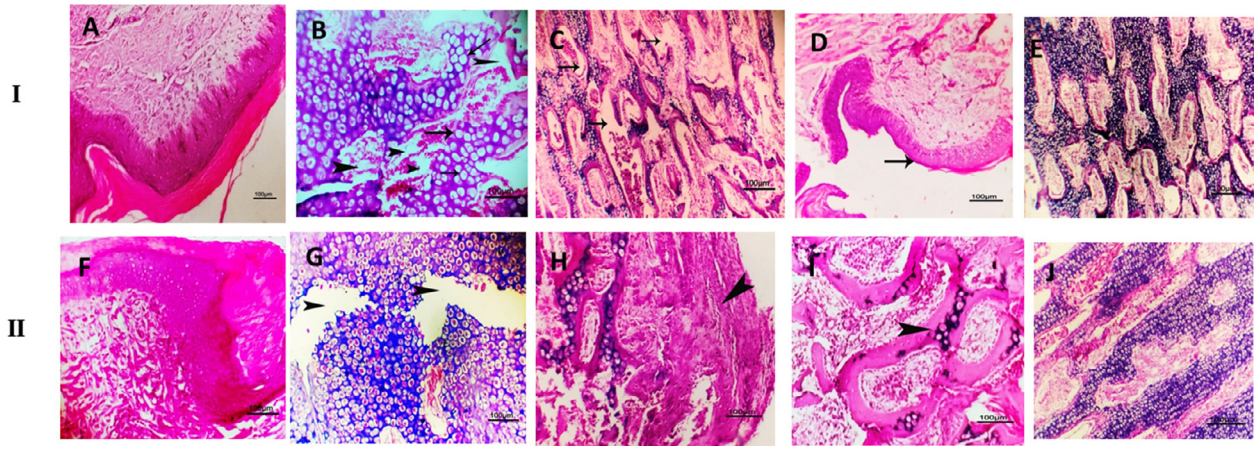

Figure 10. Histopathological pictures of joint samples obtained from broiler chickens (I) and ducks (II) in negative control (A and F), positive control (B and G) and infected and marjoram (C and H), antibiotic (D and I) and marjoram/antibiotic (E and J) treated experimental groups (H&E stain, magnification = ×400, Scale bar = 100 μm). A and F: negative controls (noninfected and nontreated birds); normal dermis, epidermis and connective tissue layers. B and G: positive controls (chickens and ducks experimentally infected with vancomycin resistant *S. aureus* (VRSA) and *S.* Typhimurium, respectively); partial necrosis of chondrocytes (arrows) and chondromalacia (arrows' heads). C and H: VRSA and *S.* Typhimurium infected and oral marjoram treated chickens and ducks, respectively; poor ossification, erosions and ulceration (C, arrows) and focal area of hyperosification (H, arrow head). D and I: VRSA and *S.* Typhimurium infected and intramuscular amoxicillin/clavulanic acid and oral florfenicol treated chickens and ducks, respectively; focal areas of hypokeratinization (D, arrow) and regular joint ossification (I, arrow head). E and J: chickens and ducks experimentally infected with VRSA and *S.* Typhimurium and treated with intramuscular amoxicillin/clavulanic acid or oral florfenicol in combination with oral marjoram, respectively; regular progressive ossification with normal cartilage.

Moreover, up to 10^3 and 10^4 CFU/mL were observed in the synovial fluids of experimentally infected birds following treatments with amoxicillin/clavulanic acid or florfenicol and marjoram, respectively.

Histopathological Findings The histopathological pictures of joint samples obtained from chickens and ducks in all experimental groups are illustrated in Figure 10. Histopathological examination of chicken joint samples in the negative control group revealed no noticeable microscopic alterations with normal dermis, epidermis and connective tissue layers. Meanwhile, the positive control group displayed the most apparent changes in the examined joint tissues in the form of partial necrosis of chondrocytes and poor ossification of bony materials. All the applied treatments in the present study thrived in reliving the histopathological lesions in all VRSA-infected treated groups. Following marjoram and amoxicillin/clavulanic acid treatments, the joint histopathological photographs were improved with poor ossification, erosions and ulceration and focal areas of hypokeratinization, respectively. Interestingly, treatment with marjoram and amoxicillin/clavulanic acid in combination caused no histopathological indication of joint deterioration as the joint architecture showed normal cartilage with regular ossification.

With regard to the histopathological investigation of duck joint samples in the noninfected and nontreated group, normal dermis, epidermis and connective tissue layers were evident. Meanwhile, *S.* Typhimurium infected group revealed chondromalacia with poor ossification. The treatment with marjoram led to ameliorating the joint histopathological findings in infected treated ducks with only a focal area of hyperosification. Moreover, florfenicol treatment resulted also in a promotion in the joint structure in infected treated ducks in the form of apparently normal layers with regular ossification. Notably, a better improvement of the joint histopathological image was attained in infected ducks following treatment with marjoram and florfenicol in combination till exhibiting regular progressive ossification with normal cartilage.

DISCUSSION

Leg disorders are symptoms of a condition caused by a variety of etiological agents including several bacterial pathogens. They have a negative impact on poultry performance resulting in significant economic losses in poultry flocks. The most common clinical forms of this condition in chickens and ducks are arthritis, synovitis, tenosynovitis, osteomyelitis, bumble foot and femoral head necrosis with subsequent lameness. Antibiotic treatment of leg affection still represents a challenge in the poultry field and it is threatened with increasing the prevalence of MDR bacterial pathogens (Hriouech et al., 2020). Therefore, there is an urgent need to find novel plant-derived alternatives for overcoming this developing antibiotic resistance. From this point, the present study aimed to identify some bacterial pathogens incriminated in leg affections in chickens and ducks and their antimicrobial susceptibility patterns with emphasis on evaluating the *in vivo* antibacterial effectiveness of the most effective antibiotics and EOs against the highly prevalent MDR and multivirulent recovered bacterial isolates.

Herein, the observable clinical signs of the investigated naturally infected chickens and ducks were swollen footpad and hock joint, lameness, reluctance to move and gradual emaciation. The PM examination of the freshly dead and sacrificed birds revealed caseous exudates in the swollen joints in addition to congestion of internal organs, pneumonia and airsaculitis in some

cases. The previous clinical signs and PM lesions are consistent with those observed in naturally infected birds suffering from leg affections in previous researches (Bakheet, 2011; Youssef et al., 2019).

Several bacterial pathogens are involved in leg dysfunction leading to great economic losses in poultry farms. According to the bacteriological and molecular investigation results of the current study, *Salmonella, E. coli, S. aureus* and *M. synoviae* were recovered from chickens with leg affections. Similar to our findings, *S . aureus, E. coli* and *Salmonella* isolates were previously recovered from chickens suffering from arthritis and bumble foot (Lebdah et al., 2015; Tawfik et al., 2016). Interestingly, *S. aureus* isolates were the most frequently bacterial species encountered in the present study among diseased chicken cases either singly or mixed with *E. coli*. The overall incidence level of *S. aureus* from chickens was close to those stated earlier in previous studies investigating chicken cases with leg affections; 66% (Abd EL-Tawab et al., 2017) and 62% (Bakheet, 2011). Differently, higher prevalence rates of *S. aureus* were recorded from broilers with septic arthritis in Pakistan; up to 81% (Nazia et al., 2015) and Iran; 85.7% (Feizi et al., 2012) and lower isolation percentages of *S. aureus* were documented from broilers suffering from bumble foot lesions; 45.8% (Youssef et al., 2019) and arthritis; 36.6% (Omayma, 2005) in Egypt. In line with our findings regarding the predominant mixed bacterial profile (*S. aureus* with *E. coli*) in the naturally diseased chickens, a previous study also recorded that *S. aureus* was recovered in mixed form with *E. coli* (26.7%) from bumble foot diseased chicken cases (Youssef et al., 2019). Observations of the current study illustrated a low isolation percentage of *M. synoviae* from diseased chickens, which is consistent with a previous study conducted in Egypt recording the same occurrence rate of this pathogen in chickens (Abdanaser et al., 2019). However, this isolation rate is higher than that reported in another study carried out in Egypt; 1.6% (Emam et al., 2020) and it is lower than those recorded in previous studies in Egypt; 22.7% (Abd-El-Tawab et al., 2020) and Turkey; 12.9% (Yilmaz et al., 2011). Analyzing the phenotypic and genotypic identification results of the current study, *Salmonella, E. coli*, and *S. aureus* were isolated from ducks with leg disorders. Similar findings were noticed in an earlier study conducted in Denmark (Bisgaard, 1981), where *Salmonella, E. coli* and *S. aureus* were recovered from ducks with arthritis. Another study in India isolated *S. aureus* and *E. coli* from a duck with a bumble foot (Choudhury, 2019). The variations in the occurrence rates of the incriminated avian bacterial pathogens among various studies in different countries might reflect substantial discrepancies in geographic location, sample types, identification protocols, infection control practices, antibiotic usage and seasonal and environmental factors. Notably, *M. synoviae* did not be isolated from the investigated ducks in the present study. This could be attributed to the explanation suggested previously (Bencina et al., 1988), where *Mycoplasma* species could infect ducks when they were reared in close contact with *Mycoplasma* infected chickens. Meanwhile, the examined ducks in the current study were obtained from farms rearing ducks only.

Herein, serotyping of all investigated *E. coli* isolates revealed 6 different serotypes with O78 being the most prevalent one in chickens and ducks. These findings are consistent with previous studies conducted in Egypt, where O78 was the most dominant serogroup identified from chickens with leg affections; 26.7% (Lebdah et al., 2015) and 31.3% (Youssef et al., 2019). Moreover, this result is supported by another study carried out earlier in Denmark, where O78 was the most prevalent serotype from ducks with arthritis (Bisgaard, 1981). Comparably, other *E. coli* serotypes (O88, O25, O12, and O45) were recovered from broilers with osteomyelitis and arthritis in Brazil (Braga et al., 2016). Regarding *Salmonella* serotyping, *S.* Typhimurium serotype exhibited the highest occurrence rates among the investigated chickens (50%) and ducks (44.4%). Another study in Mexico reported an outbreak in chickens with lameness caused by *S.* Typhimurium (Padron, 1990). In Denmark, *S.* Typhimurium dominated among the isolated *Salmonella* serogroups identified from ducks with arthritis (Bisgaard, 1981). Conversely, Guo et al., (2019) identified *S.* Pullorum serotype from broilers with lameness and arthritis. Serotyping results reflect that *E. coli* O78 and *S.* Typhimurium serogroups are frequently accompanied with avian leg affections confirming their contribution in extra intestinal infections. Meanwhile, other serotypes identified in previous studies could be related to variations in geographical and period aspects.

In the current study, *E. coli* isolates recovered from chickens and ducks displayed high resistance levels to oxytetracycline, amoxicillin/clavulanic acid, doxycycline and ampicillin. These resistance rates are comparable to those formerly reported for *E. coli* isolates originating from chickens with leg affections (Lebdah et al., 2015), where the recovered isolates were resistant to amoxicillin/clavulanic acid, doxycycline and ampicillin. Likewise, a high resistance rate to amoxicillin (73.3%) has been reported for *E. coli* isolates recovered from broilers with osteomyelitis and arthritis in Brazil (Braga et al., 2016). Another investigation conducted in Egypt documented that duck *E. coli* isolates were resistant to ampicillin and penicillin (Eid et al., 2019). However, contrary to our results, *E. coli* isolates recovered from chickens with leg affections in earlier studies conducted in Brazil (Braga et al., 2016) and Egypt (Youssef et al., 2019) were highly sensitive to tetracycline. Herein, the high sensitivity rates of duck *E. coli* isolates to streptomycin and chicken *E. coli* ones to trimethoprim/sulfamethoxazole are in contradiction with those recorded in former studies in Egypt (Eid et al., 2019; Youssef et al., 2019).

Analyzing the antibiogram results of chicken *Salmonella* isolates in our study demonstrated that they showed high resistance rates to amoxicillin/clavulanic acid, ampicillin, erythromycin and doxycycline. A similar trend in the occurrence of ampicillin and doxycycline-resistant chicken *Salmonella* isolates was observed

in previous Chinese (Guo et al., 2019) and Egyptian (Lebdah et al., 2015) reports, but in contrast to our research, *Salmonella* isolates recovered previously from chickens with leg affections in Egypt were sensitive to amoxicillin (Lebdah et al., 2015). With regard to the antimicrobial susceptibility results of duck *Salmonella* isolates in the current study, high resistance rates of amoxicillin/clavulanic acid, ampicillin and erythromycin and excellent activities of trimethoprim/sulfamethoxazole, florfenicol and neomycin were observed. This is comparable with a previous study performed in South Korea (Cha et al., 2013), where Pekin duck *Salmonella* isolates were resistant to amoxicillin/clavulanic acid, ampicillin and trimethoprim/sulfamethoxazole. Another previous investigation in Taiwan (Tsai and Hsiang, 2005) reported sensitivity patterns of duck *Salmonella* isolates to trimethoprim/sulfamethoxazole, florfenicol, neomycin, and amoxicillin/clavulanic acid.

Our antimicrobial susceptibility findings of chicken *S. aureus* isolates revealed high susceptibility rates to amoxicillin/clavulanic acid as previously documented for *S. aureus* isolates recovered from chickens with arthritis (Abd El Tawab et al., 2018) and bumble foot (Youssef et al., 2019). On contrary, a higher resistance rate was observed for amoxicillin/clavulanic acid (87.7%) in an earlier study carried out in Egypt (Bakheet et al., 2018). Herein, the high resistance rate observed for oxacillin (52.6%) is in harmony with those reported in previous studies in Egypt (Bakheet et al., 2018; Youssef et al., 2019). Moreover, higher resistance rates of our *S. aureus* isolates were recorded against trimethoprim/sulfamethoxazole (84.2%) and oxyetracycline (73.7%). In another study carried out in Egypt (Lebdah et al., 2015), *S. aureus* isolates from chickens with leg affections were sensitive to sulfamethoxazole/trimethoprim and resistant to doxycycline. Regarding duck *S. aureus* isolates, the resistance was mostly observed for oxacillin and trimethoprim/sulfamethoxazole; meanwhile, they were highly susceptible to streptomycin and erythromycin. An investigation on antimicrobial resistance profiles of *S. aureus* isolates recovered from ducks revealed higher resistance rates to ampicillin, amoxicillin, and penicillin (100% each), streptomycin (90%) and erythromycin (80%) (Eid et al., 2019). Moreover, higher cloxacillin sensitivity and sulfamethizole resistance rates of ducklings *S. aureus* isolates were previously reported in India (Mondal and Sahoo, 2014). Notably, higher occurrence rates of VRSA isolates were observed among chickens and ducks. Comparably, a previous research carried out in Egypt demonstrated vancomycin resistance among *S. aureus* isolates recovered from chickens with arthritis with a percentage of 10.5% (Abd El Tawab et al., 2018). A remarkable observation in the current study was that all chicken *M. synoviae* isolates were resistant to erythromycin, doxycycline, oxytetracycline, tilmicosin, and tylosin and sensitive to spectinomycin, spiramycin, and tiamulin. Another recent investigation from Egypt found similar results, where a higher resistance rate of *M. synoviae* isolated from diseased chickens was recorded to tetracycline (Abd-El-Tawab et al., 2020). Moreover, several previous studies in Egypt demonstrated higher sensitivity levels among chicken *M. synoviae* isolates to spiramycin (Emam et al., 2020) and tiamulin (Abdanaser et al., 2019; Abd El-Hamid et al., 2019a). Seemingly, the great discrepancies in the antimicrobial resistance rates of the recovered isolates among various studies may be well contributed to the type and dose of antimicrobials prescribed for treating avian leg bacterial infections in various geographical areas, management practices in avian production and variances in legislation controlling the use of antimicrobial agents.

Notably, the present study demonstrated alarming MDR frequencies for chicken *S. aureus, Salmonella*, and *E. coli* and duck *E. coli* and *Salmonella* isolates as they exhibited resistance to at least one drug in 3 or more different antimicrobial classes. Of interest, MDR among chicken and duck isolates causing leg disorders constituted a major subject of concern worldwide, where 26.3% of chicken *S. aureus* isolates in Egypt (Abd El Tawab et al., 2018), 73% of chicken *E. coli* strains in Brazil (Braga et al., 2016), 80% of chicken *Salmonella* isolates in China (Guo et al., 2019) and 50.5% of duck *Salmonella* isolates in South Korea (Cha et al., 2013) showed MDR phenotypes. Totally, the outcome, which is common between our research and previous studies (Ahmed et al., 2021; Ammar et al., 2021a) is the increasing trend of MDR among avian isolates. This could be attributable to the uncontrollable usage of antimicrobial agents for controlling bacterial pathogens in the poultry industry in many geographical localities because of their availability. Therefore, appropriate legislation and judicious usage of antimicrobials in poultry production must be adopted to overcome this serious situation.

Regarding the distribution of particular virulence genes among the selected isolates, our results demonstrated full occurrence of *spa* and *cna* genes among chicken *S. aureus* isolates and *fimH* and *spvC* genes among duck *S.* Typhimurium isolates. Likewise, a wide distribution of *spa* and *cna* genes (100%) had also been documented earlier among chicken *S. aureus* isolates associated with arthritis (Abd El Tawab et al., 2018) and bumble foot (Tawfik et al., 2016). This strengthens the findings of the current study confirming their roles in the pathogenesis of *S. aureus* arthritis. Both genes are notable members of the microbial surface components recognizing adhesive matrix molecules that are anchored on the cell wall peptidoglycan, where *spa* is a vital virulence factor important for adherence and invasion of host cells and also enables *S. aureus* to evade the host immune response and *cna* mediates *S. aureus* collagen adhesion (Foster et al., 2014; Abd El-Hamid and Bendary, 2015). The high incidence rates of *fimH* and *spvC* genes among duck *S.* Typhimurium isolates were also reported by other authors in South Korea (Cha et al., 2013) and Egypt (Nasser et al., 2018) emphasizing their ability to promote the persistence of *S.* Typhimurium at extra-intestinal sites, where *fimH* encodes an adhesive protein that plays important roles in *S.* Typhimurium adhesion and *spvC* has a phosphothreonine lyase

activity and inhibits mitogen-activated protein kinase signaling (Ammar et al., 2016b).

Considering the previous scenario for the high prevalence of MDR and multivirulent bacterial isolates, there is a dire necessity for developing alternative strategies to fight the infections caused by MDR and multivirulent strains (Ammar et al., 2021b; Ibrahim et al., 2021a,b, 2022a,b,c). Plant-derived compounds are gaining rekindled attention as reasonable novel antimicrobials. So, we evaluated the effects of marjoram, garlic, ginger and cinnamon EOs against MDR and multivirulent chicken VRSA and duck *S.* Typhimurium isolates. Critical analysis of our results demonstrated promising *in vitro* antibacterial activities for the used EOs with prominent potential for marjoram. Indeed, a previous study had focused on the potent antibacterial aspects of garlic and ginger against both *Salmonella* and *S. aureus* strains (Chand, 2013). Other studies carried out in Egypt (Elmowalid et al., 2019) and USA (Robinson et al., 2022) confirmed that garlic and ginger had effective antibacterial properties against *S. aureus* and *Salmonella* strains. Like the findings of the current study, the considerable antibacterial activity of cinnamon EO had been proved against *S.* Typhimurium (Angienda et al., 2010) and *S. aureus* (Liu et al., 2017). Another previous evidence (Indu et al., 2006) supported our antibacterial findings of garlic and ginger EOs against *Salmonella* strains. Notably, the promising *in vitro* antibacterial efficacy of marjoram against the investigated isolates is in a link with an earlier study conducted in Iran (Moghadam and Ahanjan, 2015), where marjoram exhibited strong antibacterial effects against *S. aureus* and *S. enterica* strains. We speculate reasonable various explanations by which the EOs mediated their favorable antibacterial effects such as alteration of cell wall permeability, interference with cell wall synthesis and membrane functions, interaction with membrane proteins and functional alteration in DNA synthesis. Other anticipated scenarios clarifying the antimicrobial actions of EOs might be related to the inactivation of extracellular enzymes or the generation of free toxic radicals. Finally, the inhibition of bacterial efflux pumps could not be excluded (Abd El-Hamid et al., 2019c; Elmowalid et al., 2019).

The *in vitro* findings attained in the current study encouraged us to implement 2 independent *in vivo* trials to investigate, for the first time, the therapeutic efficacy of marjoram EO in combination with florfenicol or amoxicillin/clavulanic acid compared with marjoram, florfenicol or amoxicillin/clavulanic acid alone in chicken and duck bacterial arthritis models. Our successful experimental models of VRSA and *S.* Typhimurium arthritis in experimental birds were supported by previous comparable studies (Gu et al., 2013; Guo et al., 2019), where arthritis was established after intra-articular injection of *S. aureus* and intra-muscular injection of *S.* Pullorum in chickens. Regarding the efficacy of treatment regimens, amoxicillin/clavulanic acid or florfenicol in combination with marjoram EO treatments were able to reserve the arthiritis signs, reduce the mortalities, joint macroscopic lesions and viable VRSA and *S.* Typhimurium counts and improve the joints histopathological pictures rendering these protocols as potent approaches for experimental avian VRSA and *S.* Typhimurium arthritis. Similarly, a previous study carried out in Iran demonstrated that birds treated with florfenicol had better outcomes in the histopathological picture of the tibiotarsal joint compared with the positive control group, especially relating to the thickness of reparative cartilage (Mosleh et al., 2016). With regard to the effectiveness of intramuscular administration of amoxicillin/clavulanic acid in chickens, it was reported that antibiotics administration intramuscularly could lead to ultimate serum concentrations within minutes (Soranoglou et al., 2017). Additionally, the bioavailability of amoxicillin-clavulanic acid after the intramuscular injection was high in chickens (Carceles et al., 1995). Concerning the effectiveness of florfenicol in ducks, it was proved that the oral route was efficient for its administration in the field owing to rapid absorption and bioavailability. The concurrent administration of 2 antibiotics is among the probable policies for controlling the bacterial related diseases in poultry, but this leads to the appearance of numerous MDR strains (Rondevaldova et al., 2018). Therefore, there is a growing attention in replacing one of the 2 antibiotics by natural antimicrobials. This replacement leads to a reduction in the effective doses of drugs with a consequence of diminishing their potential side effects, fighting the resistance phenomenon and decreasing the treatment costs (Fadli et al., 2012). In the current study, we emphasized, for the first time, the augmented antibacterial effects of amoxicillin/clavulanic acid or florfenicol upon the combination with marjoram EO in the treatment of induced VRSA and *S.* Typhimurium experimental leg infections in chickens and ducks, respectively. Only one previous study in Morocco stated that 1,8-cineole EO showed a synergistic effect when combined with amoxicillin/clavulanic acid for the treatment of *S. aureus* osteomyelitis in experimental rabbits (Hriouech et al., 2020). The synergistic activity of amoxicillin and 1,8-cineole EO considerably reinforced the antibacterial effect of amoxicillin. This could be attributed to the protecting role of 1,8- cineole EO for the antibiotic against β-lactamases action in resistant bacterial pathogens and its substantial capacity to disrupt the bacterial cell membrane or affect its respiration (Chaves et al., 2018). Notably, reviewing the accessible preceding literature, we found that there were no *in vivo* prospective applications of florfenicol and EOs alone or in combination as therapeutic agents against avian bacterial arthritis. These outcomes should guide future *in vivo* studies for further assessing the efficacy of EOs alone or in combination with antibiotics in treating bacterial pathogens included in leg disorders in poultry.

In conclusion, based upon the findings of the current investigation, we could conclude that *S. aureus, Salmonella* species, *E. coli* and *M. synoviae* are major pathogens of chickens and ducks with leg disorders in Egypt. Moreover, our outcomes offer further evidence of the

emergence of MDR and multivirulent avian bacterial isolates calling for crucial approaches to successfully treat these worrisome pathogens. To our knowledge, it is striking to highlight that this is the first in-depth report assessing the *in vitro* and *in vivo* synergistic antibacterial effects of marjoram in combination with florfenicol or amoxicillin/clavulanic acid in controlling the induced experimental *S.* Typhimurium or VRSA leg infections. In light of the data described herein, enhancing the antimicrobial influence of antibiotics using marjoram seems to be a certainly promising approach to explore novel pathways in developing new antimicrobial drugs for the treatment of bacterial pathogens associated with leg infections in the field alongside navigating successful opportunities for antimicrobials usage reduction and/or antibiotics-free poultry production.

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DISCLOSURES

The authors have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102889.

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