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In vitro and *In vivo* appraisement of the potency of different antibiotics against experimental *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections as well as the effectiveness of Guava (*Psidium guajava* L.) leaves extract against *Mycoplasma gallisepticum* as a natural antibiotic alternative

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ABSTRACT

Mycoplasma synoviae (MS) and *Mycoplasma gallisepticum* (MG) are two of the most serious infections in poultry, causing financial losses across the globe. Antibiotics treatment can lessen the clinical signs of MG and MS infection in birds and help restrict the spread of these infections. However, the rise of antibiotics multidrug resistance (MDR) among poultry diseases is still a problem for the world's health. Minimum inhibitory concentration (MIC) is crucial to resolving this issue since it helps ensure correct antibiotic use and fights the development of antibiotic resistance. This investigation aims to assess the *In vitro* and *In vivo* efficacy of several antibiotics (tilmicosin, tylosin, erythromycin, spiramycin, lincomycin, and doxycycline) against MG and MS field isolates, in addition to guava leaves extract (GLE) against MG. For *In vivo* study, 510, one day old chicks were randomly split into 17 groups the birds were experimentally infected at 3 days old and supplied with different treatments in drinking waters for 5 successive days at 18 days old. The results of *In vitro* study showed that tilmicosin was the most effective against MG isolates with MIC values ranging from 0.0078 to 0.0156 µg/ml and spiramycin considered effective against MS with MIC values ranging from 0.015625 to16µg/ml and MIC value of GLE was 0.25 µg/ml that was considered as moderate sensitive to MG infection. The *In vivo* study revealed that tilmicosin and spiramycin were able to significantly lower the clinical score, lesion score, and re-isolation rate of MG and MS, respectively in the treated birds at p-value < 0.05 in contrast with control positive infected untreated groups. Additionally, the treated groups with tilmicosin in MG infection and spiramycin in MS infection showed significantly higher mean body weights (MBW) compared to the infected untreated ones at p-value < 0.05. The current study demonstrates that MG and MS's sensitivity to many of the most popular drugs changed as in tylosin become low sensitive and erythromycin become resistant. GLE has moderate sensitivity antibacterial effect against MG isolates both *In vivo* and *In vitro* and can be used as a natural antibiotic alternative. Therefore, we recommend periodical monitoring of the sensitivity of the circulation MG and MS strains using MIC test to overcome MDR of avian mycoplasmas with further suggestion to evaluate GLE as nano-preparation against MG infection.

Introduction

During 2020, 35% of the world's meat production was chicken according to FAO (2020). *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG) are common bacterial pathogens that frighten the poultry industry (Ferguson-Noel, 2013; Marouf *et al*., 2022a, b). These two Mycoplasma species are on the International Organization for Animal Health's list due to their potential negative effects on birds' health and the economy of poultry production (OIE, 2018). In industrial poultry species, MG is a well-known avian pathogen that can produce chronic respiratory disease (CRD) (Júnior *et al*., 2017). Avian mycoplasmas belong to the Mollicutes class of bacteria, which includes fastidious, wall less, minimalist organisms (Armour, 2020). Avian mycoplasmas infections resulting in an increase in processing condemnations, downgrading of carcasses, decreased egg productivity, feed conversion rate, and decreased egg hatchability (Bottinelli *et al*., 2022). MS is characterized clinically by arthritis associated with respiratory problems in infected chickens, and in commercial egg layers, it resulted in a decrease in egg production and hatchability with abnormalities in the eggshell apex (Wei *et al*., 2023). The presence of other infections exacerbates the severity of the clinical symptoms, which can range from mild to severe forms (Landman, 2014). *Mycoplasma* species can spread vertically from the hens to progeny through eggs and horizontally by susceptible birds who contact with contaminated surfaces either directly or indirectly, and the severity of the infection exaggerated with the increased intense industry and stress-related factors (Razin & Hayflick, 2010). The poultry industry uses multiple approaches to keep healthy flocks (Mehdi *et al*., 2018). The three primary approaches for controlling the disease are eradication, vaccine, and medicine (Garmyn *et al*., 2017). Despite the availability of vaccinations, using antibiotics remains the most cost-effective strategy (Abd El-Ghany, 2009), medication can be a quick and efficient strategy to lessen the economic losses by decreasing egg transfer and severity clinical symptoms, but eradication and vaccination give long-term solutions for the control of avian mycoplasmosis (Kleven, 2008). To maximize treatment effectiveness, an antibiotic susceptibility profile should be established first and reduced for reasons of antimicrobial resistance (AMR) (Landman *et al*., 2014). The intrinsic resistance of Mollicutes, which is related to the bacterium's lack of antibiotic targets, limits the selection of antimicrobials that can be used to treat MG and MS infections (Browning and Citti, 2014). Because they lack cell walls, Mollicutes are insensitive to betalactams, glycopeptide antibiotics, and bacitracin (Browning and Citti, 2014), and due to the lack of a folic acid production enzyme, they are not sensitive to sulphonamides or trimethoprim (Bottinelli *et al*., 2022). Additionally, RNA polymerase conservative mutations have made Mollicutes resistant to rifampicin (Bottinelli *et al*., 2022). It is customary, MG and MS have demonstrated *In vitro* and *In vivo* sensitivities to many antimicrobials drugs that penetrate cells could be used to achieve a successful treatment, including macrolides, tetracyclines, and quinolones (Forrester *et al*., 2011). For instant of anti-mycoplasma drugs that inhibit protein synthesis, tetracyclines (chlortetracycline, oxytetracycline, and doxycycline), lincosamides (lincomycin), macrolides (tilmicosin, tylosin, erythromycin, and spiramycin), pleuromutilins (tiamulin), and quinolones (enrofloxacin) (Kreizinger *et al*.,

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2017). While advising treatment for avian mycoplasmosis, veterinarians frequently overlook the negative consequences of synthetic medicines, a further issue is the emergence of drug resistance to substances currently in use (Grózner *et al*., 2016). It's interesting to observe that Mollicutes of animal origin have more mutations in target protein-coding genes than Mollicutes of human origin (Gautier-Bouchardon, 2018). This observation might result from the widespread use of antibiotics in the poultry and animal production sectors (Koike *et al*., 2017). It is also accompanied by the threat of commensal organisms developing AMR, an international health problem (Nhung *et al*., 2017) So, it became more necessary to use new and alternative antimicrobials due to their high costs (Erfan and Marouf, 2019). In the fight against *Mycoplasma* infections, a method for the creation of novel antibiotics alternatives primarily from fruit leaves offers a significant contribution (Maiyo *et al*., 2010). Few researchers have, to our knowledge, discussed the effectiveness of therapeutic fruit leaves against various *Mycoplasma* species especially GLE which shown a considerable amount of antibacterial action against MG (Hemeg *et al*., 2020). Guava (*Psidium guajava* L.) is a fruit plant from the *Myttaceae* family. Guava leaves, roots, and fruit are used to treat and prevent diarrhea (Karnikowski *et al*., 1995). Guava also shown significant antibacterial action against the *Shigella, Staphylococcus* species that cause food-borne diarrhoea and *Pseudomonas* (Jaiarj *et al*., 1999). In addition to, guava is employed in the treatment of diabetes, hypertension, pain, fever, respiratory problems, gastroenteritis, diarrhoea, and dysentery, it also acts as an anti-inflammatory (Gutiérrez *et al*., 2008). The purpose of this investigation was to evaluate the *In vitro* and *In vivo* inhibitory effects of different commercial antimicrobials against MS and MG field isolates and evaluate the effectiveness of GLE against MG infection using MIC assay that sought to lower AMR.

Materials and methods

Ethical approval

This work is ethically approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University (Vet. CU. IACUC) with number Vet CU 2009 2022519.

Antibiotics

The susceptibility tests were conducted using the following antibiotics from three distinct groups: Doxycycline (ATCO Pharma®, Egypt Company) each 1 gram contains 831.91mg Doxycycline hyclate in a dose of 0.27 g/L , lincomycin (ATCO Pharma®, Egypt Company) each 1 gram contains 453.6 mg lincomycin hydrochloride in a dose of 0.125 g/L, spiramycin (ATCO Pharma®, Egypt Company) each 100 grams contains 69.75 mg spiramycin adipate in a dose of 38.65 mg/kg body weight , erthromycin (ATCO Pharma®, Egypt Company) each 100 grams contains 20 gm erthromycin thiocynate in a dose of 2.20 gm/L, Tylogran® (EGYEURO Company) each gram contains 86.54 mg tylosin tartrate in a dose of 1.25 g/L , and Tildosin® (EGYEURO Company) 250 mg/ml tilmicosin phosphate in a dose of 0.3 ml/L . Al treatments were supplied to the birds in drinking water at 18 days of age (15 days post infection and existence of clear clinical signs) for five successive days for 2 h daily.

Guava leaves extract

The guava leaves extract was obtained as a commercial drug (MEPACO-MEDIFOOD®) was administered five days in a dose of 3 ml/L of drinking water daily for 2 h daily.

Minimum inhibitory concentration test (MIC)

Each evaluated antimicrobial compound was two-fold serial dilution with a varied concentration in accordance with the antibiotic that is used. Tylogran 0.4 mg base/ml, erythromycin 180 mg/ml, lincomycin 125 mg/ml, spiramycin 207,000,000 IU, doxycycline 270 mg/ml, tilmicosin 250 mg/ml and GLE 500 µg/ml.

Mycoplasma strains were diluted to a concentration of 10^5 CFU per 0.2 mL (Bastamy *et al*., 2022), using media that only contained phenol red indicator, hours serum, and PPLO broth.

100µl of each diluted substance and 100 µl of each diluted *Mycoplasma* stains were combined in the 96-well microtiter plates, a growth control (tested field *Mycoplasma* strains cultivated in broth media with no tested substances), sterility control (broth media without either tested substances or *Mycoplasma* inoculum), and negative control (substances with broth medium and PH adjusted to 6.8) were all included in each plate. After being sealed, the plates were incubated at 37°C and checked periodically for any changes in the color of indicator, to ensure that the findings were accurate; each experiment was carried out in duplicate then repeated twice according to Hannan (2000). The initial MIC, often known as the lowest antimicrobial concentration at which the colour of the *Mycoplasma* growth control remained unchanged when no further colour change was seen in the growth control wells, the final MIC was determined in the broth containing the antimicrobials, using a micro-pipette and filter tip, add 2 mL of each *Mycoplasma* dilution (in duplicate for higher accuracy) to the surface of freshly and dried *Mycoplasma* agar plates. Incubate the plates at 37°C under the proper environmental conditions (aerobically, 95% N_2 + 5% CO_2) in sealed containers once the droplets of each suspension have been absorbed into the agar. The seeded plates should be incubated until the *Mycoplasma* colonies on the plates that were inoculated with the lower diluted sample of the *Mycoplasma* suspension are fully developed. The count of colonies that are formed by each 2 mL droplet and interpretation was adopted using ordinary microscope (Olympus) according to Hannan (2000) and modified technique of Boeder *et al*. (2018).

The antimicrobial degree of sensitivity and/ or resistance were adopted following standard of Gerchman *et al*. (2011) who stated that when the gap between susceptible and resistant populations is greater than (0.5) considered resistant.

Requirements for Mycoplasma strains and culture

A recent 4 chosen *Mycoplasma* stains (2 MG and 2 MS) obtained from previous surveillance studies (Qoraa *et al*., 2023a, b).

MG selected strain showed MIC value of 0.00781 g/mL for tilmicosin, and MS strain with a MIC of 0.03125 g/mL for spiramycin were used for the *Mycoplasma* experimental *In vivo* challenge.

The strain was kept at -70°C in *Mycoplasma* broth (PPLO broth from Oxoid), which was supplemented with 1% glucose monohydrate, 0.5% phenol red indicator, 10% yeast extract, 25% inactivated horse serum, and penicillin G sodium (one mg/mL), thallium acetate for MG but MS add NAD and l- cysteine to this supplement (Bradbury, 1998). 20 mL of *Mycoplasma* broth and one mL of stored broth (-70°C) were combined, and the mixture was then incubated for two days at 37°C until a change in color from red to yellow was visible (Mekkes and Feberwee, 2005).

For MIC, two-fold serial dilutions in MG and MS *Mycoplasma* broth were adopted then check on the inoculum's bacterial content, and the colony forming units (CFU) was calculated (Hannan, 2000).

For MG challenge, each bird challenge with 100 µl intraocularly and intranasally, the inoculum had about $1x10^6$ CFU/ml/bird (Abd El Aziz *et al*., 2014). For MS challenge, each bird received 100 µl intraocularly and subcutaneous injection (S/C) via foot pad inoculation, the inoculum had about $3x\ 10^5$ CFU/ml/bird (Lockaby *et al*., 1998).

Chickens

Five hundred and fifteen, one day old ROSS male broiler chicks were

obtained from an Egyptian local company. The birds were grown using standard procedures and they were raised on deep litter system using wood shaving in previously cleaned and disinfected separated experimental rooms of the Poultry Disease Department of the Faculty of Veterinary Medicine Cairo University. The birds have free access to balanced ration that was free from any anti-mycoplasma food and clean water. At the bird's arrival, trachea and lungs were taken from five randomly chicks to ensure the absence of MG and MS infections by conventional PCR analysis to detect 16S-rRNA (Cetinkaya *et al*., 2009) and the rest 510 birds were then allocated in different experimental groups. At three days old, birds challenged with MG and MS, at day 18 of age the birds supplied with different treatments in drinking water for 5 successive days.

Experimental design and grouping

As shown in Table 1, 510 chicks were randomly assigned in 17 experimental groups with 30 birds each which were subsequently subdivided into two replicates/group (15 birds/replicate).

The birds were vaccinated against, infectious bronchitis and Newcastle disease (ND) at 3 days of age, infectious bursal disease using a mild strain vaccine at 13 days of age, The Lasota vaccination against ND was administered at 18 days and repeated at 28 days, while the inactivated avian influenza vaccine (H5N2) was administered at 10 days of age. All vaccinations were administered via eye drop instillation except AI vaccine were administered by S/C injection at the base of the neck.

During the experiment time 35 days, birds were checked daily for the observation of clinical signs and mortalities, while the average body weight and feed consumption were estimated weekly. At 7, 14, 18, 23, 29 and 35 days of age, four birds from each group (2 birds/replicate) were sacrificed, weighed, and necropsied to check for postmortem (PM) lesions, and re-isolation.

For clinical signs score, each respiratory symptom was assessed separately, and signs scores was evaluated according to guidelines of Kempf and Gesbert (1998) as a following:

0 = no respiratory symptoms.
1 = Slight symptoms (Few Tracheal reflex as sneezing).
2 = Moderate symptoms (Breathing noise).
3 = Severe symptoms (Dyspnea just after manipulation or overt dyspneic symptoms prior to manipulation).

The amount of fibrous exudate present on the serous membrane of the air sacs was used to assess the PM lesion score, on a scale from 0 (no lesions) to 4 (severe bilateral lesions) (Kleven *et al*., 1972; Czifra *et al*., 2000) as follow:

Lesion score = (0) No air sac lesions were found; the bird's air sac membranes were totally transparent without significant changes.
Lesion score = (1) The membranes had a minor cloudiness but no significant changes.
Lesion score = (2) The membranes were typically slightly thicker and covered in tiny exudates that resembled cheesy substances.
Lesion score = (3) The membranes had a distinct thickening and meaty consistency, and an extensive accumulation of clotted exudates were restricted to a single air-sac.
Lesion score = (4) The membranes had score No. 3 gross significant pathological changes, however lesions were discovered in two or more air sacs.

For MS, the degree of arthritis was observed, and clinical signs and PM lesion score was performed following the scale of Kleven *et al.* (1975).

Statistical analysis

All statistical analysis and graphs were performed using RStudiov1.3.1093 (RStudio Team, 2020) and R programming language v4.0.3 (R Core Team, 2020). Statistical significance was tested between groups using one way analysis of variance (ANOVA) test. First, ANOVA assumptions were validated by using the Shapiro-Wilk test for normal distribution and the Levene's test for homogeneity of variance. Then, ANOVA was conducted followed by multiple comparisons between groups with the Tukey post-hoc test. For non-parametric data (lesions scores and MIC), Kruskal-Wallis one way analysis of variance was used, and the ¬p-value was adjusted according to the Benjamini-Hochberg procedure. Regarding nominal data (mortality percentage), the Fisher's exact test was conducted since most of the expected frequencies were less than 5. Finally, for illustrative purposes, data was plotted with a letter or more over each group. Groups without shared letters indicate a significant difference between them with a p-value < 0.05.

Table 1. Expermentail design.

Experimental trails (Challenge at 3 days old)	Experimental groups (30 birds/group)	Treatments & dosage (From 18th to 22nd days of age)
MG (Each bird challenge with 100 µl of MG intraocularly and intranasally, the inoculum had about $1x10^6$ CFU/ml/bird)	A	Control +ve
	B	Control -ve
	C	Tylosin (1.25 g/L
	D	Tilmicosin (0.3 ml/L)
	E	Spiramycin (0.5 g/L)
	F	Lincomycin (0.125 g/L)
	G	Doxycyclin (0.27 g/L)
	H	Erthromycin (2.20 g/L)
	I	GLE (3 ml/L)
MS (Each bird challenged with 100 µl intraocularly & S/C injection in foot pad, the inoculum had about $3x\ 10^5$ CFU/ml/bird)	A	Control +ve
	B	Control -ve
	C	Tylosin (1.25 g/L)
	D	Tilmicosin (0.3 ml/L)
	E	Spiramycin (0.5 g/L)
	F	Lincomycin (0.125 g/L)
	G	Doxycyclin (0.27 g/L)
	H	Erthromycin (2.20 g/L)

Results

In vitro antibacterial activities of antibiotics and GLE

Broth microdilution test

Our results conducted on 4 chosen *Mycoplasma* stains (2 MG and 2 MS). By using the broth microdilution technique, the *In vitro* activity of one fruit leaves extract and six antibiotics from three groups against the tested strains were determined. Therefore, initial MIC values were used in evaluating the investigated *Mycoplasma* strains sensitivity to various antimicrobials. The macrolide category of antibiotics showed the widest ranges of MIC values, with MICs ranging from 0.0078 to 0.5 /mL. The most effective antibiotic in the test was tilmicosin, which had the lowest MIC values from 0.00781 to 16 µg/mL against MG isolates and from 0.03125 to16/mL against MS isolates) among the four macrolides that were investigated. Moreover, tylosin developed high MICs against MG and MS isolates, ranging from 0. 25 to16µg/mL in MG and from 0.125 to16 µg/mL in MS. Moreover, spiramycin has MICs values ranging from 0.03125 to16µg/mL in MG and from 0.015625 to16 µg/mL in MS. But Erythromycin have highest MIC value against MG and MS with rang from 4 to 8 µg/ml for MG and MS isolates. Doxycycline's MIC values among tetracyclines ranged widely (0.0625–16 /mL for MG and 0.125–16 µg/ml, for MS). The MIC values of lincomycin for the various lincosamides ranged from 0.125 to 16 µg /mL for MG and 0.25 to 16 µg /mL for MS these values were explained in Table 2.

Figure 1 shows that statistical analysis ensures that antibiotics without shared letters indicate a significant difference between them with a p-value < 0.05. It was noticeable that the MS isolate was almost susceptible to every tested antibiotic except for erythromycin. In contrast, all MG isolates were susceptible to the antibiotics tilmicosin, doxycycline, lincomycin, and spiramycin and less susceptible to tylosin and erythromycin. Besides, GLE have moderate sensitivity against MG isolate ranged from 0.125 to 16 µg /mL. The result of MIC was concluded that erythromycin is deemed resistant to both MG and MS infection, while spiramycin and tilmicosin are deemed very sensitive to MS and MG infection, respectively, Doxycycline, spiramycin, lincomycin, and GLE are regarded as moderately sensitive to MG infection, while Doxycycline, tilmicosin, and tylosin are regarded as moderately sensitive to MS infection. Tylosin is regarded as low sensitive to MG infection, but lincomycin is regarded as low sensitive to MS infection.

In vivo assessment the effectiveness of various anti-mycoplasma drugs

Mortalities

Each group's mortality rate was observed daily till the end of the study (35 days of age). PM examination was performed on the day of the death to record the PM lesions. The cumulative mortalities were recorded in Table 3 and Mortalities didn't show statistically significant differences between all the MG experimental groups at p-value = 0.73 and in the MS experimental groups at p-value = 0.77.

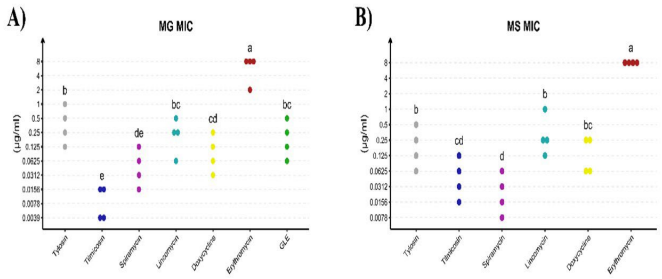

Fig. 1. MIC of different antibiotics was compared using the Kruskal-Wallis test followed by post-hoc analysis according to the Benjamini-Hochberg procedure. Antibiotics without shared letters indicate a significant difference between them with a p-value < 0.05 in case of MG and MS else.

Clinical signs

The five examined birds at the zero point were PCR negative for MG & MS. During the experiment sneezing, nasal discharge, gasping, difficulty breathing, ocular signs such as conjunctivitis or ocular secretion with bubbles, and tracheal rales were also noted. These symptoms were followed by poor growth and decreased feed intake in the inoculated chicken group at 7 days post-challenge, gradually worsened until the infection's last day (18 days post challenge), and then gradually subsided in the treated groups as seen in Table 3. The control group showed no respiratory symptoms as seen in Figure 2.

Fig. 2. Showing A: conjunctivitis and watery eye in MG infected group 10 days post challenge, B: lameness and set on hock joint in MS infected group 11days post challenge and C: uneven growth between MG infected non treated group and tilmicosin treated group at the end of study.

Infected MS birds showed stunting, joint swelling, lameness, ruffled feathers, slightly respiratory symptoms, and decreased spontaneous activity. and then gradually subsided in the treated groups as seen in Table

Table 2. Microbroth antimicrobial sensitivity test.

Antibiotics	Minimum MIC mean range value. (µg/ml) Isolate1 (MG)	Minimum MIC mean range value. (µg/ml) Isolate2 (MG)	Dilution means range value. (µg/ml)	Minimum MIC range mean value. (µg/ml) IsolateA (MS)	Minimum MIC mean range value. (µg/ml) Isolate B (MS)	Dilution range (µg/ml)	Degree of sensitivity	
							MG	MS
Doxycycline	0.06	0.13	0.0625-16	0.13	0.13	0.125-16	M	M
Lincomycin	0.25	0.13	0.125-16	0.5	0. 25	0.25-16	M	L
Spiramycin	0.03	0.06	0.03125-16	0.02	0.03	0.015625-16	M	H
Erythromycin	4	8	4-8	8	8	4-8	R	R
Tilmicosin	0.01	0.01	0.00781-16	0.03	0.06	0.03125-16	H	M
Tylosin	0.25	0.5	0. 25-16	0.25	0.13	0.125-16	L	M
Guava leaves extract	0.25	0.13	0.125-16	----------	----------	----------	M	-------

MG: *Mycoplasma gallisepticum*; MS: *Mycoplasma synoviae*; H: Highly sensitive; M: Moderately sensitive; L: Low sensitive; R: Resistant

3 and Figure 2. The control group showed neither mortalities nor symptoms all over the experimental period.

Post-mortem lesions and lesions scoring

The air sacs from the MG-infected positive control group and the erythromycin-treated groups have the highest lesion ratings, which ranged from (3 to 4) as shown in Table 3. Figure 3 showed that the lesion scores were compared between groups; groups without shared letters indicate a significant difference between them with a p-value < 0.05. (Post infection, post treatment and the end of study). Tilmicosin-treated groups specifically displayed scores from (o to 1) and seen some PM lesion in erythromycin and positive control groups as seen in Figure 5.

Table 3 and Figures 6 & 7 list the results of the PM lesion of MS infection, which include varying degrees of arthritis, foamy air saculitis, sternal bursitis, and an increased condemnation rate. The MS-infected positive control group and the erythromycin-treated groups have the greatest lesion ratings; Groups without shared letters indicate a significant difference between them with a p-value < 0.05. (Post infection, post treatment and the end of study) as spiramycin-treated groups particularly decrease the severity of arthritis and seen some PM lesion in erythromycin and positive control groups in Figure 3.

Post-mortem investigation of slaughtered or freshly dead chickens

On days 18, 23, and 35 of age. Four birds from each group were sacrificed, one after the onset of clinical symptoms, one following the therapy, and one at the end of the experiment. Birds were opened and

Table 3. Clinical score, PM lesion score, mortalities, and re-isolation.

Groups of MG (trail1)	Groups of MS (trail2)	The applied drug (Number of birds in each Group)	Signs score		Lesions score		Mortalities During the period of study (35 days)		Re-isolation% Post treatment	
			Post infection		Post infection					
			Post treatment		Post treatment					
			End of study		End of study					
A	1	Control +ve -30	MG	MS	MS	MG	MS	MG	MS 100%	MG 100%
			2	+2 Arthritis	+3 Arthritis	+3	2/30	3/30		
			No treatment	No treatment	No treatment	No treatment				
			3	+3 Arthritis	+4 Arthritis	4				
B	2	Control -ve -30	MS	MG	MS	MG	MS	MG	MS 0%	MG 0%
			0	0	0	0	2/30	3/30		
			No treatment	No treatment	No treatment	No treatment				
			0	0	0	0				
C	3	Tylosin -30	MS	MG	MS	MG	MS	MG	MS 80%	MG 80%
			3 Arthritis+ airsaculitis sternal bursitis	3	2 Arthritis	2	1/30	2/30		
			+2 Arthritis +foamy airsaculitis sternal bursitis	+3	2	2				
			2	2	1	2				
D	4	Tilmicosin -30	MS	MG	MS	MG	MS	MG	MS 30%	MG 20%
			MS	MG	MS	MG	0/30	0/30		
			+3 Arthritis	+3	+2 Arthritis	+2				
			2	1	1	1				
E	5	Spiramycin -30	MS	MG	MS	MG	MS	MG	MS 20%	MG 25%
			3	3	+2 Arthritis	+2	0/30	1/30		
			2	2	1	1				
			0	1	0	1				
F	6	Lincomycin -30	MS	MG	MS	MG	MS	MG	MS 40%	MG 40%
			3	3	2 Arthritis	2	1/30	1/30		
			2	2	2	2				
			2	1	2	1				
G	7	Doxycycline -30	MS	MG	MS	MG	MS	MG	MS 40%	MG 30%
			3	3	2 Arthritis	2	1/30	0/30		
			2	2	2	1				
			2	1	1	1				
H	8	Erythromycin -30	MS	MG	MS	MG	MS	MG	MS 100%	MG 100%
			3	3	2 Arthritis	2	2/30	2/30		
			3	+4+ lung pneumonia	2	2				
			4	4	3	3				
I	-----	GLE -30	3		2		60%		1/30	
			2		1					
			2		1					

MG: *Mycoplasma gallisepticum*; MS: *Mycoplasma synoviae*

macroscopically checked for any lesions, particularly for CRD in MG infection (fibrinous pericarditis, perihepatitis, air saculitis) and arthritis, foamy air saculitis and sternal bursitis in case of MS infection.

Feed conversion rate (FCR) and average body weights (ABW)

The productivity of the birds was estimated weekly and the ABW, FCR, and feed consumption were measured as seen in Table 4. We make statistical analysis on final body weight (FBW) to each group and compere between treated groups as seen in Figure 4 Groups without shared letters indicate a significant difference between them with a p-value < 0.05.

Tilmicosin treated birds in MG groups showed a significant improvement in FBW which have value =1880 and FCR= 163. On the same way, spiramycin and tilmicosin showed a significant improvement in FBW

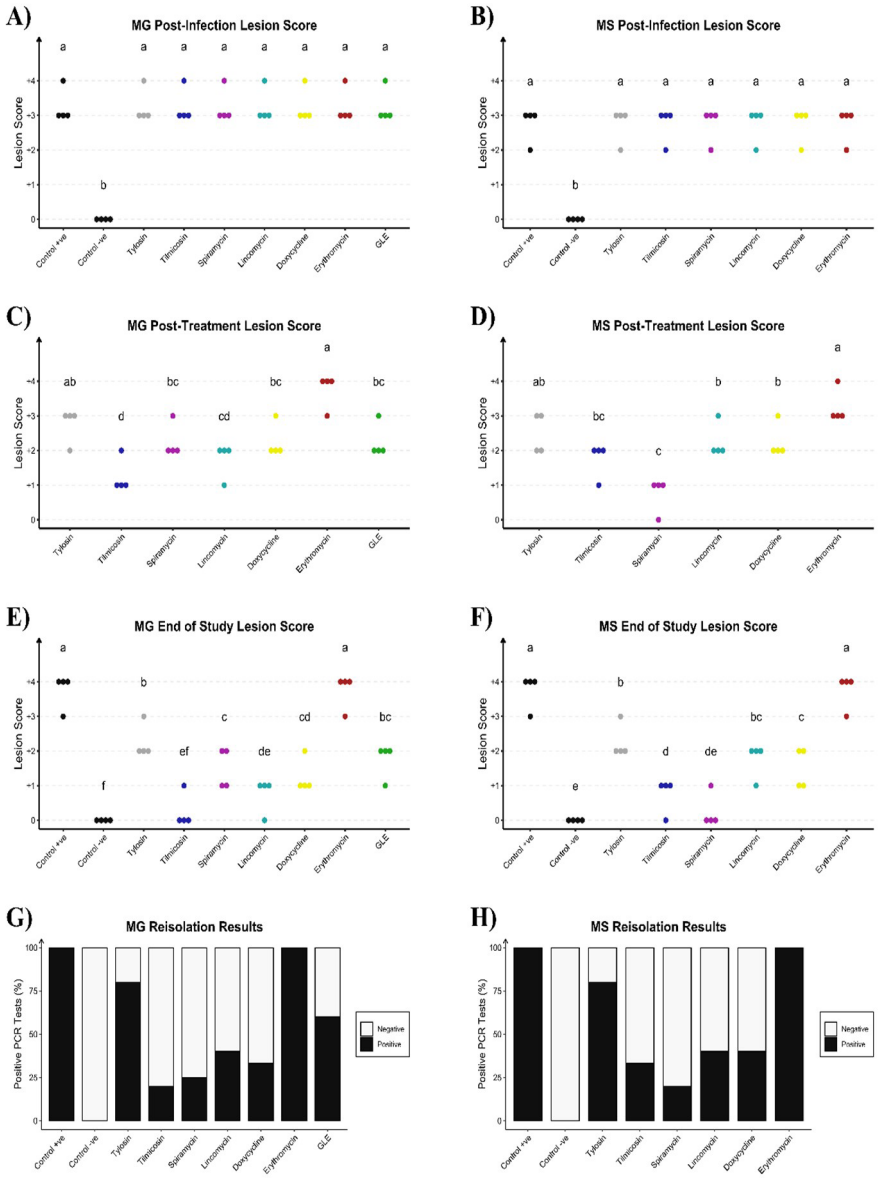

Fig. 3. Showing lesion scores and re isolation rates were compared between groups using the Kruskal-Wallis test followed by post-hoc analysis according to the Benjamini-Hochberg procedure. Groups without shared letters in case of lesion score indicate a significant difference between them with a p-value < 0.05 and re isolation rates in case of MG infection showing tilmicosine treated group nearly to negative group but in MS infection showing spiramycin treated group nearly to negative control group.

Table 4. Birds productivity and feed conversion rate in different experimental groups.

		Tilmicosin	Tylosin	Spiramycin	Licomycin	Doxycycline	Erthromycin	GLE	+ve	-ve
MG	IBW	45	45	45	45	45	45	45	45	45
	FBW	1880	1480	1750	1800	1800	1360	1780	1390	2300
	TFC	3000	3000	3000	3100	2900	2800	3200	3200	3500
	FCR	1.63	2.09	1.75	1.77	1.65	2.13	1.84	2.38	1.55
MS	IBW	45	45	45	45	45	45	-	45	45
	FBW	1750	1387	1790	1545	1600	1400	-	1300	2100
	TFC	2900	2890	3000	2800	2800	3000	-	3100	3350
	FCR	1.70	2.15	1.72	1.87	1.81	2.21	-	2.47	1.63

MG: *Mycoplasma gallisepticum*; MS: *Mycoplasma synoviae*; IBW: initial body weight; FBW: final body weight; TFC: total feed consumption; FCR: feed conversion rate; +ve: positive control group(infected); -ve: negative control group(non-infected)

which have value =1790,1750 and FCR= 1.719,1.70 respectively in MS groups at a p-value < 0.05.On the other hand, erythromycin revealed a significant reduction in FBW which have value =1360,1400 and significant increase in FCR= 2.12, 2.21 in both MG and MS respectively infected-treated groups a p-value < 0.05 (Table 4 and Figure 4).

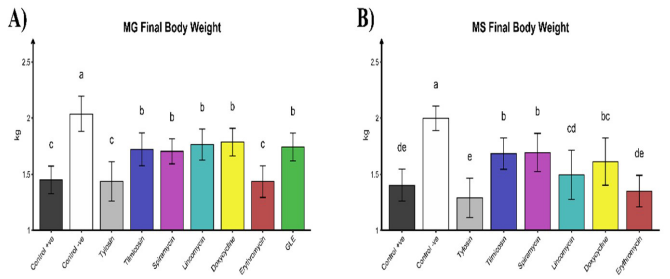

Fig. 4. Showing ANOVA was used to compare the different groups after checking its assumptions: normality and homogeneity of variance. Then, the Tukey post-hoc test was used to perform multiple comparisons between groups. Data shown as mean ± standard deviation. Groups without shared letters indicate a significant difference between them with a p-value < 0.05 in case of MG and MS else.

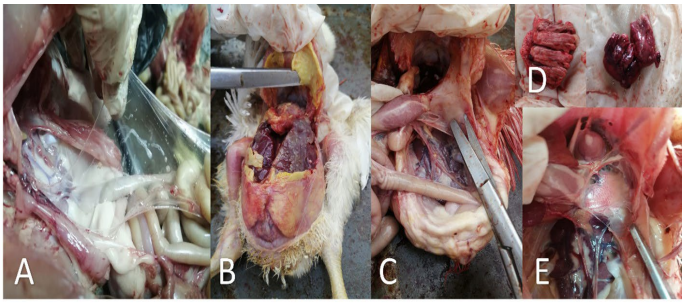

Fig. 5. Showing A: foamy abdominal air saculitis result from MG infected group10 dpi; B: caseous air saculitis and poly serositis; C: fibrinous air saculitis; D: degree of pneumonia in positive control group and seen else in erythromycin treated group; E: Control negative group normal lung and normal air sac.

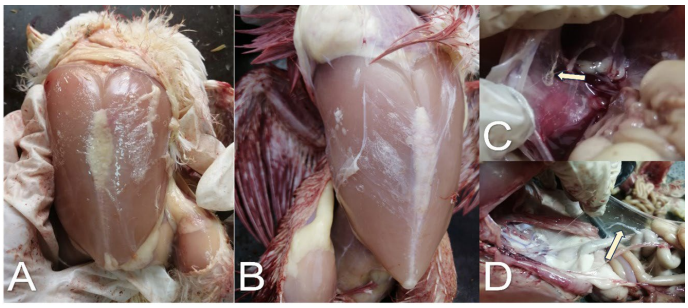

Fig. 6. Showing A and B: degree of foamy sternal bursitis in MS infected group C: foamy airsaculitis (thoracic air sac) in MS infected group non treated; C and D: foamy airsaculitis (Abdominal air sac) in MS infected group non treated and treated group with erythromycin.

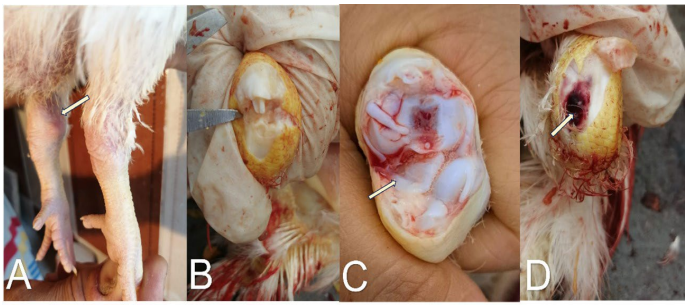

Fig. 7. Showing A: highly degree of arthritis in hock joint which seen in Erythromycin MS treated group and positive control group; B, C and D: opened hock joint containing caseous and hight amount of synovial fluid (bloody exudate).

MG and MS's re-isolation

A greater frequency of pathogen re-isolation was seen in infected non-treated groups following days post infection (dpi) compared to non-infected birds across all testing periods. Infected groups treated with tilmicosin were bacteriologically nearly negative to MG, and infected groups treated with spiramycin were bacteriologically nearly negative to MS, the frequency of re-isolation was therefore fully decreased to (1/5) 20% one case positive from five examined samples and the rest was negative as seen in Table 3.

Discussion

Avian mycoplasmosis, particularly MG and MS, are serious avian pathogens and to reduce the financial losses caused by these bacteria, appropriate treatment should be selected (Armour *et al*., 2020; El-Naggar *et al*., 2022). Due to the enormous populations of poultry, multi-age farms, and numerous potential linkages (people, feed trucks, etc.) between the meat and layer sectors, it is difficult to completely control MG and MS infections (Kleven *et al*., 2008; Marouf *et al*., 2020). Beside MG and MS can alter its surface proteins, which enables the pathogen to lessen the effectiveness of antimicrobials, particularly those that target surface proteins (Beylefeld *et al*., 2018) Though, avian mycoplasmosis cannot be completely eradicated, antibiotic treatment can be regarded of as a good choice to reduce the death rate (Taiyari *et al*., 2021). Antimicrobial resistance is a critical problem (Gautier-Bouchardon, 2018). A useful strategy to prevent AMR is to monitor the *In vitro* antibiotic susceptibility profile MIC of field isolates by using the broth microdilution method to develop a successful antimicrobial treatment for MG and MS infections (Gigueré *et al*., 2013). Moreover, the absence of specified standards makes it difficult to evaluate the results (Gigueré, 2013). The Clinical and Laboratory Standards Institute (CLSI, 2018; Hannan, 2000) has established approved breakpoints for several antibiotics in the case of the animal infection mycoplasmas. Tetracyclines, macrolides, pleuromutilins, and fluoroquinolones are the antimicrobials that are most frequently used because they are effective against mycoplasmas (Bradbury *et al*., 1994). Because several *Mycoplasma*-active antimicrobials lack interpretation criteria (cutoff values for sensitivity/resistance), it is challenging to assess the efficiency of the drugs *In vivo* only using data on *In vitro* susceptibility profiles. Because of this, the authors avoided using the words "sensitive," intermediate" and "resistant" as much as possible and instead chose to describe the MIC values as low, intermediate, or high. additionally, per Taiyari and associates' recommendations (Taiyari *et al*., 2021). So, we use both *In vivo* and *In vitro* to evaluate the efficacy of various antibiotics against MG and MS infection to reach an effective treatment.

The *in-vitro* assessment of tested anti-mycoplasma drug efficacy were discussed as the MIC values for all macrolides showed a wide range such as (tilmicosin, tyloin, spiramycin and erythromycin) (Hofacre *et al*., 2013).

From our result, tilimicosin is highly active against MG with MIC value from 0.00781 to 16 µg/ml which agree with result of de Jong *et al*. (2021) with MIC value from 0.008 to 32 µg/ml spiramycin, on the other hand, spiramycin was the best antibiotic in preventing MS from growing when using MIC *In vitro* with MIC value range from 0.015625 to 16 µg/ml which agree with (Emam *et al*., 2020).

Erythromycin showed resistant MIC values against both MG and MS with value 4-8 µg/ml. These results are in concur with those conducted by Tanner and Wu (1992); Bradbury *et al*. (1994); Elbehiry *et al*. (2016) and Khatoon *et al*. (2018).

These observations could be contributed to the massive usage of antibiotics as prophylaxis during the incubation period throughout the first life of broiler raring in almost farm of poultry (Wu *et al*., 2005).

Regarding tylosin, it had a low effect on MG and moderate effect on MS with MIC value range from 0.25 to 16 µg/ml for MG and 0.125-16 µg/ml for MS this can lead to resist of tylosin and these result agrees with Elbehiry *et al*. (2016) and Khatoon *et al*. (2018) and that may be due to the development of macrolide resistance seems to be caused by the A2059 G mutation (Beylefeld *et al*., 2018).

The only lincosamides medication approved for usage in poultry is lincomycin, a moderate spectrum antibiotic that has demonstrated efficacy in treatment *Mycoplasma* infections in birds (Hofacre *et al*., 2013). From our data, lincomycin sensitivity ranged from 0.125 to 16 µg/ml for the MG and 0. 25 to 16 µg/ml for MS strains which agree with (Pakpinyo and Sasipreeyajan, 2007).

Doxacycline show sensitivity to MG higher than MS with MIC value range from 0.0625-16 µg/ml as seen in Table 2 and agree with de Jong *et*

al. (2021) MIC result.

The world is currently facing a major threat to global public health because of the widespread development of acquired bacterial resistance to antibiotics, which is one of the major causes of this situation (Chopra, 2000; Osman *et al*., 2012). Attention is now being focused on biologically active components derived from plant and fruit species commonly used as herbal medicine due to the issue of resistance against antibiotics, since they may offer a new potent source of antibacterial and antifungal properties (Erfan and Marouf, 2019). The fruit plant guava (*Psidium guajava* L.) is a member of the *Myttaceae* family, guava leaves, roots, and fruit have an antibacterial action against MG (Hemeg *et al*., 2020). Therefore, MIC value of guava leaves extract showed moderate sensitivity to MG with range 0.25-16 µg/ml and this need to further investigation. The in-vivo assessment of tested anti-mycoplasma drug efficacy according to Table 3.

The observed clinical signs and score were sneezing, slight lacrimal and nasal discharge, and moderate decrease of appetite, delayed growth, and mouth breathing among the MG-infected untreated group (control positive).

Similar clinical indications have also been documented by Stipkovit, *et al*. (2012), but MS infected untreated group (control positive) showing lower growth rate, walking difficulty, swelling joints, and poor-quality chicks. Which have also been documented by Lockaby, *et al*. (1998).

The clinical symptoms in the tilmicosin-treated groups with MG infections gradually decreased till disappeared following the course of treatment. Likewise, (Guo *et al*., 2004), but in MS infection can reduce clinical signs but not disappeared according to (Yan *et al*., 2023). When the course of treatment for MS infections was utilized for 7 days, the clinical symptoms in the spiramycin-treated groups steadily subsided till they disappeared likewise, Elazab *et al*. (2021). Majima (2004) reviewed the clinical advantages of macrolides in the treatment of chronic sinusitis. These advantages include a reduction in discharge from the nose and nasal secretions, a reduction in nasal obstruction, an impact on mucus production, and an impact on the transportability of airway secretions.

Regarding the groups treated with erythromycin, there is no available research to prove the extent of its resistance *In vivo*, but the results, proves that the groups treated with erythromycin have the picture of MG and MS infection signs as control positive group as mucous exudates were found in the trachea, air sacs, and nasal passages in the MG-infected untreated group, both light-colored foci and congestion could be seen in some lungs and most damaged air sacs received scores of 2 or 3. Mycoplasmosis is generally a respiratory disease that affects the air sacs first before spreading to the trachea and upper respiratory passages (Much *et al*., 2002).

MS-challenged birds exhibited sternal bursitis, low-grade air sacculitis, and inflamed hock joints (arthritis) similar lesions were recorded by Amer *et al*. (2019). Also, Abd El-Daeem (2008) mentioned similar lesions (tracheitis and air sacculitis) are the main signs of a specific infection in poultry because the infection begins with colonisation of the respiratory system.

MG chicken exposed to tilmicosin had a range of air sac lesions, ranging from mild turbidity to seeming normal, and they scored (1) in some slaughtered manifestations before falling to a score of (0) at the end of the trial. In the same way, Charleston *et al*. (1998) demonstrated that 5-day; "in water" tilmicosin therapy was highly effective for treating experimental MG infection and significantly decreased the incidence and degree of air sacculitis lesions produced by MG. A treatment regimen of 20 mg tilmicosin/kg BW for five consecutive days seems to be the best advised regimen for treating clinical outbreaks of *M. gallisepticum* in broilers (Garmyn *et al*., 2019).

Our findings enhanced those made by Amer *et al*. (2009), who discovered that tilmicosin and lincomycin are still effective in preventing mycoplasmosis in broilers by causing the mean gross air sacs and microscopic tracheal lesions scores of the treated group to be markedly lower than those of the untreated infected group. Our results enriched those previously recorded by Elazab *et al*. (2021) who discovered that spiramcin is still effective in preventing MS in broilers, reducing the severity of arthritis, and making lesions scores of the air sac of the treated group significantly lower than those of the infected untreated group which taken by dose 17 mg /kg/day for 7 days.

Tilmicosin un-significantly lowered MG mortality, but significantly lowered macroscopic lesions as well as it reduced the re-isolation rate up to (one positive birds/5 negative birds) 20 %. These results were parallel to that recorded by Abd El Ghany (2009) and Zakeri and Kashefi (2011) they also confirmed that tilmicosin decreased re-isolation up to 0% at the end of study.

The records described above provided evidence that MG and MS had become resistant to numerous antimicrobials over a long period of time, although tilmicosin and spiramycin are unrelated to these drugs and are still quite effective against MG and MS resistant strains respectively. Both drugs are now the ones that are advised for controlling resistant mycoplasmas because they also very slowly generate *Mycoplasma* resistance.

Group treated with GLE showed moderate to satisfactory benefits against MG infection by reductions in symptoms and lesions, re-isolation rates, and satisfied FCR results, but additional research is required. Hemeg *et al*. (2020) confirmed that GLE has an *In vitro* antibacterial effect against MG. Up till now; no data about the *In vivo* evaluation of the antibacterial effect of GLE against MG was available. But much research revealed the effect of GLE on other system and different microbe, Rahman *et al*. (2013) explain that the guava leaf is an excellent source of nutrients and has a notable impact on broiler fat levels, mortality rate, and antibacterial activity against E. coli, followed by *Streptococcus* sp. and *Staphylococcus* sp. Morsy *et al*. (2019) observed that In Egyptian environmental conditions, GLE could be successfully added to the diet of developing rabbits up to 3.0 ml/kg food, which boosted production performance without having a negative impact on health during the growth phase.

Conclusion

For proper antibiotic selection and to avoid the emergence of AMR, MIC data collection is essential. Many MG and MS strains exhibited low sensitivity of tylosin and erythromycin due to the abuse of this antibiotics as well as existence of mutations. Tilmicosin is consider the most appropriate antibiotic against MG strains and spiramycin against MS isolate both *In vivo* and *In vitro* which reduce signs, PM lesion, mortalities, maintains body weight and FCR of birds and re-isolation rate. Since different nations execute their own prevention and treatment policies against MG and MS infection, routine standard antimicrobial MIC reports should be undertaken on a logical number of MG and MS field isolates in varied geographical distributions such programmes can offer a useful tool to reduce the cost of MG infection prevention and treatment programmes. GLE is one of the natural herbal extracts that is thought to be a real solution for antimicrobial resistance but require more research to use its nano-preparation alone or with other herbal extracts with different treatments doses for different time periods to overcome AMR with the usage of safe natural products.

Conflict of interest

The authors declare that they have no conflict of interest.

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