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ORIGINAL ARTICLE

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Antibacterial Effect of Different Vitamin C Formulations on Multi-drug Resistant Salmonella Serovars Recovered from Broiler Chickens

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Abstract
There is a great exertion to look for the alternative therapeutic methods that assist in controlling the antibacterial resistance of Salmonellae. The aim of this study was to evaluate the antibacterial effect of vitamin C (ascorbic acid) different formulations at different concentrations. Serotyping of the isolates revealed that 10 belonged to *S. Enteritidis* 1,9,12:g,m, 7 *S. Virchow* 6,7,14:r:1,2, and 3 *S. Montevideo* 6,7,14:g,m[p],s :[1,2,7]. The isolates were highly resistant (100%) to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, and doxycycline, and 90% of the isolates were susceptible to amikacin. PCR revealed that 10 *S. Enteriditis* isolates harbored 3 virulence-associated genes *invA*, *sefA*, and *fimH,* while 7 isolates harbored *stn* gene. Also, 10 *S. Enteriditis* isolates harbored *sul1* and bla_{TEM} genes. Biofilm formation was 100%, 50%, and 42.8% in *S. Montevideo*, *S. Enteritidis*, and *S. Virchow* respectively. Vit C (formulation 1) completely inhibited the growth of all isolates at concentrations 0.3%, 0.5% and 1%. Formulation 2 inhibited the growth of the tested isolates at concentration 0.3%. While, concentration 0.1% inhibited only the growth of *S. Virchow.* Formulation 3 inhibited the growth of *S. Enteritidis* and *S. Virchow* at concentrations 0.3 and 0.5%. Formulation 4 inhibited the growth *S. Enteritidis, S. Virchow* and *S. Montevideo* at concentration 1%. Formulation 5 inhibited the growth of *S. Enteritidis* and *S. Virchow* at concentrations 0.1%, 0.5% and 1%. Formulations 4 and 1 inhibited effectively the biofilm-forming ability of *S. Enteritidis* (80% and 100%) and *S. Virchow* (60% and 66.6%).

Keywords
Antimicrobials, Organic Acids, Salmonella, Vitamin C

1. Introduction
Salmonellosis is a zoonotic disease, hence Salmonella is considered as one of the dangerous foodborne pathogens among chickens that causing a worldwide public health disaster **(Punchihewage-Don et al., 2024).** *Salmonella enterica* serovars are a varied group of pathogens which have the ability to live in an environment with low pH (including; cecum (of poultry), colon, and small intestine) so that Salmonella is able to colonize multiple host species including chickens. In which poultry and poultry products are regarded as an important source of Salmonellosis outbreaks in the human populations **(Foley et al., 2013; Talukder et al., 2021)**. Salmonella harbors Salmonella pathogenicity islands (SPIs) in which SPI1 is responsible for the invasion of host cells **(Peng, 2016)**. *Salmonella Enteritidis* is one of the foodborne pathogens that threaten the safety of broiler chickens whereas it's harboring a diversity of virulence factors that participate in the pathogenicity **(Foley et al., 2008).**

The frequent usage of antimicrobial agents for (the treatment in sub-therapeutic doses, the prevention of diseases, and as a growth promoter), it has an important role in the rapid spreading of MDR Salmonella strains **(Lamichhane et al., 2024).** Salmonella is harboring the anti-microbial resistance genes on mobile genetic elements (MGEs) including (plasmids, transposons, and phages) through conjugation, transformation, and transduction mechanisms which contribute in the rapid distribution of MDR among Salmonella serotypes or other different bacterial genera **(Frost et al., 2005; Nazari Moghadam et al., 2023).**

Salmonella frequently present in nature as planktonic and sessile forms that have the ability for biofilm formation on biotic and abiotic surfaces that are embedded in extracellular matrix of polymeric substances (EPS) that result in prevention of the passage of physical and chemical substances into the bacterial cells and also resist the host immune system **(Peng, 2016; Karygianni et al., 2020).**

Great exertions have been made through the usage of antibiotic alternatives due to the swift dissemination of MDR Salmonella including; organic acids, essential oils, prebiotics, probiotics, quorum sensing (QS)/virulence inhibitors, phage therapy, antimicrobial peptides, and small molecule growth inhibitors that may be used separately or in combination **(Lamichhane et al., 2024).**

Vitamin C (ascorbic acid) is a necessary micronutrient which composed of two isomers; the ascorbic acid (reduced form) and the dehydroascorbic acid (oxidized form). It has antimicrobial activities alternative to antibiotics, decreasing the threat of the infection, anti-biofilm, and anti-oxidant effects and also it has immunomodulatory role. It is accessible, cheap, and has a little or no side-effects. The antibacterial activity of vitamin C was attributable to the lowering effect of pH **(Mousavi et al., 2019; Abdelraheem et al., 2022).** Vitamin C was also found to inhibit the injurious impact *S. Enteritidis* **(Sharma et al., 2018).**
Organic acids are organic carboxylic acid (amino acids and fatty acids) comprising; carboxylic acids bearing a hydroxyl group such as (lactic, citric, tartaric, and malic acids) and simple monocarboxylic acids such as (acetic, butyric, propionic, and formic acids) which have

antimicrobial property depending on pH (Al-Kassi and Mohssen, 2009). This study aimed to inspect the effect of vitamin C (ascorbic acid) in combination with (organic acids, EOs, astragalus oil) on the growth and anti-biofilm formation of Salmonella serovars isolated from broiler chickens *in vitro*. Also the effect of vitamin C in combination with astragalus oil (formulation 5) on antimicrobial susceptibility of some isolates was studied.

2. Materials and Methods

2.1. Ethical Statement

Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Beni-Suef University (BSU-IACUC), Egypt. The ethical approval number was BSU-IACUC-022-246.

2.2. Sampling

All isolates were completely identified according to (Koneman et al., 1992; Collee et al., 1996; Quinn et al., 2011).

2.3. Serotyping of Salmonella Isolates

Biochemically identified Salmonella isolates were serotyped using Kauffmann-White complete plate agglutination test (Kauffmann, 1974) at the Serology Unit, Animal Health Research Institute, Dokki, Giza, according to Grimont and Weill, (2007) for the determination of somatic antigen (O) and flagellar antigen (H).

2.4. Antimicrobial Susceptibility Testing of Salmonella Isolates

In vitro antimicrobial susceptibility testing of 20 Salmonella isolates including (10 *S. Enteritidis*, 7 *S. Virchow* and 3 *S. Montevideo*) to fourteen different antimicrobials from seven different classes (Oxoid, England) was estimated using the disc diffusion method on Mueller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2021) were as follow; amoxicillin-clavulanic acid (30µg), ceftriaxone (30µg), amikacin (30µg), apramycin (15µg), gentamycin (10µg), streptomycin (10µg), ciprofloxacin (5µg), doxycycline (30µg), fosfomycin (200µg), ofloxacin (5µg), colistin-sulphate (10µg), erythromycin (15µg), clindamycin (2µg), and sulfamethoxazole-trimethoprim (25µg). Zone of inhibition results were interpreted according to (CLSI, 2021).

2.5. Molecular Characterization of Virulence and Resistance Genes in *Salmonella Enteritidis*

Deoxyribonucleic acid (DNA) from 10 *Salmonella* Enteritidis isolates was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) from overnight tryptone soya broth cultures according to the manufacturer's instructions. The polymerase chain reaction (PCR) amplification of Salmonella isolates using specific primers (Table, 1) was conducted using 25µl Volume/reaction mixture including 12.5µl Emerald Amp GT PCR master mix (2x premix) (Takara, Japan), 5.5µl PCR grade water, 1µl forward primer (20pmol), 1µl reverse primer (20pmol), and 5µl template DNA. The cycling conditions of the PCR protocol were 35 cycles for each: primary denaturation at 94˚C for 5min, secondary denaturation at 94˚C for 30sec, Annealing at 52-60˚C for 30-40sec (depending on the primer), extension at 72˚C for 30 sec and finally final extension at 72˚C for 7min. A 1.5% gel was prepared and electrophoresed and then photographed using a gel documentation system.

Table1. Oligonucleotide primer sequences were used for the identification of virulence and resistance genes used in this study.

Gene	Sequence	Amplified product	Reference
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	Oliveira et al., (2003)
sefA	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	310 bp	Akbarmehr et al., (2010)
fimH	TGTGACAGGGACATTTAGCG GTGCCAATTCCTCTTACCGTT	164 bp	Hojati et al., (2015)
stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617 bp	Murugkar et al., (2003)
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom et al., (2003)
sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433 bp	Ibekwe et al., (2011)

2.6. Detection of Biofilm Formation

By using Congo red (CR) assay to evaluate curli production (Zhou et al., 2013). The pure colonies of 20 Salmonella isolates including (10 *S. Enteritidis*, 7 *S. Virchow* and 3 *S.* Montevideo) were streaked onto Luria bertani (LB) agar plates and incubated for 48hr at 37°C. Single colonies were picked and streaked onto YESCA CR agar plates and incubated at 25°C for 48-72hr. The red colonies were positive for biofilm formation and the pink or white colonies were negative for biofilm formation.

2.7. Detection of Antimicrobial Activity of Vitamin C Formulation

The antimicrobial activity of vitamin C formulations (Table, 2) against 20 Salmonella isolates were performed by using the agar dilution method (Koneman et al., 1992; Mekonnen et al., 2016) as follow; the tested Salmonella isolates were streaked onto tryptone soya agar (Oxoid) at 37˚C for 24 hours. Colonies were suspended in saline, and the suspension was adjusted to a turbidity equivalent to 0.5 McFarland (1.5×10^8CFU/ml). A stock solution of ascorbic acid was prepared by dissolving commercially purchased ascorbic acid (L-ascorbic acid, sigma) (Fisher Science Education, Hanover Park, IL) in sterile distilled water and sterilized through cellulose filter membrane (0.45µm). Concentrations (0.1, 0.3, 0.5 and 1%) of vit C formulations (1), concentrations (0.05, 0.1, and 0.3%) of vit C formulation (2), concentrations (0.1, 0.3, and 0.5%) of vit C formulation (3), concentrations (0.5 and 1%) of vit C formulation (4), and concentrations (0.05, 0.1, 0.5 and 1%) of vit C formulation (5), were added to the sterilized cooled tryptone soya agar (~55°C) separately, then poured in Petri dishes and left to solidify. The suspended colonies in saline streaked onto the agar plates and incubated at 37 c for 24 hours and then examined for the bacterial growth.

2.8. Effect of Vitamin C Formulation 5 on Antimicrobial Susceptibility Profile

Six Salmonella isolates including (2 *S. Enteritidis*, 2 *S. Virchow*, and 2 *S. Montevideo*) from highly resistant bacteria that could grow in the presence of vitamin C formulation 5 at concentration of 0.5% were selected to investigate the potential effect on the antimicrobial resistance profile. The test was conducted using the method already described (Collee et al., 1996; CLSI., 2021).

Table2. Vitamin C formulations.

	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5
Vitamin C	20%	20%	20%	20%	20%
Citric acid	10%	10%	-	10%	-
Lactic acid	-	10%	-	10%	-
Eucalptys oil	1%	1%	1%	-	-
Saccharomyces Cerevisae (1×10^6 cfu/ml)	-	-	5%	-	-
Astragalus oil	-	-	-	-	10%

2.9. Detection of Biofilm Formation of Salmonella Isolates Post Exposure to Vitamin C Formulations

Salmonella isolates which were positive for biofilm formation (5 *S. Enteritidis*, 3 *S. Virchow* and 3 *S. Montevideo*) were selected to evaluate their ability for forming biofilm post exposure to vitamin C at conc (0.1%, 0.05%, 0.1%, 0.5, and 0.05%) of formulations (1, 2, 3, 4, and 5) respectively **(Radwan et al., 2022)**.

3. Results

3.1. Serological Identification of Salmonella Isolated from Broilers

Twenty Salmonella isolates were serotyped as ten *S. Enteritidis* 1,9,12:g,m , seven *S. Virchow* 6,7,14:r:1,2, and three *S. Montevideo* 6,7,14:g,m[p],s :[1,2,7].

3.2. Antimicrobial Susceptibility Testing of Salmonella Isolates

In vitro antimicrobial susceptibility of the 20 Salmonella isolates from broiler chickens to 14 antimicrobial agents. The results revealed that the Salmonella isolates were highly resistant to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, and doxycycline with an incidence of (100%), followed by 85% for fosfomycin and sulfametho-xazole-trimethoprim, 80% for ceftriaxone and ofloxacin, apramycin (75%), respectively. Whereas the isolates were sensitive to amikacin (90%) followed by 55% and 50% for colistin-sulphate and ciprofloxacin, respectively as indicated in **Table (3).** The antimicrobial resistance rates of *S.* Montevideo were higher than those *S. Enteritidis* and *S. Virchow*. Among Salmonella isolates 35% were MDR**;** the proportion of MDR isolates among *S.* Montevideo isolates was the highest (78%), followed by *S. Enteritidis* (50%) and *S. Virchow* (35%). Concerning the antimicrobial resistance rates based on the serovars; all isolates were highly resistant to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, and doxycycline with an incidence (100%). While *S.* Montevideo was also resistant to sulfamethoxazole/trimethoprim, ofloxacin, fosfomycin, ciproflo-xacin, ceftriaxone, and gentamycin with an incidence (100%).

3.3. Molecular Characterization of Virulence and Resistance Genes in *S. Enteritidis*

All *S. Enteritidis* isolates were investigated for four virulence genes: *invA, sefA fimH*, and *stn* by PCR. In *S. Enteritidis* isolates the most commonly detected virulence genes were *invA*, *sefA*, and *fimH* with an incidence of 100%, followed by *stn* (70%) arranged as (6 out of 8 from cecum) and (1 out of 2 from liver). The isolates were investigated for two antimicrobial resistance genes; *sul*1 gene and ESBL-producing gene (bla_{TEM}) and the results revealed that the isolates 100% were positive for each one, as shown in **(Table 4 and Fig. 1).**

3.4. Biofilm Formation

The biofilm formation on YESCA CR agar, 11 (5 *S. Enteritidis*, 3 *S. Virchow*, and 3 *S. Montevideo*) isolates were positive biofilm formation with a percentage of 50%, 42.8%, and 100%, respectively. The strong biofilm formation was 40%, 66.6%, 33.3%, respectively. As shown in **(Table, 5).**

Table 3. Antimicrobial sensitivity testing results of 20 Salmonella isolates.

Antimicrobial agent	Concentrations(µg)	Salmonella isolates			
		Resistance		Susceptible	
		No.	%	No.	%
Amoxicillin-Clavulanic acid (AMC)	30	20	100	0	0
Ceftriaxone (CTR)	30	16	80	4	20
Amikacin (AK)	30	2	10	18	90
Apramycin (APR)	15	15	75	5	25
Gentamycin (Gen)	10	13	65	7	35
Streptomycin (S)	10	20	100	0	0
Ciprofloxacin (Cip)	5	10	50	10	50
Ofloxacin (OF)	5	16	80	4	20
Erythromycin (E)	15	20	100	0	0
Clindamycin (DA)	2	20	100	0	0
Doxycycline (Do)	30	20	100	0	0
Colistin sulphate (CL)	10	9	45	11	55
Fosfomycin (Fo)	200	17	85	3	15
Sulfamethoxazole- Trimethoprim (sxt)	25	17	85	3	15

Table 4. Virulence and antimicrobial resistance genes of *S. Enteritidis* isolates

Isolate no. (origin)		*invA*	*sefA*	*stn*	*fimH*	*Sul1*	bla_{TEM}
1	Cecum	+	+	+	+	+	+
2		+	+	+	+	+	+
3		+	+	-	+	+	+
4		+	+	+	+	+	+
5		+	+	+	+	+	+
6		+	+	-	+	+	+
7		+	+	+	+	+	+
8		+	+	+	+	+	+
9	Liver	+	+	+	+	+	+
10		+	+	-	+	+	+

Table 5. Results of biofilm formation.

Examined serovars	Positive biofilm producers		NO. of Positive biofilm isolates	% of Positive biofilm
	Strong	Moderate		
S. Enteritidis **(n=10)**	**2**	**3**	**5**	**50**
S. Virchow **(n=7)**	**2**	**1**	**3**	**42.8**
S. Montevideo **(n=3)**	**1**	**2**	**3**	**100**

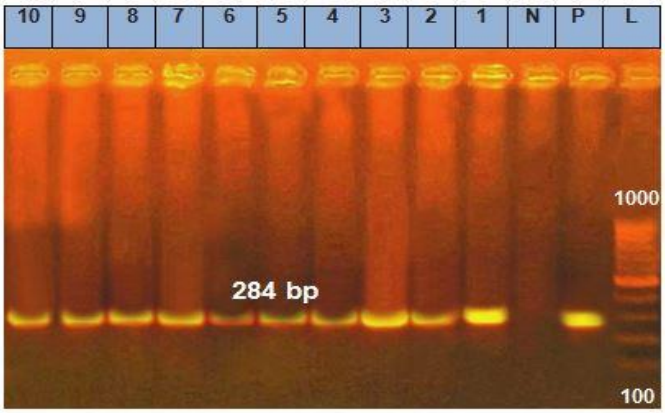

Fig 1A. PCR results of *invA* gene at amplicon of 284 bp. N: Negative control, P: Positive control (*S. Enteritidis* ATCC American type culture collection), L: (100-1000 bp) DNA ladder. Lanes (1-10): tested *S. Enteritidis* isolates for *invA*.

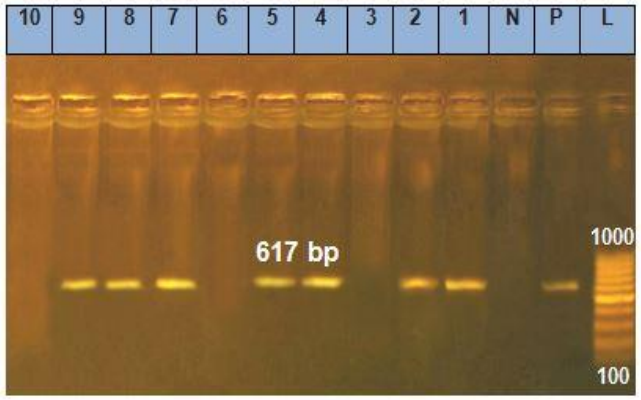

Fig 1B. PCR results of *stn* gene at amplicon617bp**.** N: Negative control, P: Positive control (*S. Enteritidis* ATCC American type culture collection), L: (100-1000 bp) DNA ladder. Lanes (1, 2, 4, 5, 7, 8, and 9): tested *S. Enteritidis* isolates for *stn*.

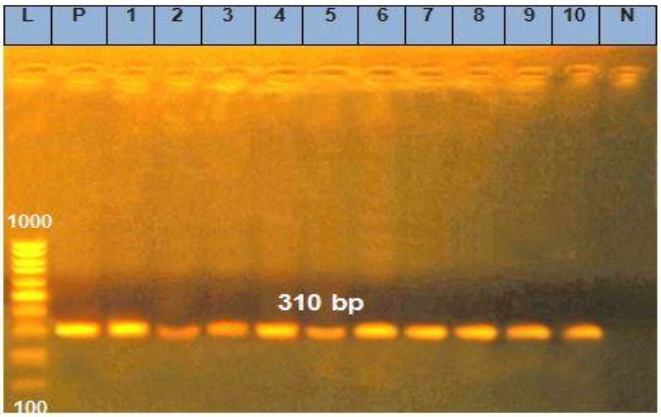

Fig 1C. PCR results of *sefA* gene at amplicon of 310 at bp. N: Negative control, P: Positive control (*S. Enteritidis* ATCC American type culture collection), L: (100-1000 bp) DNA ladder. Lanes (1-10): tested *S. Enteritidis* isolates for *sefA*.

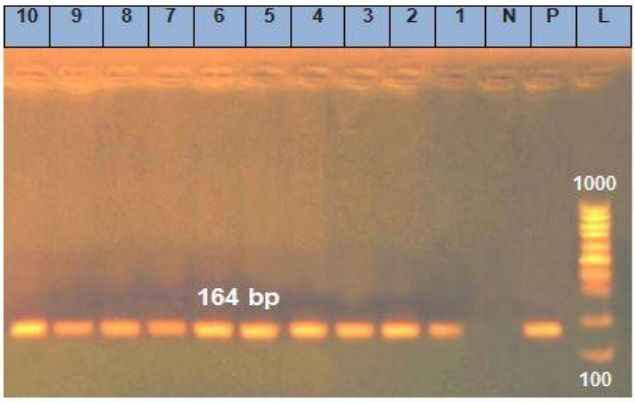

Fig 1D. PCR results of *fimH* gene at amplicon of 164 bp. N: Negative control, P: Positive control (*S. Enteritidis* ATCC American type culture collection), L: (100-1000 bp) DNA ladder. Lanes (1-10): tested *S. Enteritidis* isolates for *fimH*.

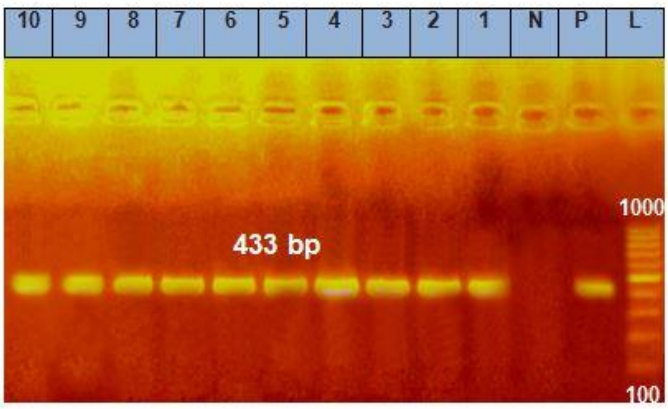

Fig 1E. PCR results of *sul1* gene at amplicon of **433** bp**.** N: Negative control, P: Positive control (*S.* Enteritidis ATCC American type culture collection), L: (100-1000 bp) DNA ladder. Lanes (1-10): tested *S.* Enteritidis isolates for *sul1.*

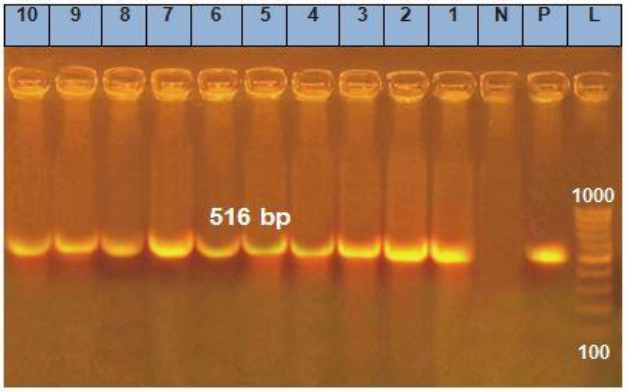

Fig 1F. PCR results of bla_{TEM} gene at amplicon of 516 bp**.** N: Negative control, P: Positive control (*S.* Enteritidis ATCC American type culture collection), L: (100-1000 bp) DNA Ladder. Lanes (1-10): tested S. Enteritidis isolates for bla_{TEM}.

3.5. Antimicrobial Activity of Vitamin C Formulation

The antimicrobial effects of five different vitamin C formulations against 20 Salmonella isolates were assessed using agar dilution assay are summarized in **Table (6).** Vitamin C formulation 1 at concentration of 0.1% didn't inhibit the growth of all Salmonella isolates, while at concentrations of 0.3, 0.5, and 1% effectively inhibited the growth of all isolates (100%). Vitamin C formulation 2 at concentration of 0.3% inhibited the growth of (80% *S. Enteritidis*, 71.4% *S. Virchow*, and 100% *S. Montevideo*), only at concentration of 0.1% inhibited the growth of *S. Virchow* (42.8%), while at concentration of 0.05% didn't inhibit the growth. Vitamin C formulation 3 inhibited the growth of (20%, 30% *S. Enteritidis* and 14.2%, 14.2% *S. Virchow*) at concentrations of 0.3 and 0.5%, respectively although at concentration of 0.1% didn't inhibit the growth. Vitamin C formulation 4 at conc 1% effectively inhibited the growth 100% of all Salmonella isolates but the growth of all isolates didn't inhibit at concentration of 0.5%. Vitamin C formulation 5 at concentration of 0.05% didn't inhibit the growth of all isolates while at concentrations of 0.1, 0.5 and 1% inhibited the growth of *S. Enteritidis* (30%) and *S. Virchow* (42.8%) but didn't inhibit the growth of *S. Montevideo*.

3.6. Antimicrobial Susceptibility of Some Isolates Post Treatment with Vitamin C Formulation 5

Post exposure to vit C formulation 5, 2 *S. Enteritidis* changed to be susceptible to amikacin, apramycin, streptomycin, colistin-sulfate, ciprofloxacin, and ofloxacin. 2 *S. Virchow* changed to be susceptible to streptomycin, ciprofloxacin, and ofloxacin. 2 *S.* Montevideo isolate changed to be susceptible to streptomycin and ciprofloxacin **(Table, 7).**

3.7. Effect of Vitamin C Formulations on Biofilm Formation of Salmonella Isolates

The results of vit C effect at non-bactericidal concentrations (0.1%, 0.05%, 0.1%, 0.5, and 0.05%) of formulations (1, 2, 3, 4, and 5) respectively revealed that vit C formulation 4 effectively inhibited biofilm formation of 4 *S. Enteritidis* and 3 *S. Virchow* isolates with a percentage of 80% and 100% respectively, followed by vit C formulation 1 inhibited biofilm formation of 3 *S. Enteritidis* and 2 *S. Virchow* isolates with a percentage of 60% and 66.6%, respectively and there was no effect on biofilm formation of *S.* Montevideo isolates of both formulations. No significant effect vit C formulations (2, 3, 5) on all tested isolates **(Table, 8).**

Table 6. Antimicrobial effect of vitamin C formulations against multidrug resistant Salmonella serovars.

Formulations	Conc.	NO growth / Growth	*S. Enteritidis* (n=10)	*S. Virchow* (n=7)	*S. Montevideo* (n=3)
Formulation 1	0.1	NO growth	0	0	0
		Growth	10	7	3
	0.3	NO growth	10	7	3
		Growth	0	0	0
	0.5	NO growth	10	7	3
		Growth	0	0	0
	1	NO growth	10	7	3
		Growth	0	0	0
Formulation 2	0.05	NO growth	0	0	0
		Growth	10	7	3
	0.1	NO growth	0	3	0
		Growth	10	4	3
	0.3	NO growth	8	5	3
		Growth	2	2	0
Formulation 3	0.1	NO growth	0	0	0
		Growth	10	7	3
	0.3	NO growth	2	1	0
		Growth	8	6	3
	0.5	NO growth	3	1	0
		Growth	7	6	3
Formulation 4	0.5	NO growth	0	0	0
		Growth	10	7	3
	1	NO growth	10	7	3
		Growth	0	0	0
Formulation 5	0.05	NO growth	0	0	0
		Growth	10	7	3
	0.1	NO growth	3	3	0
		Growth	7	4	3
	0.5	NO growth	3	3	0
		Growth	7	4	3
	1	NO growth	3	3	0
		Growth	7	4	3

Table 7. Antimicrobial susceptibility of some isolates post treatment with vitamin C formulation 5.

Pre- exposure / Post- exposure	Antimicrobial Agents													
	AMC	CTR	AK	APR	GEN	S	CL	E	DA	CIP	DO	FO	OF	SXT
Pre-exposure	*S. Enteritidis* (n=2)													
	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Post exposure (Vitamin C Formulation 5)	R	R	S	S	R	S	S	R	R	S	R	R	S	R
Pre-exposure	*S. Virchow* (n=2)													
	R	R	S	R	S	R	S	R	R	R	R	R	R	R
Post exposure (Vitamin C Formulation 5)	R	R	S	R	S	S	S	R	R	S	R	R	S	R
Pre-exposure	*S. Montevideo* (n=2)													
	R	R	S	R	R	R	S	R	R	R	R	R	R	R
Post exposure (Vitamin C Formulation 5)	R	R	S	R	R	S	S	R	R	S	R	R	R	R

Table 8. Effect of vitamin C formulations on biofilm formation.

Formulations		NO. of Positive biofilm producers	% of Positive biofilm	NO. of Negative biofilm	% of Negative biofilm
			S. Enteritidis (n=5)		
	1	2	40	3	60
	2	5	100	0	0
	3	5	100	0	0
	4	1	20	4	80
	5	5	100	0	0
			S. Virchow (n=3)		
	1	1	33.3	2	66.6
Post- exposure to vitamin C Formulations	2	3	100	0	0
	3	3	100	0	0
	4	0	0	3	100
	5	3	100	0	0
			S. Montevideo (n=3)		
	1	3	100	0	0
	2	3	100	0	0
	3	3	100	0	0
	4	3	100	0	0
	5	3	100	0	0

4. Discussion

Avian Salmonellosis is the main menace for each of poultry sector and human health through the consumption of the undercooked contaminated poultry meat and poultry products that may be transmitted to human populations **(Talukder et al., 2021).**

In the present study, serotyping of the isolated Salmonella spp. revealed the presence of three different serovars among 20 Salmonella isolates, 10 *S. Enteritidis* 1,9,12:g,m, 7 *S. Virchow* 6,7,14:r:1,2, and 3 *S. Montevideo* 6,7,14:g,m[p],s :[1,2,7]. This result was compatible with **Saad et al., (2017)** who recovered *S. Enteritidis* 1,9,12:g,m, *S. Virchow* 6,7,14:r:1,2 and *S.* Montevideo 7, 6, 14; 9,m,{p},s; (1,27) from broiler chickens. *S. Enteritidis* was the most commonly isolated serovar from cecum and liver and this result contrasts with the previous study which reported that *S. Montevideo* and *S. Virchow* are the most prevalent Salmonella serotypes in chickens in Korea **(Lee et al., 2019).**

The usage of antimicrobial agents for the purpose of enhancing growth performance**,** prophylactic, and treatment of chickens lead to increase the incidence of multi-drug resistance among the enteric bacteria **(Oguttu, 2008).** Antimicrobial resistance may be attributed to the mutation of various chromosomal loci which are a portion of a core set of genes, such as genomic islands. Genomic islands are preserved zones in the accessory districts in the genome of Salmonella. They are essential for the evolution of this genus as they have granted a number of advantages to their host via virulence and multi-drug resistance genes **(Seth-Smith et al., 2012; Castro-Vargas et al., 2020).**

The antimicrobial susceptibility of Salmonella isolates were examined against some antimicrobial agents that usually used in the farms of poultry. The current study revealed that Salmonella isolates recovered from broiler chickens were highly resistant to amoxicillin-clavulanic acid, streptomycin, erythromycin, clindamycin, and doxycycline, while *S. Montevideo* is also highly resistant to sulfamethoxazole-trimethoprim, ofloxacin, fosfomycin, ciprofloxacin, ceftriaxone, and gentamycin. In contrast, they were highly susceptible to amikacin. The antimicrobial resistance rates of *S. Montevideo* were higher than those *S. Enteritidis* and *S. Virchow*. This result was in agreement with **Hassan et al., (2018)** who reported high resistance of Salmonella isolates against erythromycin (100%) followed by streptomycin**.** The highest antimicrobial resistance to amoxicillin/clavulanic acid was reported by **Ammar et al., (2016). Radwan et al., (2022)** and **Raheel et al., (2023)** recorded the susceptibility to amikacin with 75% and 90% respectively.

In the present study, the isolates carried the virulence-associated genes *invA*, *sefA* and *fimH* with a percentage of 100% of each, followed by *stn* (70%). It has been reported that all Salmonella serovars approximately harboring the *invA* gene that comprising specific DNA sequence **(Yanestria et al., 2019; Tawyabur et al., 2020).** *stn and sefA* virulence genes are responsible for Salmonella enterotoxin and *S. Enteritidis* fimbriae, respectively **(Prager et al., 2000; Skyberg et al., 2006).** The prevalence of *sefA* and *stn* genes among the Salmonella isolates was similar to the findings of **Mir et al., (2010)** who reported the presence of *sefA* and *stn* genes in all of *S. Enteritidis* isolated from poultry. In accordance with the resistance genes, all isolates were harbored *sul1* and bla_{TEM} genes (100%), which is similar to 97.8% and 97.3% that reported by **Zhu et al., (2017)** respectively.

The bacterial biofilm production through promoting symbiotic with other organisms that renders a steady environment for the bacteria **(Harrell et al., 2021).** Our results showed that the highest biofilm formation was in *S. Montevideo* (100%), followed by *S. Enteritidis* (50%) and *S. Virchow* (42.8%). The highest prevalence of biofilm production was 70.6% in *S. Enteritidis* isolates reported by **Borges et al., (2017)** from avian origin. **Abd El-basit et al., (2019)** reported that 52.6% of Salmonella isolates from chickens were able to produce biofilm on CRA.

Vitamin C has antibacterial effect that depended on (bacterial strain and vit C concentration), antifungal, antiviral, and anti-parasitic effects. Several bacteria can ferment vitamin C in which the presence of vit C exposes the bacteria to oxidative stress that may lead to bacterial growth inhibition. In which the combination of natural products is appropriate way to overcome the multi-drug resistance of bacteria **(Mousavi et al., 2019).** In the current study, formulation 1; inhibited the growth of all isolates (100%) at concentrations of 0.3, 0.5, and 1%, but at concentration of 0.1% didn't inhibit the growth. Formulation 2; inhibited the growth of the tested serovars (80% *S. Enteritidis*, 71.4% *S.* Virchow, and 100% *S.* Montevideo) at concentration of 0.3%, at concentration of 0.1% inhibited only the growth of *S. Virchow* (42.8%) but at concentration of 0.05% there was no effect on the growth. Formulation 3; inhibited the growth of 20%, 30% *S. Enteritidis* and 14.2%, 14.2% *S. Virchow* at concentrations of 0.3 and 0.5%, respectively while at concentration of 0.1% didn't inhibit the growth. Formulation 4; effectively inhibited the growth 100% of all Salmonella isolates at concentration of 1% but at concentration of 0.5% the growth of all Salmonella isolates didn't inhibit. Formulation 5; inhibited the growth of 30% *S. Enteritidis* and 42.8% *S. Virchow* at concentrations of at concentrations of 0.1, 0.5 and 1% but didn't inhibit the growth of *S.*

Montevideo although at concentration of 0.05% there was no effect on the growth of all Salmonella isolates. **Hernandez-Patlan et al., (2018)** recorded that vitamin C has antibacterial effect against *S. Enteritidis* using a model of broiler-digestive *in vitro*. Decreasing *S. Enteritidis* count in the crop of broiler chickens post dietary supplementation of ascorbic acid at concentration of 0.1% into the feed as a result of the ability of ascorbic acid to lower pH in the crop by releasing of protons **(Hernandez-Patlan et al., 2019). Sharma et al., (2018)** reported a significant inhibition effect of vit C on *S. Enteritidis*. The variation of high, low or no antimicrobial effect of vitamin C may be attributed to the differences of the methodology that applied during the study including; the concentration of vitamin C, bacterial cell density, and culture media composition.

The present study focusing on the effect of antimicrobial susceptibility on multiple antibiotic-resistances of some isolates post treatment with vitamin C formulation 5. The treated resistant *S. Enteritidis*, *S. Virchow* and *S.* Montevideo isolates were changed to be susceptible to some antimicrobial agents.

Vitamin C formulation 4 followed by formulation 1 inhibited the biofilm-forming ability of the tested *S. Enteritidis* and *S. Virchow* isolates with a percentage of 80%, 100% and 60%, 66.6%, respectively; however, both have no significant effect on *S.* Montevideo biofilm-forming ability.

5. Conclusion

The current study showed that vitamin C formulations at different concentrations exert an inhibitory effect on *S. Enteritidis*, *S. Virchow*, and *S. Montevideo*. On the other hand, the combined vitamin C with (citric acid and eucalptys oil) and organic acids were effective on the biofilm formation of *S. Enteritidis* and *S. Virchow*, but ineffective on *S. Montevideo.*

6. Authors Contributions

All authors participated equally to the design of the research, methodology, and writing of the manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

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