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RESEARCH ARTICLE

Impact of the non-antibiotic compound vitamin C on ciprofloxacin efficacy: An in vitro study

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Objective: Antimicrobial resistance has become a worldwide health challenge due to antibiotic misuse; thus, there is a rising interest in repurposing non-antibiotic substances, such as vitamin C. Whether these compounds can alter antibiotic efficacy remains insufficiently investigated, especially alongside commonly used antibiotics like ciprofloxacin. This study aims to evaluate the impact of vitamin C on ciprofloxacin activity in standard bacterial strains.

Methods: Ciprofloxacin and vitamin C were assessed by checkerboard assay on six ATCC strains: methicillin-susceptible *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli*, and *Klebsiella pneumoniae*. The research was conducted in triplicate to ascertain minimum inhibitory concentrations and calculate the fractional inhibitory concentration index (FICI). Data were summarized with means and standard deviations, classified by outcome, and analyzed with Fisher's exact test. Figures were created using R software.

Results: For both methicillin-susceptible and methicillin-resistant *Staphylococcus aureus, Enterococcus faecalis*, and *Klebsiella pneumoniae*, all combinations showed indifference (FICI range 0.83-4), while *Pseudomonas aeruginosa* showed one antagonistic outcome (FICI=5). In *Escherichia coli*, antagonism was predominant (n=9, FICI range 4.001-6), with a statistically significant reduction in complete inhibition compared to ciprofloxacin alone (p=0.037), suggesting that vitamin C reduces ciprofloxacin efficacy at higher concentrations, while synergy occurred at lower concentrations (n=4, FICI range 0.064-0.281).

Conclusions: To our knowledge, this is the first systematic checkerboard analysis of ciprofloxacin-vitamin C on multiple ATCC strains, underscoring the impact of non-antibiotic compounds. These findings are significant because they support the need for further studies on how non-antibiotic compounds may influence antibiotic therapy in patients.

Keywords: vitamin C, ciprofloxacin, checkerboard assay, fractional inhibitory concentration index, in vitro study

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Introduction

Nowadays, the antibiotic resistance phenomenon, a major threat to modern human health, hastens the need for new antimicrobial agents or adjuvants. Among various non-antibiotic candidates, vitamin C (ascorbic acid) has been investigated for its antimicrobial potential, but conflicting reports exist in the literature concerning the role of this vitamin.

Vitamin C is generally well tolerated and has been considered a potentially useful adjuvant for the treatment of infections, partly through its contribution to immune defense [1]. Additionally, several studies have demonstrated that exposing bacteria to vitamin C inhibits their growth by increasing oxidative stress in their cells [2,3]. Such a mechanism may be especially significant in the context of urinary tract infections [2], given the fact that vitamin C is excreted through the renal system and accumulates in high levels in urine [4].

However, despite its proposed benefits, other studies have reported contrasting results showing that vitamin C could hinder the efficacy of specific antibiotics, such as fluoroquinolones, by reducing their bactericidal properties. These contradictory findings raise questions about how vitamin C may influence antibiotic therapy. Previous studies were often limited by testing only a few strains, using non-standardized methods, or working with concentrations that were not applicable in clinical settings. For instance, Masadeh et al. 2012 [5] reported that pre-treatment with vitamin C at fixed, relatively high concentrations diminishes the antibacterial efficacy of ciprofloxacin. In contrast, Rahim et al. 2025 [6] found that vitamin C, also at high concentrations, interferes with biofilm development in multidrug-resistant *Escherichia coli,* thereby enhancing susceptibility to ciprofloxacin.

It remains unclear whether vitamin C modifies ciprofloxacin activity across different bacteria and concentration ranges when tested with standardized methods (e.g., checkerboard assay, FICI). Exploring this interaction is practical, as ciprofloxacin is commonly prescribed for numerous infections, and vitamin C is routinely taken by patients.

Ciprofloxacin was chosen for this study due to its broad-spectrum activity against both Gram-negative and, to a diminished extent, Gram-positive bacteria. Urinary tract infections (UTIs) are caused by a range of pathogens, but most commonly by *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis* [7]. *Pseudomonas aeruginosa* has the capacity to form biofilms on catheter surfaces and compromised bladder epithelium [8]. UTIs can be treated using a variety of antimicrobial agents, among which is

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ciprofloxacin [9], although resistance to fluoroquinolones within uropathogens has been emerging lately [10].

This study aims to investigate the effect of vitamin C alone and in combination with ciprofloxacin on *Escherichia coli*, *Klebsiella pneumoniae, Enterococcus faecalis*, *Pseudomonas aeruginosa*, methicillin-susceptible *Staphylococcus aureus* (MSSA), and methicillin-resistant *Staphylococcus aureus* (MRSA), under the hypothesis that vitamin C could modify the activity in a manner influenced by both the concentration of ciprofloxacin and the bacterial species being examined.

Methods

Tested bacterial strains and serial concentrations

To achieve the aim of this study, the combined effect of vitamin C (A) and ciprofloxacin (B) was studied using a checkerboard assay in sterile, flat 96-well polystyrene microplates.

The following reference strains were used:

– methicillin-susceptible *Staphylococcus aureus* (ATCC 29213);
– methicillin-resistant *Staphylococcus aureus (*ATCC 43300);
– *Enterococcus faecalis (*ATCC 29212);
– *Pseudomonas aeruginosa (*ATCC 27853);
– *Escherichia coli (*ATCC 25922);
– *Klebsiella pneumoniae* (ATCC 13883).

The six ATCC strains were selected to cover a broad range of clinically important bacteria: Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and Gram-positive (*MSSA, MRSA, Enterococcus faecalis*), and to enable comparison with previous studies that focused on fewer species. Vitamin C stock solutions were prepared in sterile distilled water. To minimize oxidative degradation and light-induced instability, vitamin C dilutions were prepared freshly and covered with foil during handling.

From each bacterial strain, a 0.5 McFarland (1×10^8 CFU/mL) standard suspension was prepared. To obtain a 1:100 dilution, 100 µL of each inoculum was pipetted into 9.9 mL of Mueller-Hinton (MH) 2X broth.

Serial dilutions of ciprofloxacin were performed in 2.0 mL Eppendorf tubes using a two-fold dilution scheme, starting from 2 mL of a 4 µg/mL stock solution of ciprofloxacin. Each subsequent dilution was prepared by mixing 1 mL of the previous dilution with 1 mL of distilled water, resulting in the following concentrations: 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL, and 0.0625 µg/mL, as illustrated in Figure 1.

Similarly, a two-fold serial dilution of vitamin C was performed across the columns of a 96-well microtiter plate, starting with 100 µL in column 1 and transferring 50 µL into successive wells containing 50 µL of distilled water, leaving 50 µL in each well after dilution, up to column 12, obtaining the following serial concentrations: 2×10^4 µg/mL, 1×10^4 µg/mL, 5×10^3 µg/mL, 2.50×10^3 µg/mL, 1.25×10^3 µg/mL, 6.25×10^2 µg/mL, 3.13×10^2 µg/mL, 1.56×10^2 µg/mL, 7.81 x 10 µg/mL, 3.91 x 10 µg/mL, 1.95 x 10 µg/mL and 9.77 µg/mL.

Pre-diluted ciprofloxacin solutions were added along the rows to the microplate by pipetting 50 µL of each dilution in rows A to G. Row H served as a control (i.e., positive control - 200 µL from each of the tested bacterial inoculum and negative control - 200 µL of MH 2X broth) - Figure 1. Subsequently, 100 µL of the bacterial suspension prepared in Mueller-Hinton broth was inoculated into each well of the microtiter plate. Final well volume was 200 µL (50 µL vitamin C + 50 µL ciprofloxacin + 100 µL inoculum),

Fig. 1. Schematic representation of the different concentrations of vitamin C (ascorbic acid) (A) and ciprofloxacin (B) diluted in the 96-well microtiter plate. Vitamin C was diluted across the columns (1-12), while pre-diluted ciprofloxacin solutions were added along the rows (A-G). Abbreviations: NC - negative control; PC - positive control.

yielding an inoculum of ~5 x 10^5 CFU/mL per well, in line with EUCAST recommendations for broth microdilution assays [11]. The 96-well microplates were incubated at 37°C for 24 hours under static conditions, after which the wells were inspected visually (turbidity assessment by the naked eye) for bacterial growth.

Serial dilutions of ciprofloxacin were prepared starting from 4 μg/mL, according to the EUCAST (2025)[12] epidemiological cut-off values. The vitamin C range was chosen to reflect both dietary supplement levels and high-dose regimens described *in vitro* and previous clinical studies.

Minimum inhibitory concentration (MIC)

Additionally, both substances (vitamin C and ciprofloxacin) were tested separately on each bacterial strain to obtain their individual MICs. For ciprofloxacin-only testing, 200 μL of ciprofloxacin solution (4 μg/mL) was added to the wells of column 1 in a separate microplate. The remaining wells in columns 2–12 were filled with 100 μL sterile distilled water. Serial two-fold dilutions were performed by transferring 100 μL from one column to the next, mixing after each transfer. Then, 100 μL of bacterial inoculum in broth was added to each well. Each row was assigned to one strain: row A – MSSA, row B – MRSA, row C – *Pseudomonas aeruginosa*, row D – *E. coli*, row E – *Enterococcus faecalis,* and row F - *Klebsiella pneumoniae*. A similar protocol was applied to vitamin C-only testing, starting from 200 μL of 2 x 10^4 μg/mL vitamin C.

Minimum bactericidal concentration (MBC)

For determining the minimum bactericidal concentration (MBC), 5 μL from the wells without visible growth were transferred to MH agar plates and incubated for 24 hours at 37°C in a normal atmosphere, then checked for colony formation. MBC was defined as the lowest concentration with no visible colony growth after subculture.

Determination of the Fractional Inhibitory Concentration (FIC) Index

The assays were run in triplicate, and the fractional inhibitory concentration index (FICI) was determined to assess the interaction between compounds, using the following formula:

$FICI = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$, where FIC_A and FIC_B represent the fractional inhibitory concentrations of vitamin C (A) and ciprofloxacin (B), respectively; [A] and [B] represent the concentrations of substance A and B, respectively; MIC_A and MIC_B represent the minimum inhibitory concentration of substances A and B.

The FIC index is based on Loewe's additivity zero interaction theory. Briefly, this theory is based on the fact that a substance cannot interact with itself; therefore, the effect of a self-substance combination will always be additive, with an FIC index of 1. In the checkerboard method, the FIC index can be interpreted as follows:

- ≤0.5 represents synergy;
- between >0.5 and ≤4 represents additivity/indifference;
- >4 represents antagonism [13].

Statistical analysis

FICI results were tabulated as means ± standard deviation (SD) and were expressed with three decimal places to reflect subtle variations and the precision of calculations. Statistical analysis was carried out using GraphPad Prism 9, and the data were assessed by Fisher's Exact Test to determine the significance of observed patterns across replicate experiments, as this test is appropriate for small sample sizes and expected frequencies <5, unlike the Chi-square test. For this analysis, only positions that exhibited either full inhibition (all three replicates with clear wells) or partial inhibition (one or two clear wells) were included. Wells with growth in all three replicates were excluded from this comparison, as they represent a complete lack of antibacterial effect and do not contribute information about the consistency of inhibitory response across replicates. This comparison was limited to positions with either full or partial inhibition and does not reflect concentration-dependent effects. This Fisher's exact test analysis was intended as a secondary, descriptive approach to highlight patterns of replicate consistency and should not be interpreted as a substitute for concentration–response modeling.

The isobolographic analysis was generated in RStudio (v4.5.1) with ggplot2 to visualize the interaction between ciprofloxacin and vitamin C. The heatmap was created in RStudio (v4.4.1) to visualize the distribution of interaction effects between ciprofloxacin and vitamin C.

Results

General results overview

Overall, most combinations tested yielded indifferent outcomes (FICI >0.5–≤4), as summarized in Table I. The findings demonstrate the comprehensive interaction profile between ciprofloxacin and vitamin C across all strains tested. The highest frequency of antagonistic outcomes was recorded for *E. coli* (n=9), followed by *Pseudomonas aer-*

Table I. Summary of the interactions recorded for each strain, derived from the calculated FICI value

Bacteria	FICI >4 (antagonism)	FICI >0.5 and ≤4 (indifference)	FICI ≤0.5 (synergy)
Escherichia coli	9	8	4
Enterococcus faecalis	0	10	0
MSSA	0	16	0
MRSA	0	20	0
Pseudomonas aeruginosa	1	17	0
Klebsiella pneumoniae	0	16	0

uginosa (n=1). The highest number of the tested bacteria showed FIC indexes between 0.5 and 4, corresponding to indifference, therefore suggesting that vitamin C and ciprofloxacin did not have any interaction and can likely be administered together, even in high concentrations. Synergy was present in a very low number of tests, only in *Escherichia coli*. The interactions will be further detailed in the following sections, with complete numerical data provided in Tables II-VII.

Bactericidal activity was determined by visual inspection (absence of turbidity) followed by subculture to confirm the minimum bactericidal concentration (MBC). Each outcome was categorized based on the number of replicates (out of three) in which no regrowth was observed on agar:

– 3/3 = no visible growth in all three replicates;
– 2/3 = no visible growth in two of three replicates;
– 1/3 = no visible growth in one of three replicates.

For *Escherichia coli*, a total of 21 wells showed complete bactericidal activity (3/3 wells without growth), moderate bactericidal activity (2/3 wells) was observed in 27 wells, and partial activity (1/3 wells) was found in 19 wells. For *Enterococcus faecalis*, 10 wells showed full activity (3/3), while partial activity (1/3) was observed in only 7 wells.

For MSSA, full bactericidal activity was observed in 16 wells, while partial activity (1/3) was seen in 10 wells. For MRSA, full bactericidal activity was observed in 16 wells. Moderate activity (2/3) was recorded in 3 wells, and partial activity (1/3) was seen in 5 wells.

For *Pseudomonas aeruginosa*, full bactericidal activity was observed in 18 wells, moderate activity (2/3) was recorded in 8 wells, and partial activity (1/3) was seen in 6 wells. For *Klebsiella pneumoniae*, full bactericidal activity was observed in 16 wells, moderate activity (2/3) was recorded in 5 wells, and partial activity (1/3) was seen in 5 wells.

Escherichia coli (ATCC 25922)

Table II presents an overview of the FICI values obtained by testing different combinations of ciprofloxacin and vitamin C on the standard *Escherichia coli* strain. At the highest concentration of ciprofloxacin (4 μg/mL), most combinations with vitamin C yielded antagonistic interactions (FICI>4), indicating that vitamin C has a detrimental effect on ciprofloxacin activity. As ciprofloxacin concentrations decreased, the interaction shifted predominantly to indifference (FICI >0.5–≤4). At the lowest ciprofloxacin levels, synergistic effects were observed in certain specific wells (E7, F6, F12, and G11), suggesting a variation in interaction that is dependent on concentration.

To test if the combination of ciprofloxacin and vitamin C produced a significant effect of synergy or antagonism, Fisher's test was applied. The analysis revealed a statistically significant decrease in the proportion of full inhibition due to the combination of compounds compared to ciprofloxacin alone (p=0.037).

An isobologram (Figure 2) was also made to better illustrate the interaction between ciprofloxacin and vitamin

Table II. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
Escherichia coli	A1	6	6	6	6	0	Antagonism
	A2	5	5	5	5	0	Antagonism
	A3	4.5	4.5	4.5	4.5	0	Antagonism
	A6	4.062	4.062	4.062	4.062	0	Antagonism
	A7	4.031	4.031	4.031	4.031	0	Antagonism
	A8	4.015	4.015	4.015	4.015	0	Antagonism
	A9	4.007	4.007	4.007	4.007	0	Antagonism
	A10	4.003	4.003	4.003	4.003	0	Antagonism
	A11	4.001	4.001	4.001	4.001	0	Antagonism
	B1	4	4	4	4	0	Indifference
	B7	2.031	2.031	2.031	2.031	0	Indifference
	B8	2.015	2.015	2.015	2.015	0	Indifference
	B9	2.007	2.007	2.007	2.007	0	Indifference
	B11	2.001	2.001	2.001	2.001	0	Indifference
	C7	1.031	1.031	1.031	1.031	0	Indifference
	D12	0.501	0.501	0.501	0.501	0	Indifference
	E7	0.281	0.281	0.281	0.281	0	Synergy
	F6	0.187	0.187	0.187	0.187	0	Synergy
	F12	0.125	0.125	0.125	0.125	0	Synergy
	G1	2.062	2.062	2.062	2.062	0	Indifference
	G11	0.064	0.064	0.064	0.064	0	Synergy

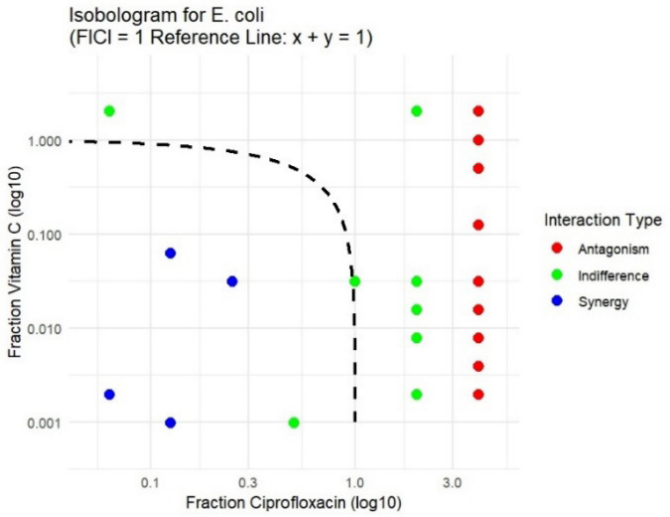

Fig. 2. Isobologram of the interactions between ciprofloxacin and vitamin C on *Escherichia coli.*

C against *Escherichia coli*, based on FICI values. Each point represents combinations of fractional inhibitory concentrations, plotted on a log10 scale. The dashed curve represents additive interaction (FICI = 1). This plot reinforces the tabular findings. Blue points that represent synergistic effects (FICI ≤ 0.5) were restricted to low fractions of both compounds, indicating that vitamin C may enhance ciprofloxacin activity only when antibiotic pressure is minimal. Antagonistic outcomes illustrated through red points (FICI > 4) predominated when ciprofloxacin was used at higher fractions, suggesting that vitamin C may undermine its antibacterial efficacy. This distribution shows that the relative concentrations of both drugs have a significant effect on the modulatory effect of vitamin C. Although it varied depending on the tested concentration combinations, the green points, which reflect indifferent outcomes (>0.5 and ≤4), were broadly distributed throughout the plot, suggesting that indifference was a regular occurrence.

A heatmap (Figure 3) was created to illustrate the interaction patterns between ciprofloxacin and vitamin C at different concentration combinations. Red signifies antagonism (FICI>4), green denotes indifference (FICI>0.5 – ≤4), and blue represents synergy (FICI≤0.5). This graphical representation supports the isobologram, facilitating the understanding of how antagonism was more common at higher ciprofloxacin concentrations, whereas synergy occurred sporadically at reduced doses of both agents.

Enterococcus faecalis (ATCC 29212)

For *Enterococcus faecalis*, mean FICI values ranged from 0.834 and 3.437, indicating exclusively indifferent outcomes, as summarized in Table III. Neither synergy nor antagonism was observed, suggesting that vitamin C did not influence ciprofloxacin activity in this strain. Unlike *Escherichia coli*, which showed both antagonism and occasional synergy, the lack of variability in *Enterococcus faecalis* points to a species-specific response.

Moreover, Fisher's exact test (GraphPad) was applied to compare the number of wells with no growth versus those with partial growth, between ciprofloxacin alone and its combination with vitamin C. The test yielded a two-tailed

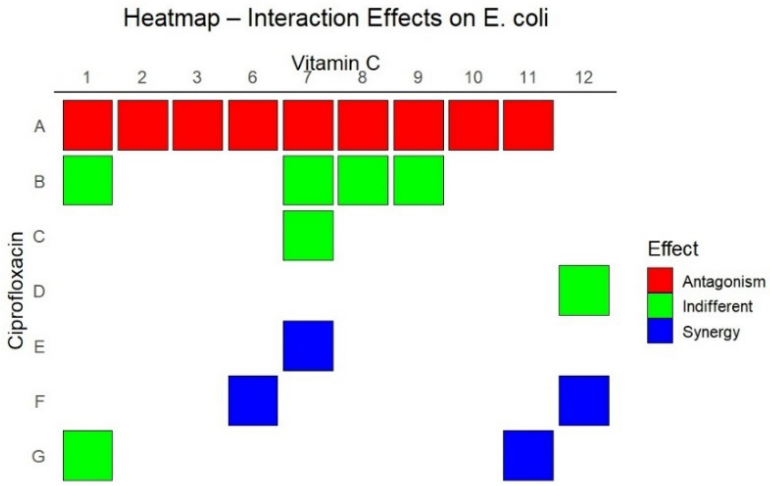

Fig. 3. Heatmap illustrating the interaction effects between ciprofloxacin and vitamin C on *Escherichia coli*

Table III. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
Enterococcus faecalis	A5	4.125	4.062	2.125	3.437	1.13	Indifference
	A6	4.062	4.031	2.062	3.385	1.14	Indifference
	A7	4.031	4.015	2.031	3.359	1.15	Indifference
	A10	4.003	4.001	2.003	3.336	1.15	Indifference
	A11	4.001	4.000	2.001	3.334	1.15	Indifference
	A12	4.000	4.000	2.000	3.334	1.15	Indifference
	B7	2.031	2.015	1.031	1.692	0.57	Indifference
	C9	1.007	1.003	0.507	0.839	0.28	Indifference
	C10	1.003	1.001	0.503	0.836	0.28	Indifference
	C11	1.001	1.000	0.501	0.834	0.28	Indifference

p-value of 0.0978, indicating no statistically significant difference between the two groups (p>0.05).

Methicillin-susceptible *Staphylococcus aureus* (ATCC 29213)

For MSSA, none of the 16 wells that showed complete growth inhibition had mean FICI values greater than 4 or less than or equal to 0.5, indicating the absence of both antagonistic and synergistic interactions, as shown in Table IV, contrasting with the variability seen in *Escherichia coli*.

According to Fisher's exact test, the difference in growth inhibition between ciprofloxacin alone and in combination with vitamin C against the *MSSA* strain was not statistically significant (p=0.5624).

Methicillin-resistant *Staphylococcus aureus* (ATCC 43300)

For MRSA, none of the replicates exhibited an FICI value above 4, or below or equal to 0.5, which indicates no synergistic or antagonistic interaction, as presented in Table V. This consistent outcome suggests that vitamin C did not influence ciprofloxacin activity in this strain.

Fisher's exact test showed no statistically significant difference (p=1.0000) when comparing ciprofloxacin alone and in combination with vitamin C.

Pseudomonas aeruginosa (ATCC 27853)

In the case of *Pseudomonas aeruginosa*, although nine wells had FICI values greater than 4 in two of the three replicates, their mean FICI remained below the antagonism threshold. Only one well (A1) was considered antagonistic, with a mean FICI of 5 (SD = 1.73), as reported in Table VI, suggesting that antagonism was rare and much less consistent than in *Escherichia coli*.

For *Pseudomonas aeruginosa*, Fisher's exact test showed no significant association (p=1.0) between ciprofloxacin alone and the combination with vitamin C, the result being consistent with the FICI values.

Klebsiella pneumoniae (ATCC 13883)

For *Klebsiella pneumoniae*, none of the 16 wells that showed complete growth inhibition had mean FICI values greater than 4 or less than or equal to 0.5, which overall indicated an indifferent interaction, as presented in Table VII. This uniform response contrasts with the variability observed in *Escherichia coli*, and suggests that vitamin C did not influence ciprofloxacin activity in this strain under the tested conditions.

The addition of vitamin C to ciprofloxacin showed no significant effect (p=0.5320), according to Fisher's exact test, aligning with the FICI result (indifference).

Discussions

Among all tested strains, only *Escherichia coli* showed a clear antagonistic response to the association of ciprofloxacin and vitamin C. All other strains showed indifference, except *Pseudomonas aeruginosa*, which demonstrated one antagonistic response, although not significant. These re-

Table IV. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
MSSA	A4	4.25	2.125	4.25	3.54	1.22	Indifference
	A5	4.125	2.062	4.125	3.43	1.19	Indifference
	A6	4.062	2.031	4.062	3.38	1.17	Indifference
	A7	4.031	2.015	4.031	3.359	1.16	Indifference
	A8	4.015	2.007	4.015	3.346	1.15	Indifference
	A10	4.003	2.001	4.003	3.336	1.15	Indifference
	A11	4.001	2.000	4.001	3.334	1.15	Indifference
	A12	4.000	2.000	4.001	3.334	1.15	Indifference
	B5	2.125	1.062	2.125	1.770	0.61	Indifference
	B6	2.062	1.031	2.062	1.718	0.59	Indifference
	B9	2.007	1.003	2.007	1.673	0.57	Indifference
	B10	2.003	1.001	2.003	1.669	0.57	Indifference
	B11	2.001	1.000	2.001	1.668	0.57	Indifference
	B12	2.000	1.000	2.000	1.667	0.57	Indifference
	C8	1.015	0.507	1.015	0.846	0.29	Indifference
	F1	2.125	1.062	2.125	1.770	0.61	Indifference

Table V. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
MRSA	A1	4	4	4	4	0	Indifference
	A5	2.125	2.125	2.125	2.125	0	Indifference
	A6	2.062	2.062	2.062	2.062	0	Indifference
	A7	2.031	2.031	2.031	2.031	0	Indifference
	A9	2.007	2.007	2.007	2.007	0	Indifference
	A10	2.003	2.003	2.003	2.003	0	Indifference
	A11	2.001	2.001	2.001	2.001	0	Indifference
	A12	2.000	2.000	2.000	2.000	0	Indifference
	B1	3	3	3	3	0	Indifference
	B6	1.062	1.062	1.062	1.062	0	Indifference
	B7	1.031	1.031	1.031	1.031	0	Indifference
	B9	1.007	1.007	1.007	1.007	0	Indifference
	B10	1.003	1.003	1.003	1.003	0	Indifference
	B11	1.001	1.001	1.001	1.001	0	Indifference
	B12	1.000	1.000	1.000	1.000	0	Indifference
	C1	2.5	2.5	2.5	2.5	0	Indifference
	D1	2.25	2.25	2.25	2.25	0	Indifference
	E1	2.125	2.125	2.125	2.125	0	Indifference
	F1	2.062	2.062	2.062	2.062	0	Indifference
	G1	2.031	2.031	2.031	2.031	0	Indifference

Table VI. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
Pseudomonas aeruginosa	A1	3	6	6	5	1.73	Antagonism
	A4	2.125	4.25	4.25	3.54	1.22	Indifference
	A5	2.062	4.125	4.125	3.437	1.19	Indifference
	A6	2.031	4.062	4.062	3.385	1.17	Indifference
	A7	2.015	4.031	4.031	3.359	1.16	Indifference
	A8	2.007	4.015	4.015	3.346	1.15	Indifference
	A9	2.003	4.007	4.007	3.339	1.15	Indifference
	A10	2.001	4.003	4.003	3.336	1.15	Indifference
	A11	2.000	4.001	4.001	3.334	1.15	Indifference
	A12	2.000	4.000	4.000	3.334	1.15	Indifference
	B1	2	4	4	3.333	1.15	Indifference
	B8	1.007	2.015	2.015	1.679	0.58	Indifference
	B9	1.003	2.007	2.007	1.673	0.57	Indifference
	B10	1.001	2.003	2.003	1.669	0.57	Indifference
	B11	1.000	2.001	2.001	1.668	0.57	Indifference
	B12	1.000	2.000	2.000	1.667	0.57	Indifference
	E1	1.125	2.25	2.25	1.875	0.64	Indifference
	G1	1.031	2.062	2.062	1.718	0.59	Indifference

Table VII. Mean FICI values and standard deviations for each tested combination (no growth wells only)

Bacteria	Well ID	FICI Replicate 1	FICI Replicate 2	FICI Replicate 3	Mean FICI	SD	Interpretation
Klebsiella pneumoniae	A5	4.062	4.062	2.125	3.416	1.11	Indifference
	A7	4.015	4.015	2.031	3.354	1.14	Indifference
	A8	4.007	4.007	2.015	3.343	1.15	Indifference
	A9	4.003	4.003	2.007	3.338	1.15	Indifference
	A10	4.001	4.001	2.003	3.335	1.15	Indifference
	A11	4.000	4.000	2.001	3.334	1.15	Indifference
	A12	4.000	4.000	2.000	3.333	1.15	Indifference
	B6	2.031	2.031	1.062	1.709	0.55	Indifference
	B7	2.015	2.015	1.031	1.687	0.56	Indifference
	B8	2.007	2.007	1.015	1.677	0.57	Indifference
	B11	2.000	2.000	1.001	1.667	0.57	Indifference
	B12	2.000	2.000	1.000	1.667	0.57	Indifference
	C8	1.007	1.007	0.515	0.843	0.28	Indifference
	C10	1.001	1.001	0.503	0.835	0.28	Indifference
	C11	1.000	1.000	0.501	0.834	0.28	Indifference
	C12	1.000	1.000	0.500	0.833	0.28	Indifference

sults could be of importance for patients receiving vitamin C while being treated with ciprofloxacin for *Escherichia coli* infections.

Only a limited number of studies have investigated the combined effects of ciprofloxacin and vitamin C, and their results have been inconsistent. Masadeh et al. (2012) [5] showed in their study through MIC determinations and disk diffusion assays that vitamin C reversed ciprofloxacin-induced antibacterial activity.

Before the findings reported by Masadeh et al. (2012), Goswami et al. (2006) [14] already investigated this combination using disk diffusion and viable count assays, and they reported that vitamin C provided substantial protection against ciprofloxacin. The mechanism of action of ciprofloxacin involves the inhibition of DNA gyrase and oxidative stress (ROS), which can be neutralized by vitamin C. It is important to note that both studies used a single fixed concentration of vitamin C. Unlike the earlier research, our study applied the checkerboard microdilution method, starting from a concentration of 2 x 10^4 µg/mL for vitamin C and 4 µg/mL for ciprofloxacin, which allowed us to assess the interaction of these two drugs across serially diluted concentrations and the calculation of FICI values.

Reports on the combination of vitamin C and ciprofloxacin remain contradictory, with some studies suggesting no significant interaction and others reporting modulatory effects such as reduced biofilm formation or altered gene expression. In a previous study, Verghese et al. (2017) [15] tested vitamin C and ciprofloxacin against uropathogenic *Escherichia coli* using a turbidimetric method. Vitamin C concentrations ranged from 5 to 10 mg/mL, while ciprofloxacin had a fixed 1 µg/mL concentration, and no enhancement of the antibiotic's activity was detected. Whereas Verghese et al. (2017) tested ciprofloxacin at a fixed concentration, the method used in our study allowed variations of both drugs, which enabled the identification of a broader interaction spectrum, including synergistic, indifferent, and antagonistic effects.

In contrast to previous research, our study reported synergistic interactions, but at specific concentration combinations, such as 6.25x10^2 µg/mL vitamin C with 0.125 µg/mL ciprofloxacin, 3.13x10^2 µg/mL with 0.25 µg/mL, 1.95x10 µg/mL with 0.0625 µg/mL, and 9.77 µg/mL with 0.125 µg/mL. In another study by Rahim et al. (2025) [6], multidrug-resistant *Escherichia coli* isolates exhibiting biofilm-forming ability were subjected to treatment with these two drugs. Vitamin C at 0.625 mg/mL was combined with sub-inhibitory ciprofloxacin concentrations, which suppressed the *recA* gene expression and reduced biofilm formation, indicating that vitamin C acted as a modulator. The techniques used included microtiter plate assays and qPCR, whereas our checkerboard assay analyzed bacteriostatic synergy across a dilution spectrum.

While data are scarce regarding the use of ciprofloxacin in conjunction with vitamin C against the other strains assessed in our research, existing studies suggest that vitamin C could potentially improve antimicrobial and antibiofilm efficacy against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus* spp.

Abdelraheem et al. (2022) [16] performed *in vitro* studies to evaluate the interaction between vitamin C and ciprofloxacin against multidrug-resistant *Pseudomonas aeruginosa*. They utilized broth microdilution techniques to ascertain the MIC values for vitamin C (which ranged from 156.2 to 1.250 µg/mL, with MIC_{50} = 312.5 µg/mL and MIC_{90} = 625 µg/mL) as well as for ciprofloxacin. Sub-inhibitory levels of vitamin C (19.5–312.5 µg/mL) showed total inhibition of biofilm formation. While this study reported synergy between vitamin C and ciprofloxacin at a limited number of fixed pairings, our checkerboard assay, which utilized standardized dilution series beginning with 2 x 10^4 µg/mL of vitamin C and 4 µg/mL of ciprofloxacin, predominantly demonstrated indifferent interactions, with one instance of antagonism (FICI=5). Their experiments focused on biofilm-forming clinical isolates and included gene expression analysis. This variation could stem from differences in strains' susceptibility and methodological techniques, and suggests that vitamin C may exert stronger modulatory effects in the biofilm than under planktonic conditions. These differences highlight the need for comparative studies assessing both planktonic and biofilm phenotypes.

While one recent study, Rahim et al. (2025) [6], documented the effect of vitamin C on a multidrug-resistant *Klebsiella spp.* isolates, and reported biofilm inhibition at concentrations between 0.625 and 2.5 mg/mL by microtiter plate assays, our research using the ATCC 13883 reference strain showed, through the checkerboard method, an indifferent interaction between the two drugs. An important limitation of the research conducted by the authors is that the interaction between ciprofloxacin and vitamin C was assessed solely in *Escherichia coli* that had been pretreated with vitamin C. In this case, the *recA* expression was analyzed as a marker of the SOS response, but this methodology was not applied to *Klebsiella pneumoniae* or *Staphylococcus aureus*. For these two strains, the authors assessed vitamin C in association with completely resistant antibiotics (e.g., oxacillin, amoxicillin). The lack of testing the interaction between ciprofloxacin and vitamin C left uncertainty, since fluoroquinolone resistance in this pathogen is increasing [17]. There is one study [18] that reported that levofloxacin, a fluoroquinolone closely related to ciprofloxacin, combined with vitamin C, eradicated biofilm formation in *Klebsiella* spp., but the results cannot be extrapolated to ciprofloxacin, highlighting a gap that our study begins to address.

These discrepancies may reflect inherent strain-specific variations or differing responses to oxidative stress. Bacteria associated with biofilms display unique physiological conditions and gene expression patterns when compared to planktonic cells [19,20]. In our research, the experi-

ments were conducted solely on bacteria in their planktonic form, without evaluating biofilm-related characteristics. Compounds such as vitamin C may have a more significant impact on disrupting the structure of biofilms or interfering with regulatory pathways that are specific to biofilms. Pandit et al. (2017) [21] illustrated that minimal levels of vitamin C disrupt bacterial biofilms, mainly by reducing EPS production, while having a negligible impact on the viability of planktonic cells.

As with MDR *Klebsiella* spp., the study by Rahim et al. (2025) evaluated the impact of vitamin C in association with selected antibiotics such as oxacillin and amoxicillin against *Staphylococcus* spp., but ciprofloxacin was not included. Other previous studies [5,22] focused on the independent effect of vitamin C on *Staphylococcus* spp., without exploring the interaction between ciprofloxacin and vitamin C. This represents another relevant gap in the literature, which our study helps to address, where we obtained overall indifferent outcomes. Thus, our results provide the first evidence that vitamin C does not influence ciprofloxacin activity in *Staphylococcus aureus* methicillin-susceptible and methicillin-resistant strains, as assessed through the standardized checkerboard microdilution method and FICI calculations.

Unlike *Escherichia coli* and *Pseudomonas aeruginosa*, the literature on *Enterococcus faecalis* is even more limited. While low levels of vitamin C have shown antibacterial properties against *Enterococcus faecalis* (for instance, at 0.15 mg/mL)[1], there is currently no available research assessing the combined effect or possible synergistic interaction of vitamin C and ciprofloxacin against *Enterococcus faecalis* in either planktonic or biofilm forms. In our study, the FICI values indicated an overall indifferent interaction between the two compounds.

Taken together, these findings indicate that the association of the two drugs is not uniform across bacterial species. While our study provides novel insights into ciprofloxacin-vitamin C interaction, several limitations can be noted. The research was conducted exclusively *in vitro*, without *in vivo* validation, which restricts the ability to assess pharmacodynamic or pharmacokinetic relevance. The study included standard reference strains, which may not adequately depict the behavior of clinical or multidrug-resistant isolates. Furthermore, only certain combinations of ciprofloxacin and vitamin C concentrations were analyzed, which may not fully represent the entire spectrum of possible interactions. Methodologically, the absence of pH and oxidative stress controls may have led to bias, as the acidic environment induced by vitamin C could influence both bacterial and antibiotic efficacy. In addition, the precise concentration of vitamin C that accumulates in the urinary tract cannot be accurately established, as it fluctuates significantly based on individual metabolic factors, renal filtration rate, and systemic clearance. Consequently, the *in vitro* concentrations evaluated may not directly correspond with achievable *in vivo* levels, complicating the prediction of potential antagonism or synergy in clinical situations. Finally, the research was carried out solely on planktonic bacterial cells, without evaluating biofilm-associated phenotypes, which could exhibit different behaviors regarding antimicrobial susceptibility and interaction dynamics.

Further investigations are warranted to clarify the clinical significance of these findings by testing clinical isolates, assessing biofilm-associated responses, and vitamin C pharmacokinetics.

Conclusion

Evaluation across multiple bacterial strains revealed that *Escherichia coli* demonstrated significant antagonism in several wells, while all other strains tested—*Enterococcus faecalis*, *Staphylococcus aureus* (MSSA/MRSA), *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*—exhibited mostly indifferent responses. Clinically, the antagonistic effect in *Escherichia coli* highlights a potential risk for patients who are prescribed ciprofloxacin alongside vitamin C, especially in urinary tract infections. For the other strains tested, vitamin C did not appear to compromise ciprofloxacin activity *in vitro* conditions. However, given the limitations of *in vitro* assays, further studies, particularly *in vivo*, are needed before translating these results into clinical settings. This research emphasizes the intricate and concentration-dependent interactions between ciprofloxacin and vitamin C among different bacterial species.

It is important to assess non-antibiotic substances, such as vitamin C, not only in isolation but also in conjunction with standard antimicrobials, as their effects may vary based on bacterial species, concentration, and physiological condition.

Our study is limited by its *in vitro* settings, by testing only standard reference strains, by not incorporating biofilm models, and by not including pH controls for vitamin C. Future work should integrate these aspects to refine the interpretation of results.

Future work

The authors plan to further extend their research on interactions between non-antibiotic and antibiotic compounds. Firstly, a comprehensive review of frequently administered non-antibiotic substances and their reported interactions with various antibiotic classes is planned. Secondly, the current study will be further extended by *in vitro* synergy testing on patient strains with different resistance mechanisms (i.e., MRSA, CPE, ESBL, etc.). Finally, this study provides a starting point for future work, which the authors plan to explore through a wider range of antibiotic and non-antibiotic interactions.

Authors' contribution

GMH (conceptualization, methodology, investigation, data curation, writing – original draft, writing – review and editing)

AC (conceptualization, methodology, supervision, writing – original draft, writing - review and editing)

Conflict of interest

None to declare.

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AI Tools Disclosure

Grammarly (v1.2.182.1722) was used solely for grammar and clarity improvements. All AI suggestions were reviewed and approved by the authors.

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