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Mitigating antibiotic resistance against *S. aureus* and *E. coli* in combination with organic acids under *in-vitro* studies

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ABSTRACT

In the context of the escalating challenge of antibiotic resistance, the efficacy of organic acids, including lactic acid and acetic acid, has proven to be promising. Strains of *Staphylococcus aureus* and *Escherichia coli* (n=2), recovered from clinical isolates of mastitis, were tested using the broth-dilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for lactic acid and acetic acid in combination with antibiotics such as ampicillin, cefotaxime, and imipenem, with triplicate samples for accuracy. The MIC values for ampicillin, cefotaxime, and imipenem against *S. aureus* were 1.04±0.26, 1.67±0.41, and 7.80±0, respectively, while against *E. coli*, they were 4.16±1.04, 0.05±0.01, and 10.40±2.6, respectively. Notably, these MICs were significantly reduced when combined with lactic acid, reaching 0.52±0.13, 0.83±0.21, and 5.20±1.3 against *S. aureus*, and 2.08±0.52, 0.02±0.01, and 5.20±1.3 against *E. coli*. Similarly, combinations with acetic acid yielded MICs of 0.65±0.13, 1.03±0.21, and 6.50±1.3 against *S. aureus*, and 2.60±0.52, 0.03±0.01, and 6.50±1.3 against *E. coli*. The MBC values for ampicillin, cefotaxime, and imipenem were 1.56±0.00, 2.91±1.10, and 13.00±2.60 against *S. aureus*, and 7.29±2.75, 0.11±0.02, and 18.20±6.87 against *E. coli*. When combined with lactic acid, the MBCs were 0.91±0.34, 1.67±0.41, and 6.50±1.30 against *S. aureus*, and 3.64±1.37, 0.05±0.01, and 10.40±2.60 against *E. coli*. The acetic acid combinations yielded MBCs of 1.30±0.26, 2.08±0.41, and 10.40±2.60 against *S. aureus*, and 5.20±1.04, 0.06±0.01, and 13.00±2.60 against *E. coli*. These findings provide valuable insights into the potential synergistic effects of antibiotics combined with organic acids, which could enhance antibacterial efficacy and aid in overcoming the growing issue of antimicrobial resistance.

Contribution/Originality: This study uniquely demonstrates how combining organic acids with antibiotics significantly lowers the MIC and MBC against resistant mastitis bacteria. This suggests a novel, cost-effective approach to enhance antibiotic effectiveness and address antimicrobial resistance.

1. INTRODUCTION

Staphylococcus aureus is a major cause of infection and disease in a wide range of animal hosts, leading to a significant impact on public health and agriculture [1]. *S. aureus* is one of the most critical to acquire antibiotic resistance through vertical and horizontal mechanisms [2]. The present study was conducted to promote good health and well-being (UN-SDGs) to ensure a healthy life through the mitigation of antibiotic resistance occurring in most of the pathogenic microorganisms of zoonotic importance, including *S. aureus* and *E. coli* [3]. Infections in

animals are deleterious to animal health, and animals can act as a reservoir for staphylococcal transmission to humans [4].

S. aureus, along with *Escherichia coli* and various Streptococcal species like *Streptococcus uberis* and *Streptococcus agalactiae*, is a leading cause of mastitis in dairy cows, leading to significant economic losses in terms of reduced production, cost of veterinary services and loss of milk and / or meat owing to antibiotic residues in the global dairy industry [5].

Antimicrobial medications are fundamental to modern healthcare, with antibiotics being used for decades to treat bacterial infections worldwide [6]. However, this has led to the off-label and inappropriate use of antimicrobials which is contributing to the development of microbial resistance, that has now become a global issue of significant concern [7].

Antibiotics are assessed based on their ability to inhibit microbial growth. Cefotaxime offers enhanced coverage against gram-negative bacteria while retaining moderate activity against gram-positive bacteria [8]. Most *E. coli* strains are susceptible to cefotaxime. However, several *E. coli* strains carrying extended-spectrum beta-lactamase (ESBL) genes such as CTX-M, TEM, and SHV exhibit resistance to cefotaxime [9, 10]. Ampicillin is effective against both gram-positive and gram-negative microorganisms [11]. However, ESBL strains of *E. coli* and certain resistant strains of *S. aureus* are unaffected by ampicillin [10]. Imipenem works by inactivating penicillin-binding proteins (PBPs), leading to cell wall lysis [12]. All methicillin-resistant *S. aureus* (MRSA) strains carry a copy of the mec gene, most commonly mecA primarily responsible for resistance against antibiotics [13, 14].

Different naturally obtained acids such as lactic acid (LA) and acetic acid (AA) are used both systemically as well as topically having efficient results in the treatment of infected wounds [15, 16]. Acetic acid has long served as anti-biofilm, antimicrobial and possess nontoxic qualities that can alter the membrane permeability of bacterial cell wall [17]. Lactic acid and acetic acid have a strong potential as an alternative to antibiotic against MDR strains of *E. coli*, *S. aureus* and *Klebsiella* because microorganism don't develop resistance against organic acids and have no residual time period either. In addition they are totally harmless for the living tissue as well even if exposed directly to the living tissue.

An alternative strategy includes using various non-antibiotic antimicrobial agents in combination with antibiotics [18]. Combination therapy is an appealing and alternative treatment option because it targets multiple, non-overlapping signaling pathways [19]. Therefore, using drug combinations presents a promising, straightforward, and effective alternative to address the issue of antibiotic resistance and reduce susceptibility [20]. Considering this approach, the current study has been designed to combat AMR through the use of LA and AA in combination with conventional antibiotics in the treatment of resistant strains of *E. coli* and *S. aureus*.

2. MATERIALS AND METHODS

2.1. Study Plan

The present study was conducted following the institutional biosafety and bioethical committee recommendations. Bacterial cultures of *S. aureus* and *E. coli* were procured from clinical isolates presented at National Veterinary Laboratory (NVL), Pakistan and the study was conducted from September to November 2024. Written informed consent was obtained from all individual participants included in the study. We compared the efficacy of antimicrobials Ampicillin (Amp), Cefotaxime (Ctx) and Imipenem (Imp) against these clinical isolates, alone as well as in combination with LA and AA separately in triplicate samples.

2.2. Identification of Bacterial Cultures

Culture was at first inoculated into blood agar media (Oxoid Limited, United Kingdom) and incubated aerobically [3] and confirmed by MALDI-TOF. All the isolates were preserved and stored at 4°C for further protocols.

Isolates of *E. coli* were inoculated into MacConkey Agar and the bacterial growth was identified as *E. coli* on the basis of colony morphology [21]. Confirmation was done on the basis of gram staining (Gram negative) and biochemical characteristics with help of Api-20E® (*bioMerieux, France*). All isolates were preserved and stored at 4°C for further protocols [22].

2.3. Preparation of Inoculum

Standardization of inoculum is vital for accurate and reproducible testing. The inoculum may be prepared by diluting a broth culture or by emulsifying overnight colonies from an agar medium in broth or saline [23]. Here we prepared 0.5 McFarland suspension by broth culture method which contains about 1.5×10^8 colony forming units (CFU) per ml of suspension [24].

2.4. Determination of MIC by Broth Microdilution Assay

For MIC assays to assess the antimicrobial susceptibility of bacterial infections, some clinical breakpoints have been developed for approved antibiotics which denote the MIC values below which an infection is likely to be highly treatable [25]. Bacterial isolates were run in triplicates against each antibiotic and combination of antimicrobials, and results were recorded in the form of OD_{600} values by the help of ELISA reader. Mean OD_{600} values were calculated and were evaluated in the form of mean MIC values [26, 27].

The following concentrations of antibiotics were used in the experiment in the form of stock solutions as documented earlier.

Ampicillin® (Sigma-Aldrich, USA): 100mg/L [11].

Cefotaxime® (Sigma-Aldrich, USA): 5mg/L [28].

Imipenem® (Sigma-Aldrich, USA): 1gm/L [29].

2.5. Determination of Minimum Bactericidal Concentration (MBC) by Plate Count Method

To determine the MBC, a dilution representing the MIC and at least two higher concentrations of each antibiotic / combination are plated on Muller Hinton (MH) agar. Incubate the plates at 37°C and counted to determine the number of colony forming units (CFU). The lowest concentration which can inhibit the 99.9% activity of the bacteria, is our MBC [30].

2.6. Statistical Analysis

Statistical analyses were conducted using SPSS version 28.0. One-way ANOVA was applied to assess differences in MIC and MBC values among different antibiotic groups alone as well as in combinations with lactic acid and acetic acid. Data are presented as mean ± standard deviation, and statistical significance was set at $p < 0.05$.

3. RESULTS

The MICs varied notably depending on the combination of antibiotic and bacterial isolate under consideration. Ampicillin alone revealed the maximum MIC of 1.04±0.26mg/L against S. aureus, whereas in combination with LA and AA it yielded significantly different MIC with 0.52±0.13mg/L and 0.65±0.13mg/L respectively. Whereas, ampicillin alone showed much higher MIC against E. coli with 4.16±1.04mg/L. Against E. coli, the combination of ampicillin with LA and AA, it exhibited significant variation in MIC with 2.08±0.52mg/L and 2.60±0.52mg/L, respectively as shown in Table 1.

The MIC of cefotaxime against S. aureus was 1.67±0.41mg/L, whereas its combination with LA and AA yielded significantly different MIC with 0.83±0.21mg/L and 1.03±0.21mg/L respectively. Whereas, the MIC of cefotaxime alone was much lower against E. coli with 0.05±0.01mg/L. The combinations of ampicillin with LA and

AA exhibited significant variation in terms of MIC with 0.02±0.01mg/L and 0.03±0.01mg/L, respectively as presented in Table 1. Against S. aureus the MIC of imipenem was 7.80±0mg/L and its combinations with LA and AA resulted significant variation in terms of MIC with 5.20±1.3mg/L and 6.50±1.3mg/L respectively. Whereas, against *E. coli* the MIC of imipenem alone was 10.40±2.6mg/L. The combinations of imipenem with LA and AA showed significant difference in MIC with 5.20±1.3mg/L and 6.50±1.3mg/L, respectively as given in Table 1.

Table 1. Mean MIC±SE (mg/L) values of antibiotics and Combinations against *S. aureus* and *E. coli.*

Antibiotic	*S. aureus* MIC (Mean±SE mg/L)	*E. coli* MIC (Mean±SE mg/L)
Amp	1.04±0.26[a]	4.16±1.04[a]
Amp + LA	0.52±0.13[b]	2.08±0.52[b]
Amp + AA	0.65±0.13[c]	2.60±0.52[c]
Ctx	1.67±0.41[a]	0.05±0.01[a]
Ctx + LA	0.83±0.21[b]	0.02±0.01[b]
Ctx + AA	1.03±0.21[c]	0.03±0.01[c]
Imp	7.80±0[a]	10.40±2.6[a]
Imp + LA	5.20±1.3[b]	5.20±1.3[b]
Imp + AA	6.50±1.3[c]	6.50±1.3[c]

Note: (Different superscript letters (a, b, c) within the same antibiotic group indicate statistically significant differences (**p ≤ 0.05**) as determined by post-hoc multiple comparison Tukey;s Test) (Amp = Ampicillin, Ctx = Cefotaxime, Imp = Imipenem, LA = Lactic acid, AA = Acetic acid) (p ≤ 0.05).

3.1. Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration as determined through cultural examination of second and third last dilutions from respective MIC against *E. coli* and *S. aureus.* It was found that ampicillin exhibited MBC of 1.56±0.00mg/L and 7.29±2.75mg/L against *S. aureus* and *E. coli*, respectively. When ampicillin was used in combination LA and AA against *S. aureus*, it was found that significant variation was observed in terms of MBC with 0.91±0.34mg/L and 1.30±0.26mg/L respectively. On the other hand, when ampicillin was combined with LA and AA against E. coli, it revealed significant variation in terms of MBC with 3.64±1.37mg/L and 5.20±1.04mg/L, respectively, as described in Table 2. Cefotaxime exhibited MBC of 2.91±1.10mg/L and 0.11±0.02mg/L against *S. aureus* and *E. coli*, respectively. When it was subjected to MBC analysis in combination LA and AA against *S. aureus*, it was found that significant variation was recorded at 1.67±0.41mg/L and 2.08±0.41mg/L respectively. On the other hand, when Cefotaxime was combined with LA and AA against *E. coli*, it revealed significant variation in terms of MBC with 0.05±0.01mg/L and 0.06±0.01mg/L, respectively as briefed in Table 2.

The MBC of imipenem against *S. aureus* and *E. coli* was observed at 13.00±2.60mg/L and 18.20±6.87mg/L against *S. aureus* and *E. coli*, respectively. It was found that MBC of imipenem in combination with LA and AA against *S. aureus* showed a significant variation and was recorded at 6.50±1.30mg/L and 10.40±2.60mg/L respectively. On the other hand, when Imipenem was combined with LA and AA against *E. coli*, it revealed significant variation with MBC values at 10.40±2.60mg/L and 13.00±2.60mg/L, respectively as given in Table 2.

Table 2. Mean MBC±SE (mg/L) values of antibiotics and Combinations against *S. aureus* and *E. coli.*

Antibiotic	*S. aureus* MBC (Mean±SE mg/L)	*E. coli* MBC (Mean±SE mg/L)
Amp	1.56±0.00[a]	7.29±2.75[a]
Amp + LA	0.91±0.34[b]	3.64±1.37[b]
Amp + AA	1.30±0.26[c]	5.20±1.04[c]
Ctx	2.91±1.10[a]	0.11±0.02[a]
Ctx + LA	1.67±0.41[b]	0.05±0.01[b]
Ctx + AA	2.08±0.41[c]	0.06±0.01[c]
Imp	13.00±2.60[a]	18.20±6.87[a]
Imp + LA	6.50±1.30[b]	10.40±2.60[b]
Imp + AA	10.40±2.60[c]	13.00±2.60[c]

Note: (Different superscript letters (a, b, c) within the same antibiotic group indicate statistically significant differences (**p ≤ 0.05**) as determined by post-hoc multiple comparison Tukey;s Test) (Amp = Ampicillin, Ctx = Cefotaxime, Imp = Imipenem, LA = Lactic acid, AA = Acetic acid) (p ≤ 0.05)

4. DISCUSSION

There are quite a lot of studies already proposing that organic acids like lactic acid and acetic acid present a highly promising and interesting alternative for antibiotics in human as well as veterinary medical practice [31]. Organic acids are non-dissociative and pass through the cell membrane of the bacterium, before dissociating and releasing protons to damage the bacterium from within [32]. The antimicrobial properties of organic acids depend on their pH [33]. The lower concentrations of acetic acid (0.00975% – 0.039% v/v) can be used as an anti-virulent agent for resistant *P. aeruginosa*, similarly its higher concentration (>0.156% v/v) can be used to disinfect biofilm-prone surgical instruments [17]. According to work done by Wang et al, pathogens like *E. coli* and *Salmonella enteritidis* can be inactivated completely by exposure to 0.5% lactic acid due to morphological changes inflicted in bacterial cells by lactic acid [34].

Lactic acid and acetic acid are generally regarded as safe (GRAS) substances as they have no detrimental effect on living tissue as well as are free of resistance concerns [35]. This study aimed to evaluate the MICs of ampicillin, cefotaxime, and imipenem, both alone and in combination with LA and AA, against *S. aureus* and *E. coli*. Using drug combinations presented a highly promising, straightforward, and much more effective alternative to address the issue of antibiotic resistance and reduce susceptibility [36]. The results presented valuable insights into the potential synergistic effects of combining these antibiotics with organic acids, which could enhance their antibacterial efficacy and contribute to overcoming the rising challenge of antimicrobial resistance [37]. LA is a potent Outer membrane-disintegrating agent, as evidenced by its ability to cause lipopolysaccharide (LPS) release and to sensitize bacteria to detergents or lysozyme [38].

The MICs of ampicillin, cefotaxime, and imipenem, when tested alone, revealed varied levels of susceptibility in the two bacterial strains. *E. coli* generally showed a comparatively higher resistance to ampicillin and imipenem as compared to *S. aureus*, which is consistent with the known beta-lactam resistance mechanisms in *E. coli*. However, Cefotaxime a third generation cephalosporin, exhibited the lowest MIC values against *E. coli*. This result aligns with previous studies showing that cefotaxime remains quite effective against a wide range of resistant pathogens, including MDR *E. coli* and *S. aureus* [39, 40] as shown in Figure 1.

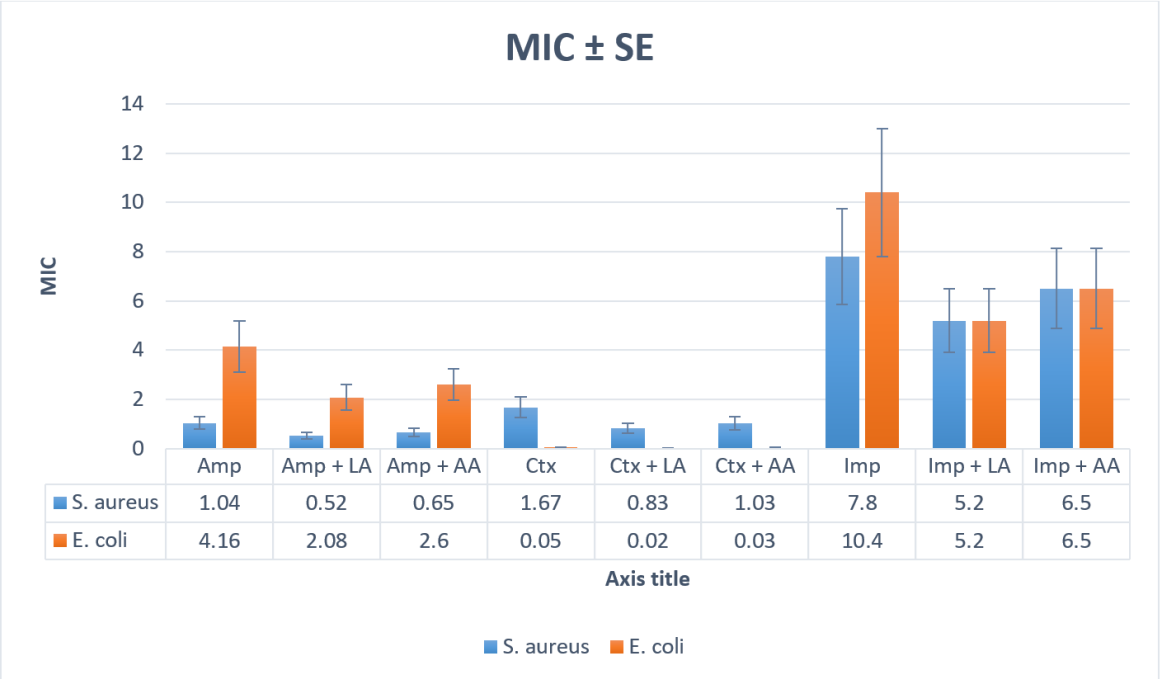

Figure 1. Bar graph showing Mean MIC±SE (mg/L) values of antibiotics and Combinations against *S. aureus* and *E. coli*.

The addition of lactic acid and acetic acid to the antibiotics resulted in a marked reduction in MICs for both *S. aureus* and *E. coli*. This suggests a synergistic effect, where the organic acids likely alter the bacterial cell membrane, making it more permeable to the antibiotics as proposed by Harrison, et al. [35] and interfere with bacterial metabolism in ways that enhance antibiotic action.

LA is authorized as an active substance for biocidal products, with applications in veterinary hygiene, food, and animal feed. Several factors can influence the antimicrobial effect of lactic acid, including the applied concentration of lactic acid, the contact time, and the organic soiling of the surface to which it was applied Commission [41]. Đurđević-Milošević, et al. [42] demonstrated the efficiency of using organic acids including LA and beefxide solution in reducing the microbial load to a level that does not cause diseases.

Comparing the combination effects of both LA and AA, we can interpret that LA has better synergism with all 3 antimicrobials, which reflects in form of their decreased MICs and MBCs against both *S. aureus* and *E. coli* as shown in Figure 2. When used in combinations, antimicrobials can produce synergistic effect and as a result can offer a higher likelihood of therapeutic responses compared to single-drug treatments [36].

In contrast, AA exhibited some synergistic effect, but the reduction in MIC values was generally less pronounced than that observed with LA. This could be due to the stronger acidity of LA leading to aggressive disruption of the bacterial membrane. These results aligns with previous work done by Kim and Rhee [33] indicating that organic acids, particularly in lower pH environments, can potentiate the effects of antibiotics by impairing bacterial defense mechanisms. However, excessive acidity could also reduce the bioavailability of the antibiotics.

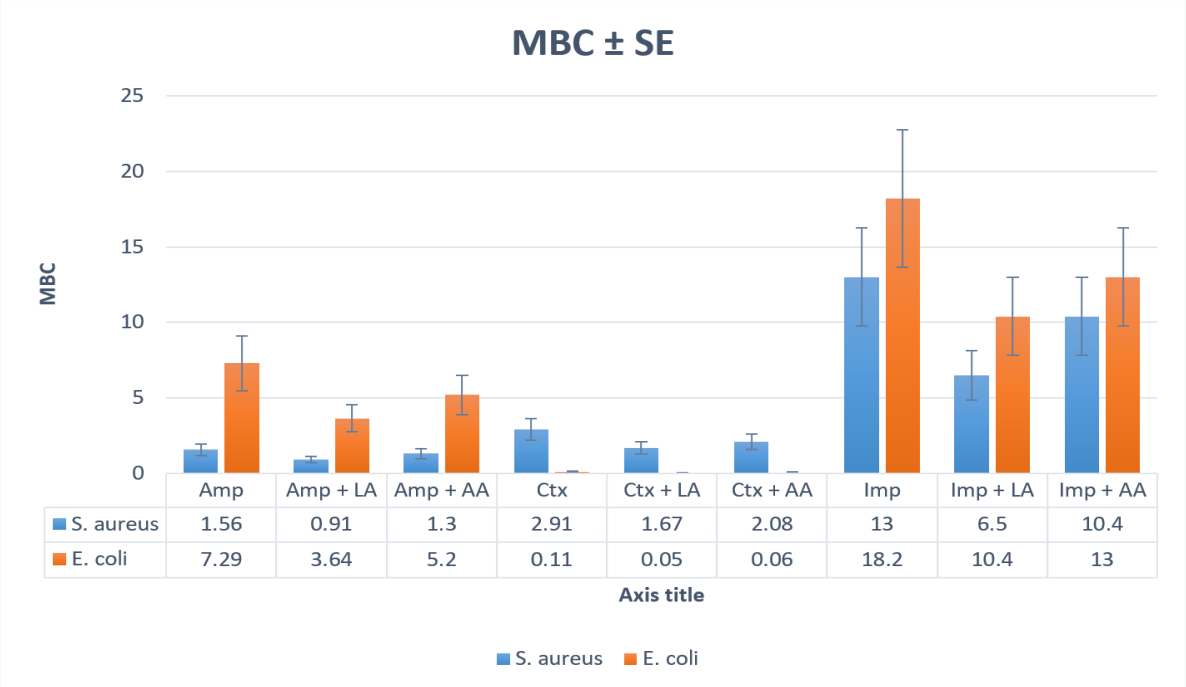

Figure 2. Bar graph showing Mean MBC±SE (mg/L) values of antibiotics and combinations against *S. aureus* and *E. coli*.

5. CONCLUSION

This study focused on the potential use of organic acids (Lactic acid and Acetic acid) as adjunctive agents to combat antibiotic resistance against ampicillin, cefotaxime and imipenem in *S. aureus* and *E. coli*. A strong synergistic effect was demonstrated in our in-vitro experiments, as results depicted that these combination significantly enhanced efficacy of all antibiotics against both bacterial strains leading to significantly reduced MIC and MBC values of all antimicrobials. However more in-vivo trials are required to confirm the clinical applicability

of these findings. This research offers promising insight into the use of lactic acid and acetic acid as novel, cost-effective way to combat antibiotic resistance in *S. aureus* and *E. coli*.

5.1. Study Limitations

The study's in-vitro design, while allowing controlled analysis of antibiotic-organic acid combinations, does not account for host factors and systemic pharmacodynamics that influence in-vivo efficacy. Furthermore, the limited bacterial strain diversity and lack of mechanistic insights restrict the generalizability and clinical translation of the findings. Future studies involving animal models and broader isolate screening are warranted to validate these results.

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Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

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