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Synergistic and Antagonistic Effects of Phenylalanine and Various Antibiotics on the Growth of Pathogenic Bacteria

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Abstract

Broad-spectrum antibiotics have been widely used in the treatment of many systemic and local infections in humans and animals. Herein, we aimed to determine the synergistic and antagonistic effects of phenylalanine with antibiotics cefoxitin, amoxicillin, vancomycin, lincomycin, and bacitracin against 14 pathogenic bacteria. The effect of antibiotics, either alone or in combination with this biomolecular liquid, was tested using the disk diffusion method against different bacteria. The addition of phenylalanine to antibiotic disks directly affected their antimicrobial activity. All the antibiotics used did not show any antimicrobial activity against *Staphylococcus haemolyticus* when used alone. However, in combination with phenylalanine, each antibiotic inhibited the growth of *S. haemolyticus*. The use of this biomolecular liquid together with amoxicillin and vancomycin also increased the antimicrobial activity against *Enterococcus durans*. The use of phenylalanine in combination with antibiotics also resulted in antagonistic effects on some pathogens. Further, the effects of antibiotics in combination with phenylalanine on different bacterial pathogens were investigated in vitro. Results provide valuable information to further our understanding of the molecular mechanism of action of antibiotics and to improve their efficacy against bacterial pathogens.

Keywords Antagonistic-synergetic effect · Pathogenic bacteria strains · Phenylalanine

1 Introduction

Antibiotics are an important class of drugs used to treat and prevent the spread of infectious diseases caused by bacterial pathogens. However, excessive use of antibiotics lowers their efficiency and renders the target bacteria antibiotic-resistant [1]. According to the World Health Organization, resistance to antibiotics is one of the leading threats to human health nowadays [2]. Antibiotic-resistant bacteria can cause severe illnesses, high mortality, disease complications, and admission of an increasing number of patients to hospitals. According to the European Centre for Disease Prevention and Control, approximately 25,000 people in Europe lose their lives each year as a result of antibiotic-resistant bacterial infections and the misuse of antibiotics. Laxminarayan and colleagues reported that an estimated 94,000 invasive methicillin-resistant *Staphylococcus aureus* (MRSA) infections required hospitalization and resulted in 19,000 deaths in the USA in 2005 [3]. Resistance to antibiotics is now a major public health concern, and the misuse of antibiotics is recognized as the major causative factor [1]. The general public, pharmacists, doctors, and hospitals should ensure the proper use of antibiotics, minimizing the development of antibiotic resistance. Because of the increasing rate of antibiotic resistance, new antimicrobial

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substances are needed. Substances isolated from various natural sources, like medical plants, and natural and organic materials should be tested for use as an alternative antibiotics alone or in combination with other chemicals, such as amino acids, several of which are essential for human health [4]. Amoxicillin is reported to be effective against only 13% of the Gram-negative bacilli [5]. In addition, *Escherichia coli* and *Klebsiella* spp. show increased resistance to the third-generation cephalosporins and other â-lactam antibiotics due to the high production of penicillinase and extended spectrum â-lactamase [5].

In a recent study, some amino acids were lipidated with non-spermidine conjugates which were connected with amide bond between amino acids and fatty acids. These lipidated conjugates show antibacterial activity against Gram-positive and Gram-negative bacteria, such as MRSA, vancomycin-resistant *Enterococcus faecium*, and β-lactam-resistant *Klebsiella pneumonia* [6]. Peng et al. showed that abundance of glucose and alanine is suppressed in the kanamycin-resistant *Edwardsiella tarda* [7]. Exogenous application of alanine or glucose renews its sensitivity to kanamycin, thus killing the multidrug-resistant *E. tarda*, possibly due to the enhancement of the TCA cycle by substrate activation mediated by exogenous glucose or alanine, resulting in the production of NADH and proton motive force and increasing the uptake of antibiotic [7].

Phenylalanine is an essential amino acid, i.e., it cannot be synthesized in the human body and must be obtained from external sources. It exists in three forms: D-phenylalanine, L-phenylalanine, and DL-phenylalanine, which is a mixture of D- and L-phenylalanine and is synthesized in the laboratory. Meat, fish, eggs, cheese, and milk are the main dietary sources of L-phenylalanine. Phenylalanine is converted into tyrosine and other amino acids, which in turn are used to biosynthesize proteins and brain chemicals. Deficiency of phenylalanine results in confusion, lack of energy, depression, memory problems, and lack of appetite. Phenylalanine is used to treat depression, attention-deficit hyperactivity disorder (ADHD), Parkinson's disease, chronic pain, osteoarthritis, rheumatoid arthritis, alcohol withdrawal symptoms, and vitiligo. Aiyelabola et al. reported that the coordination conjugates of different amino acids, such as histidine, arginine, and glutamic acid, have been produced and their antimicrobial properties studied. However, little is known about hydrophobic amino acids, such as phenylalanine [8]. In this study, we examined the synergistic and antagonistic effects of phenylalanine with five broad-spectrum antibiotics on the growth of pathogenic bacteria. These tests were conducted in vitro and analyses were performed independently of clinical use. The main objective of this study was to determine the synergistic and antagonistic effects of phenylalanine with five different antibiotics against 14 different pathogenic bacteria as shown in Table 1.

Table 1 Bacterial pathogens used in this study

Bacteria	Gram (+)	Gram (−)
K. pneumonia		X
S. aureus ATCC 25923	X	
S. hominis	X	
P. vulgaris		X
E. coli		X
S. marcescens		X
S. edidermis	X	
Streptococcus alpha haemolyticus	X	
Enterococcus faecium	X	
Pseudomonas aeruginosa		X
Listeria monocytogenes ATCC 7644	X	
Enterococcus durans	X	
Salmonella kentucky		X
Enterobacter aerogenes ATCC 13048		X

2 Material and Methods

2.1 Statistical Analysis

Experiments were replicated five times for each bacterial pathogen. Statistical significance of differences between treatments was determined at a 5% probability level with one-way analysis of variance (ANOVA) and the general linear model (GLM) using Minitab 15.

2.2 Antibiotics and Bacterial Pathogens

Five different antibiotics, including cefoxitin, 30 μg; amoxicillin, 25 μg; vancomycin, 30 μg; lincomycin, 2 μg; and bacitracin 2 μg (Bioanalyse Company, Ankara, Turkey) were tested on Gram-positive and Gram-negative bacteria (Table S1), either alone or in combination with phenylalanine (Sigma-Aldrich).

2.3 Determination of the Strength of Antibiotics

The antibacterial activity of antibiotics was determined as described previously [9]. Muller Hinton agar media was prepared, sterilized, and poured into 100 mm sterile Petri dishes with a depth of 4–5 mm [4]. Cefoxitin, amoxicillin, vancomycin, lincomycin, and bacitracin disks were loaded with phenylalanine and dried for 3 h at 30 °C under sterile conditions. The disks were then incubated at room temperature for 1–2 h to equilibrate the temperature and minimize condensation. Antibiotic disks not treated with phenylalanine were used as controls.

Five-milliliter aliquots of nutrient broth medium were inoculated with different bacterial pathogens and incubated at 37 °C for 18–24 h. Each bacterial suspension was slowly

added to the saline solution until the visible turbidity of the solution was equal to 0.5 McFarland; this is approximately corresponding to 10^8 cell forming units (CFU/ml). The prepared bacterial suspensions were then spread on Muller Hinton agar plates using sterile cotton swabs. Once the plates dried, antibiotic disks with or without phenylalanine were placed on the plates and incubated at 37 °C for 24 h. The antibacterial activity was estimated by measuring the inhibition zones (including the disk) of the bacterial pathogens. The detail of the statistical analysis is given in supporting information.

3 Results and Discussion

Most of the bacterial pathogens used in this study are pathogenic to humans and animals and cause infections of the throat, skin, prosthetic joints, catheters, and large wounds. Cefoxitin interferes with bacterial cell wall synthesis. In this study, it inhibited the growth of *S. aureus*, *E. coli*, *S. Kentucky*, *P. vulgaris*, *K. pneumonia*, *S. hominids*, *Serratia marcescens*, *and E. durans*. The application of phenylalanine on cefoxitin disks increased the sensitivity of *S. haemolyticus* to cefoxitin (Fig. 1). While the addition of phenylalanine to cefoxitin disks significantly increased the inhibition zone of *S. haemolyticus*, it had an antagonistic effect on *P. vulgaris*, *K. pneumonia*, and *S. hominids* (Fig. 1). Thus, the combination of phenylalanine and cefoxitin exhibited both synergistic and antagonistic effects on bacterial pathogens.

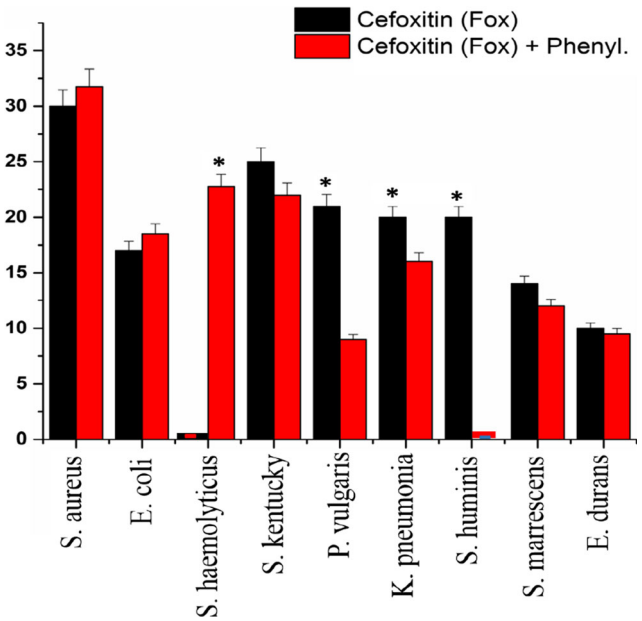

Fig. 1 Antimicrobial activity of cefoxitin with or without phenylalanine against bacterial pathogens. Data represent means ± SEM. Statistical significance of differences between cefoxitin (FOX) and cefoxitin plus phenylalanine (FOX + P) was analyzed using one-way ANOVA and is indicated with an asterisk ($P < 0.05$)

Amoxicillin is another antibiotic used in this study and is useful for the treatment of many bacterial infections. The addition of phenylalanine did not affect the antibacterial activity of amoxicillin against *E. coli*, *S. aureus*, *P. vulgaris*, *S. Kentucky*, *E. faecium*, *L. monocytogenes*, *K. pneumonia*, and *S. marcescens*. However, the activity of amoxicillin was increased by phenylalanine in the case of *S. haemolyticus* and *E. durans* but decreased in the case of *E. faecium*, *L. monocytogenes*, *K. pneumonia*, and *S. marcescens* (Fig. 2). Thus, both cefoxitin and amoxicillin in combination with phenylalanine had an antagonistic effect on *K. pneumonia*.

Vancomycin has a time-dependent bactericidal activity against most Gram-positive bacteria. In the absence of phenylalanine, vancomycin showed antibacterial activity against *E. durans*, *S. aureus*, *K. pneumonia*, *P. vulgaris*, *E. faecium*, and *S. epidermis*. In combination with phenylalanine, the antibacterial activity of vancomycin was significantly increased against *E. durans*, *S. aureus*, and *S. haemolyticus*, but lowered against *P. vulgaris*, *E. faecium*, and *S. epidermis*, compared with vancomycin alone (Fig. 3). The synergistic and antagonistic effects of phenylalanine and vancomycin were evident from the augmentation and recession of the inhibition zones, respectively.

The clinically important antibiotic, lincomycin is produced via a bifurcated biosynthetic pathway. The growth of

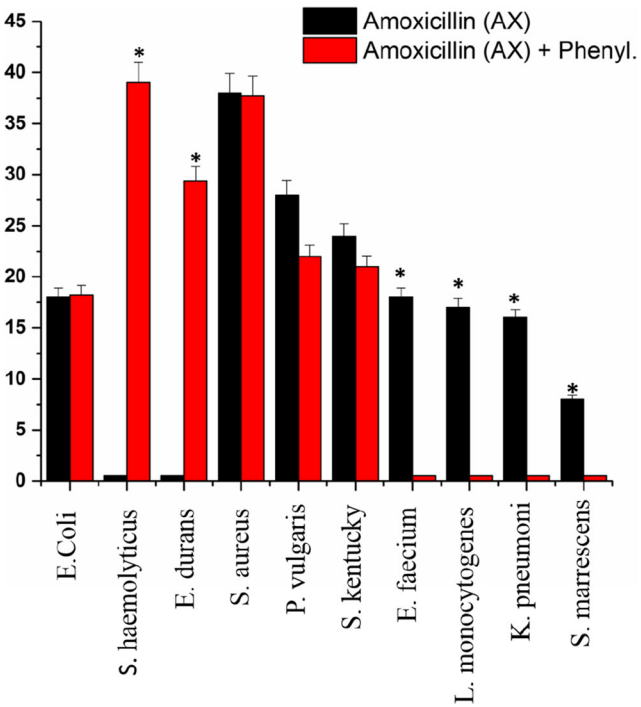

Fig. 2 Antimicrobial activity of amoxicillin with or without phenylalanine against bacterial pathogens. Data represent means ± SEM. Statistical significance of differences between amoxicillin (AX) and amoxicillin plus phenylalanine (AX + P) was analyzed using one-way ANOVA and is indicated with an asterisk ($P < 0.05$)

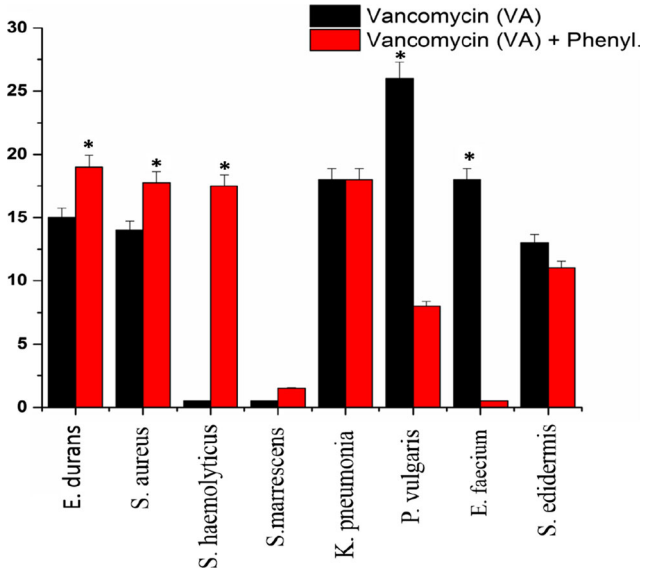

Fig. 3 Antimicrobial activity of vancomycin with or without phenylalanine against bacterial pathogens. Data represent means ± SEM. Statistical significance of differences between vancomycin (VA) and vancomycin plus phenylalanine (VA + P) was analyzed using one-way ANOVA and is indicated with an asterisk ($P < 0.05$)

E. faecium, *E. durans*, and *S. epidermis* in this study was inhibited by lincomycin alone (Fig. 4). While the addition of phenylalanine to lincomycin increased the inhibition zones of the bacterial pathogens, *S. aureus*, and *S. haemolyticus*, the combination had an antagonistic effect on *E. faecium* and *S. epidermis* (Fig. 4).

Bacitracin is composed of similar cyclic polypeptides with antibiotic properties. Like cefoxitin, bacitracin also inhibits bacterial cell wall biosynthesis. Bacitracin showed strong antibacterial activity against *S. aureus*, *E. Durans*, and *E. faecium* (Fig. 5). However, in combination with phenylalanine, the antibacterial activity of bacitracin against these pathogens was significantly reduced (Fig. 5), as indicated by the decrease in the size of the inhibition zones of these bacteria. On the other hand, the combination of bacitracin and phenylalanine was significantly more effective against *S. haemolyticus* than bacitracin alone (Fig. 5).

Overall, these data indicated that phenylalanine together with different antibiotics exhibited both synergistic and antagonistic effects on the growth of bacterial pathogens. Of the 14 bacterial pathogens tested in this study, 4 pathogens, including *S. aureus*, *E. coli*, *S. haemolyticus*, and *E. durans* were inhibited by the antibiotics, and this inhibition was stronger with the addition of phenylalanine than without. The synergistic effect of phenylalanine on all antibiotics was consistently detected against *S. haemolyticus* (Fig. 6). While *S. haemolyticus* was resistant to all five antibiotics when used alone, the addition of phenylalanine to the antibiotic disks rendered the bacterium susceptible to all antibiotics ($P < 0.05$). *S. haemolyticus* causes bacteremia/sepsis, wound infections, urinary tract infections, and conjunctivitis in humans. As the name indicates, *S. haemolyticus* causes lysis of the red blood cells (hemolysis) and is considered as one of the coagulase-negative staphylococci (CNS) species. CNS infections are difficult to treat because pathogens comprising the

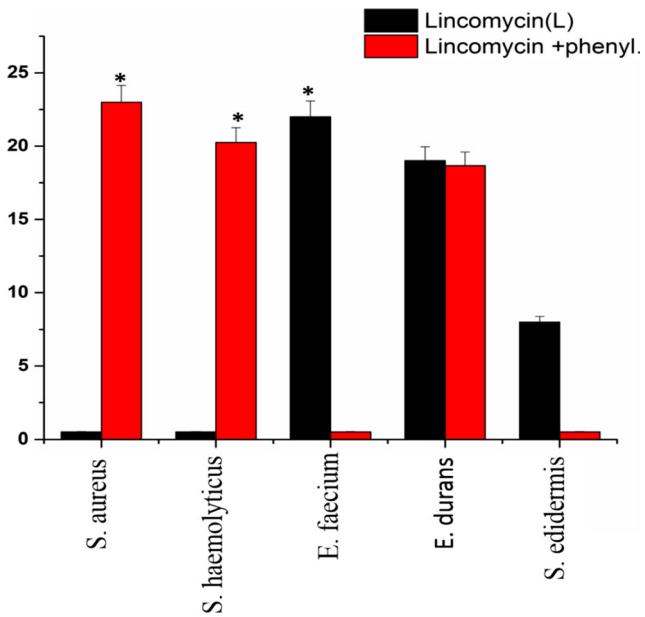

Fig. 4 Antimicrobial activity of lincomycin with or without phenylalanine against bacterial pathogens. Data represent means ± SEM. Statistical significance of differences between lincomycin (L) and lincomycin plus phenylalanine (L + P) was analyzed using one-way ANOVA and is indicated with an asterisk ($P < 0.05$)

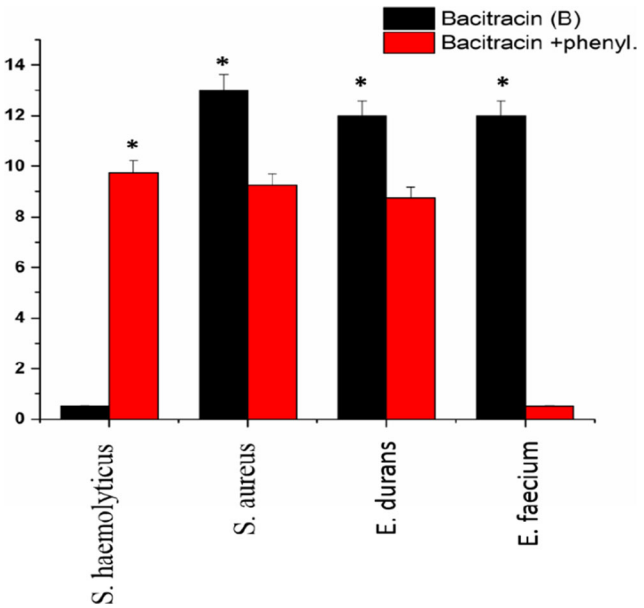

Fig. 5 Antimicrobial activity of bacitracin with or without phenylalanine against bacterial pathogens. Data represent means ± SEM. Statistical significance of differences between bacitracin (B) and bacitracin plus phenylalanine (B + P) was analyzed using one-way ANOVA and is indicated with an asterisk ($P < 0.05$)

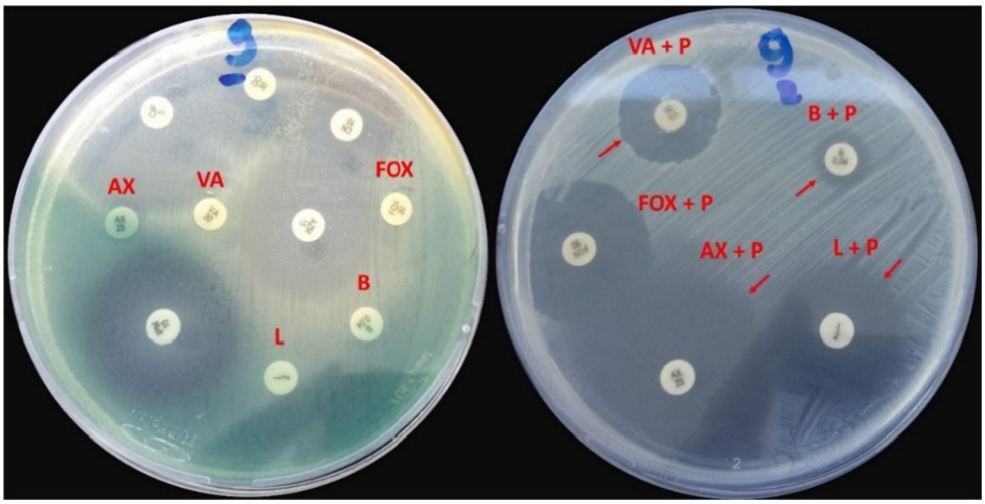

Fig. 6 Synergistic and antagonistic effects of phenylalanine and antibiotics on *S. hemolytic*. FOX, cefoxitin; AX, amoxicillin; V, vancomycin; L, lincomycin; B, bacitracin; and P, phenylalanine

CNS group are usually multidrug-resistant. A combination of vancomycin with rifampicin and/or gentamicin is suggested for the treatment of infections caused by the recalcitrant *S. haemolyticus*. Thus, our finding that the combination of phenylalanine with various antibiotics is effective against *S. haemolyticus* is highly valuable in the medical field.

By contrast, phenylalanine and antibiotics exerted antagonistic effects on *E. faecium*, *P. vulgaris*, and *S. hominids*, which were otherwise susceptible to antibiotics alone (Fig. 7). *P. vulgaris* is a Gram-negative facultative anaerobe, whose flora is located in the intestines of humans and animals, and in manure, soil, and polluted waters. *P. vulgaris* infections are treated with aztreonam, β-lactam/β-lactamase inhibitor combinations, or carbapenems [10]. Because the range of antibiotics effective against *P. vulgaris* is limited, appropriate amounts of antibiotics must be used to prevent the development of drug resistance.

Cefoxitin is a broad-spectrum antibiotic effective against many Gram-negative and Gram-positive bacteria. Because cefoxitin is a strong inducer of penicillin-binding protein (PBP) 2a, it works better than oxacillin [11]. The Clinical and Laboratory Standards Institute recommends cefoxitin disk diffusion (DD) or cefoxitin MIC analysis for *S. aureus* and *S. lugdunensis* [12]. In addition, cefoxitin DD is also a commonly used method for CNS [13]. We selected cefoxitin for this study because it is used against a variety of bacterial pathogens. Cefoxitin has been used in combination with amikacin and clarithromycin to treat the human lungs infected with *Mycobacterium abscessus* [14]. Cefoxitin DD is considered as a superior test for MRSA, which is a serious threat to the immunocompromised patients as well as to the general public [15]. Consistent with previous findings, we showed that a combination of cefoxitin with other chemicals worked better against *S. haemolyticus* than when used alone. However, the addition of phenylalanine to cefoxitin disks resulted in an insignificant increase in the inhibition of *S. aureus.*

Amoxicillin is a semi-synthetic drug belonging to the aminopenicillin group of antibiotics and is mainly used for the treatment of strep throat, pneumonia, skin infections, and urinary tract infections. In addition to its usage in humans, it is

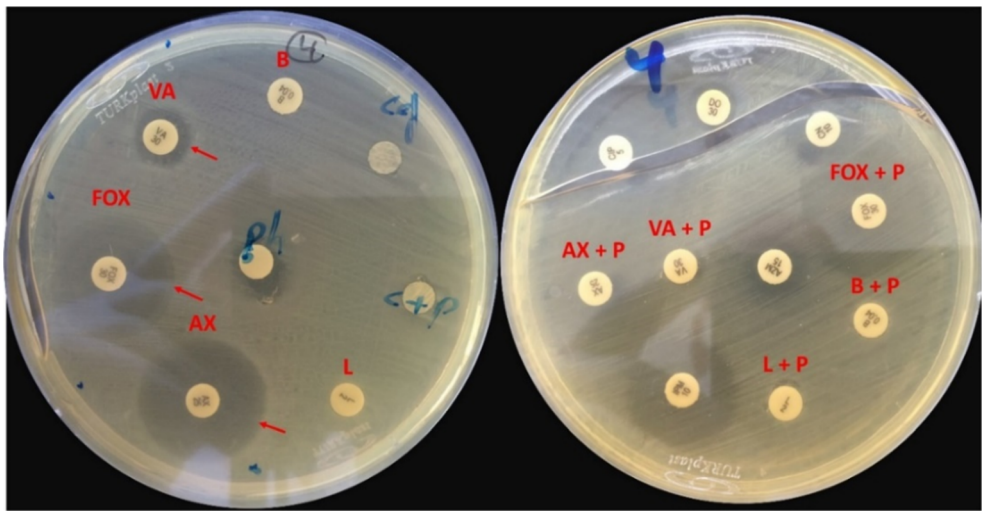

Fig. 7 Synergistic and antagonistic effects of phenylalanine and antibiotics on *P. vulgaris*. FOX, cefoxitin; AX, amoxicillin; V, vancomycin; L, lincomycin; B, bacitracin; and P, phenylalanine

also extensively utilized in treating bacterial infections in animals. Schwarz and colleagues evaluated the susceptibility of porcine respiratory tract pathogens to amoxicillin and determined the MIC of amoxicillin [16]. Recently, the synergistic effect of amoxicillin with Toll-like receptor (TLR) ligands has been examined on dendritic cells. Data shows that the combination of amoxicillin with TLR agonists causes amplification of dendritic cells and results in the proliferation of specific T cells, suggesting the role of amoxicillin in immune response [17]. Additionally, Hai et al. have shown that transferrin-amoxicillin construct is more effective against *Chlamydia trachomatis* than amoxicillin alone [18]. These data are consistent with our findings showing that the combination of amoxicillin with phenylalanine was significantly more powerful than amoxicillin alone in suppressing the growth of the bacterial pathogens, *S. haemolyticus*, and *E. durans*.

Vancomycin, which is utilized for the treatment of Gram-positive bacterial infections, has a glycopeptide structure. It is generally considered to be effective against most recalcitrant infections caused by Gram-positive bacteria [19]. Vancomycin in combination with anti-staphylococcal β-lactams against MRSA has been shown to shorten the bacteremia duration of MRSA [20]. In this study, synergistic effects of vancomycin and phenylalanine were observed, which significantly increased the inhibition zone area of *S. aureus* compared with vancomycin alone. Nephrotoxicity affecting kidney cells has been associated with vancomycin usage [21, 22]. Since the identification of MRSA in 1961 [23], the rate of vancomycin-associated nephrotoxicity has increased to 35%, which is considered to be highly toxic [24]. Many nephrotoxicity cases are believed to be caused by the administration of higher doses of vancomycin [25, 26]. Therefore, to limit nephrotoxicity, avoiding the overuse of vancomycin is essential. From this point of view, our study showed promising results; with the addition of phenylalanine, vancomycin can potentially be used to treat infections at lower doses. Despite the promising results obtained in this study, the combination of phenylalanine and vancomycin must be validated before clinical applications.

Lincomycin binds to the 50S subunit of bacterial ribosomes, thus preventing protein synthesis in Gram-positive bacteria [27]. The effect of a mixture of environmentally relevant compounds, including atenolol, bezafibrate, ciprofloxacin, and lincomycin has been examined on OVCAR3 tumor cells, *E. coli*, and HEK293 cells [28]. Results indicate that the combination of ciprofloxacin and lincomycin negatively impacts OVCAR3 proliferation but does not show a statistically significant effect on the HEK293 cell line. Additionally, lincomycin induces the proliferation of *E. coli* [28]. Consistent with these findings, we showed that the combination of lincomycin and phenylalanine was more effective than lincomycin alone in inhibiting the growth of some bacterial pathogens.

Bacitracin is generally used in clinical practice and as a growth promoter in animal husbandry [29]. Although it is being increasingly used in both human and veterinary medicine, the effect of bacitracin in combination with different substances has not been investigated previously. Here, we show that bacitracin and phenylalanine together were significantly more powerful in inhibiting the growth of *S. haemolyticus* compared with bacitracin alone.

4 Conclusions

As a conclusion, we provided strong evidence supporting the use of phenylalanine in combination with antibiotics, such as cefoxitin, amoxicillin, vancomycin, lincomycin, and bacitracin to control the growth of different Gram-positive and Gram-negative bacteria. We also provide data to limit the use of antibiotics in a more effective way. The use of phenylalanine in combination with various antibiotics has a high potential for application in the laboratory as well as in animal models and clinical trials.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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