Synergism and Efficacy of Some Naturally Occurring D-amino Acids Against Clinically Relevant Bacterial and Fungal Species

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ABSTRACT

Amino acids are predominantly synthesized and used in their L-enantiomeric form in all three kingdoms of life. However, bacteria produce diverse D-amino acids that are involved in the synthesis and cross-linking of peptidoglycan. Several studies reported possible antimicrobial activities of selected D-amino acids against Escherichia coli. The present study was undertaken to investigate the antibacterial and antifungal susceptibility patterns and growth inhibitory effects of certain D-amino acids, including D-alanine, D-lysine, D-serine, and D-proline. Our findings indicate that D-lysine is the most potent antibacterial and antifungal, among the examined D-amino acids, followed by D-alanine, whereas D-serine and D-proline had insignificant antimicrobial activities. Gram positive bacteria were more susceptible to the antibacterial effects of D-amino acids than Gram negative bacteria. Growth kinetic studies revealed that D-lysine and D-alanine resulted in extended lag phases, suggesting that the D-amino acids successfully influenced the microorganisms' ability to use nutrients efficiently and disrupted their normal biological functions. Additionally, synergism was evident between D-alanine and D-lysine when combined with either Ampicillin or Amphotericin B. These results suggest a new avenue for D-amino acids' potential as naturally occurring antimicrobial reagents for the treatment and prevention of microbial growth in food and agriculture applications.

Keywords: D-lysine, D-alanine, Antibacterial, Antifungal, Synergism.

1. INTRODUCTION

Recently, research on naturally occurring products has gained enormous global attention. Considerable amount of evidence has accumulated to reveal the promising potential of natural elements as medicinal reagents. Far and wide, secondary metabolites (natural products) from bacteria serve as lead compounds for the development of pharmaceutical drugs used to eradicate bacterial, viral and fungal infections, as well as cancer and immune system disorders.

Amino acids are best known as the building blocks of

proteins, which themselves form the biological machinery of all cells. In all three kingdoms of life, cells predominantly use the L-amino acids as building blocks for protein synthesis. Nevertheless, significant quantities of D-amino acids are naturally produced by most bacteria. It has been shown that D-alanine and D-glutamic acid are the most widely naturally produced amino acids in bacteria[1], and are incorporated into peptidoglycan in the cell walls of both Gram positive and Gram negative bacteria[2-7].

In recent years, it was shown that D-amino acids are released by diverse bacterial species in the stationary phase of growth and act as agents controlling cell wall assembly and modification[8]. The regulatory role of D-amino acids appears to be related to the transition of cells into the stationary phase of growth, which is thought to

down regulate cell wall and peptidoglycan synthesis[8]. Additionally, it has been found that a mixture of D-amino acids produced in bacteria also prevents biofilm formation and is involved in their disassembly in some bacterial populations[9].

Interestingly, potential toxicity has been linked to D-amino acids in both prokaryotes and eukaryotes[10, 11]. Although, in bacteria, D-amino acids were suggested to induce physiologically relevant alterations in peptidoglycan structure[11], the underlying mechanism of toxicity is not fully understood. In this study, in an effort to investigate the potential of D-amino acids as naturally occurring antimicrobial reagents, we examined the growth regulatory effects of certain D-amino acids on several clinically important microorganisms, including: *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeuriginosa, Xanthomonas vesicatoria, Candida albicans, Candida glabrata,* and *Candida krusei.* Furthermore, to establish the fundamental basis of the mechanism underling their antimicrobial activity, we report the growth kinetic profiles of the examined microorganisms growing in the presences of sub-lethal concentrations of D-amino acids. Additionally, to further elucidate their antimicrobial activity and to enhance their efficacy, synergistic effects between a β-lactam antibiotic; Ampicillin, as well as a polyene antifungal; Amphotericin B, with the D-amino acids under investigation were evaluated.

Methods

Chemicals

All amino acids used in this study were purchased from Sigma-Aldrich (St. Louis, USA) and included: L-alanine, D-alanine, L-lysine, D-lysine, L-serine, D-serine, L-proline, and D-proline. The antibiotic Ampicillin and the antifungal Amphotericin B were also purchased from Sigma-Aldrich. Bacteria were grown in Mueller-Hinton broth (MHB; Oxoid, Basingstoke, UK). *Candida* species were grown in Yeast Peptone Dextrose (YPD) broth (BD Difco™).

Microorganisms

The amino acids were tested against the following microorganisms: *Bacillus subtilis* ATCC 11562, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 29425, *Pseudomonas aeuriginosa* ATCC 11921, *Xanthomonas vesicatoria* ATCC 11633, *Candida albicans* ATCC 10231*, Candida glabrata* ATCC 1615, and *Candida krusei* ATCC 6258.

Inoculums preparation

Bacterial stock cultures were maintained at 4°C on slopes of nutrient agar. Cultures for experiments were prepared by transferring a sample from the stock cultures into Mueller-Hinton broth (MHB) and incubating without agitation for 24 hrs at 37 ºC. The cultures were diluted with fresh Mueller-Hinton broth to achieve optical densities corresponding to 1×10^6 colony forming units (CFU/mL). Candidal stock was kept frozen in glycerol at −70°C until thawed at room temperature. Then, *Candida* was aseptically dispersed in 5 ml of YPD broth before incubating overnight at 37°C. The turbidity of the suspension was adjusted and standardized spectrophotometrically to an optical density corresponding to 1×10^6 colony forming units (CFU/mL).

Determination of minimum inhibitory concentration (MIC)

The MICs of the tested amino acids were evaluated by the broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) (12), with minor modifications. A stock solution of 1M, of each amino acid, was prepared in Phosphate Buffered Saline, PBS, and the pH was adjusted to 7.0 using HCl or NaOH. The stock solutions were filter-sterilized by passage through 0.45 μm membranes (Billerica, MA, USA) and serial diluted with the medium to the desired concentrations. MIC tests were carried out in 96 flat bottom microtiter plates (TPP, Switzerland). Each test well was filled with 100 μL nutrient broth. A sample (100 μL) of the stock solution was added to the first test well

and mixed. A series of dilutions was then prepared across the plate. A 10 μL aliquot of the microorganism was used to inoculate each microtiter plate well to achieve a final inoculum size of 4×10^5 CFU/mL.

Positive growth controls (well with overnight culture, nutrient broth and microorganism inoculum but without amino acid treatment) and negative controls (well with broth but without inoculum) were also prepared and incubated under the same experimental conditions. Plates were incubated for 24 h at 37 ℃ for bacterial cultures, and for 48 h at 33 ℃ for yeast cultures, with shaking. The wells were examined for microbial growth by naked eye before optical densities were measured at 600 nm (OD_{600}) using a Microplate Reader (Palo Alto, CA, USA). The minimum inhibitory concentration (MIC) value was defined as the lowest concentration that inhibited ≥80% microbial growth of the tested strain. Microbial growth in the test wells was detected as turbidity, as indicated by the optical density measured at 600 nm, relative to the negative and positive controls. MIC determination was carried out in triplicate (in same 96-well plate) and repeated three times for each miroorganism and each tested amino acid or antibiotic.

Determination of median inhibitory concentration for D-amino acids (IC$_{50}$)

To further characterize the antimicrobial activity of D-amino acids, the median inhibitory concentration (IC$_{50}$) value of D-amino acids was determined. The percentage (%) of the microbial growth inhibition was determined as $[(A_c–A_t)/A_c] \times 100$, where A_c was an average of three triplicates of OD_{600} values of the positive growth controls with no amino acid treatment, and A_t was an average of three triplicates of OD_{600} values of the cultures treated with amino acids. The IC$_{50}$ value was calculated using the linear relation between the inhibitory probability and concentration logarithm according to the method of Sakuma[13]. The IC$_{50}$ value was expressed as the mean ± standard deviation of three independent experiments.

Growth kinetics

The growth kinetics of all tested microorganisms was determined in the presence of sub-MIC concentrations of either D-ala or D-lys. Briefly, Stationary-phase cultures were prepared and used to inoculate 200 mL of MHB broth or YPD broth to an initial OD_{600} of 0.05. These cultures were divided into 10.0-mL aliquots and either D-ala or D-lys solution was added to yield the desired concentration. Growth curves of the tested microorganism metabolizing D-ala or D-lys were constructed by monitoring OD_{600} of cultures growing in the presence of D-ala or D-lys. The test flasks were shaken at 37 ℃ and samples were drawn at each time point for OD_{600} readings until the stationary phase was reached. Growth kinetic assays for each microorganism were performed in triplicate from the same stationary-phase starter culture and D-amino acid stock solution. Triplicate growth curves were then repeated three times using independent stationary-phase starter cultures and D-amino acid stocks.

Chequerboard assay and synergy evaluation

The synergistic effects of a combination of Ampicillin or Amphotericin B with either D-ala or D-lys were evaluated by the chequerboard test as previously described[14, 15]. Serial 2-fold dilutions of the D-amino acids and Ampicillin or Amphotericin B were mixed in each well of a 96-well microtiter plates so that each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. Each microtiter well was inoculated with approximately 10^5 CFU/ml of microorganism, and the plates were incubated at 37 ℃ for 24 h with shaking. MICs were determined for Ampicillin and Amphotericin B at each D-amino acid concentration and for each D-amino acid at each Ampicillin and Amphotericin B concentrations. The combination inhibitory index (CI index) was calculated according to the equation: CI index = (MIC of drug A in combination/ MIC of drug A alone) + (MIC of drug B in combination/ MIC of drug B alone). The interaction was described as synergistic when CI index was ≤ 1.0, additive if the CI index was =1.0, and antagonistic if the CI index was >1.0[16, 17] The Chou-Talalay Plot illustrates the result of the chequerboard assay and the CI values.

The axis of the Chou-Talalay Plot represents the combination inhibitory index (CI) and the fraction affected (Fa) at each combination concentration.

Statistical analysis

All results were computed and expressed as mean ± standard deviation (SD) from three determinations performed in triplicate (n = 9). Statistical analysis was performed using SPSS software (version 17.0) with analysis of variance (One-Way ANOVA) and post-hoc test Dunnett's T3 were used to contrast the significant difference between the groups. A ρ-value of < 0.05 was considered as statistically significant.

Results
Antimicrobial activity

In this study, D-ala, D-lys, D-ser, D-pro, and their corresponding L-isomers were evaluated for their antibacterial activities against six bacterial species, representing both Gram positive and Gram negative bacteria. Evidently, compared to the antibacterial activities of the D-isomers, the L-isomers did not show any noteworthy activity against whichever of the tested species (MIC > 300 μg/μL). On the contrary, the D-isomers exhibited dose-dependent antibacterial activities against the bacterial species under examination. As shown in Table 1, characterized by their MIC and IC_{50} values, the examined D-amino acids fluctuate in their antibacterial activities. Our results indicate that D-lys exhibited the most potent antibacterial activity against all tested bacteria, whereas moderate activity exhibited by D-ala. Interestingly, very low activity was seen with D-ser, while D-pro did not show any significant antibacterial activity. Noteworthy, Gram positive bacteria were more susceptible than Gram negative bacteria to D-amino acids treatment (Table 1).

Table 1. Antibacterial activity of D-amino acids

Strain/D-amino acid	MIC(μg μL^{-1})					IC$_{50}$ (μg μL^{-1})			
	D-ala	*D*-lys	*D*-ser	D-pro		*D*-ala	*D*-lys	*D*-ser	*D*-pro
Bacillus subtilis ATCC 11562	13±2	2±0.4	61±9	>150		5±1	0.7±0.3	44±6	>100
Staphylococcus aureus ATCC 6538	15±5	6±2	66±4	>150		6±1	1±0.2	51±9	>100
Staphylococcus epidermidis ATCC 12228	16±3	5±1	69±6	>150		5±1	1±0.1	48±5	>100
Escherichia coli ATCC 29425	24±4	11±3	75±9	>150		15±3	5±1	55±7	>100
Pseudomonas aeuriginosa ATCC 11921	26±5	13±2	79±4	>150		13±4	7±1.5	58±5	>100
Xanthomonas vesicatoria ATCC 11633	23±4	10±1	80±10	>150		16±3	4±1	60±8	>100

The results of the antifungal testing of the examined D-amino acids against three *Candida* species are presented in Table 2. Relative to the antifungal activities of the D-isomers, the L-isomers did not show any noteworthy activity against whichever of the tested species (MIC > 200 μg/μL). Among the tested D-amino acids, D-lys revealed the highest activity against *Candida albicans* with a MIC value of 6 μg/μL. *Candida glabrata* was the least susceptible species to most D-amino acids treatment. *Candida krusie* growth was most extensively inhibited by D-lys followed by D-ala, whereas considerably higher concentration of D-ser was needed to

inhibit the growth of all tested *Candida* species. Apparently, D-pro did not exhibit any significant anticandidal activity against whichever species tested.

Table 2. Antifungal activity of D-amino acids

Strain/D-amino acid	MIC($\mu g \, \mu L^{-1}$)					IC$_{50}$ ($\mu g \, \mu L^{-1}$)			
	D-ala	*D*-lys	*D*-ser	D-pro		*D*-ala	*D*-lys	*D*-ser	*D*-pro
Candida albicans ATCC 10231	11±3	6±1	53±4	>100		7±1	2±0.5	39±5	>100
Candida glabrata ATCC 1615	25±4	18±3	68±9	>100		14±3	7±1	48±4	>100
Candida krusei ATCC 6258	17±2	10±2	59±5	>100		9±1	4±2	35±5	>100

Growth kinetics

Figure 1 demonstrates the normal growth curves of the six bacterial species (Fig. 1A) and the three candidal species (Fig. 1B) cultured under the normal, untreated growth conditions. Growth curves for all the examined microorganisms reveal typical sigmoidal kinetics with identifiable lag and logarithmic growth phases. Durations of the lag and log phases were variable among the different species. Generally, about 5 to 7 h were needed for the cells to adjust to the normal growth environment before they were ready to proliferate and hit the log phase.

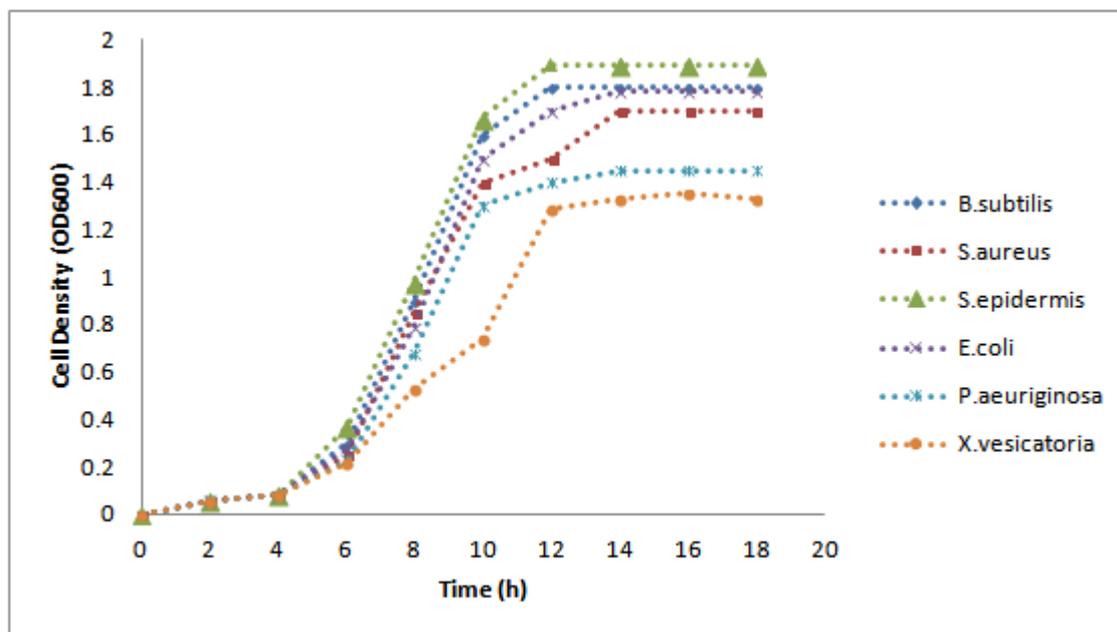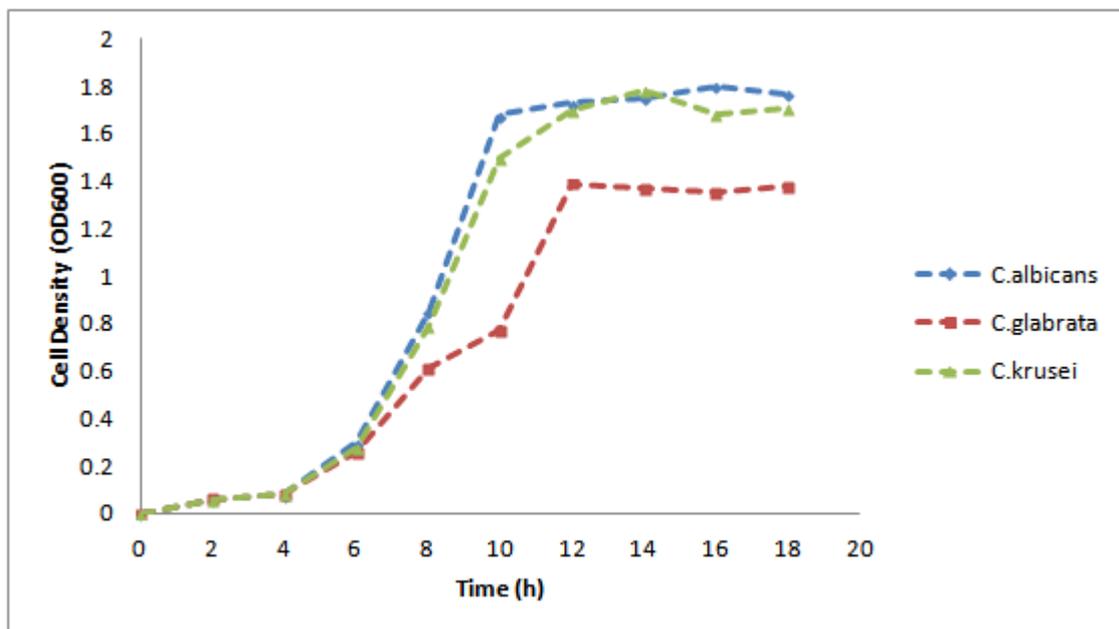**Figure 1A. Normal growth curves of six bacterial species cultured in MHB media**

Figure 1B. Normal growth curves of three *Candida* species cultured in YPD broth media

The patterns of growth curves of the all microorganisms tested were altered and showed deviations from the normal curves following treatment with either D-lys or D-ala (only data for representative microorganisms are shown in Figure 2). Growth of all microorganisms was severely inhibited when treated with sub-MIC concentrations of either D-lys or D-ala. Relative to D-lys which significantly retarded microbial growth upon treatment, D-ala treatment appears to moderately reduce the growth rate and extent of the all examined microorganisms. Specifically, a clear shift to the right due to extension of the lag phase was observed in all treated cultures.

The addition of sub-MIC concentrations of either D-lys or D-ala after completion of the lag phase (at Time=5 h) resulted in gradual growth inhibition in all tested microorganisms as illustrated in Figure 3 (only data for representative microorganisms are shown in Figure 3).

Synergistic effects

The synergistic effects upon the combination of either D-ala or D-lys with Ampicillin, on the examined bacterial species, as well as the synergistic effects upon the combination of either D-ala or D-lys with Amphoteracine B, on the examined candidal species, were evaluated. Generally, the MIC values were reduced ≥4-fold when D-ala was combined with either Ampicillin or Amphoteracin B, producing a synergistic effect on the examined bacteria and *Candida* species; as defined by CI<1.0 (Fig. 4A). Similarly, the MIC values were also reduced ≥4-fold when D-lys was combined with either Ampicillin or Amphoteracin B, yielding a synergistic effect on the examined bacteria and *Candida* species; as defined by CI<1.0 (Fig. 4B).

Discussion

Many bacteria produce and utilize D-amino acids for a variety of functions. Although potential cell toxicity has previously been linked to specific D-amino acids[10, 11, 18], the fundamental mechanism by which toxicity occurs is not fully understood. Nonetheless, several reports suggested that the observed toxicity appears to be related to the incorporation of D-amino acids in the peptidoglycan structure (4-7). The growth of *E. coli* cells

in medium supplemented with certain D-amino acids results in the incorporation of these D-amino acids into macromolecular peptidoglycan[5, 7].

Figure 2. Growth curves of representative bacteria and candida cultures treated with sub-MIC concentrations of either D-lys or D-ala
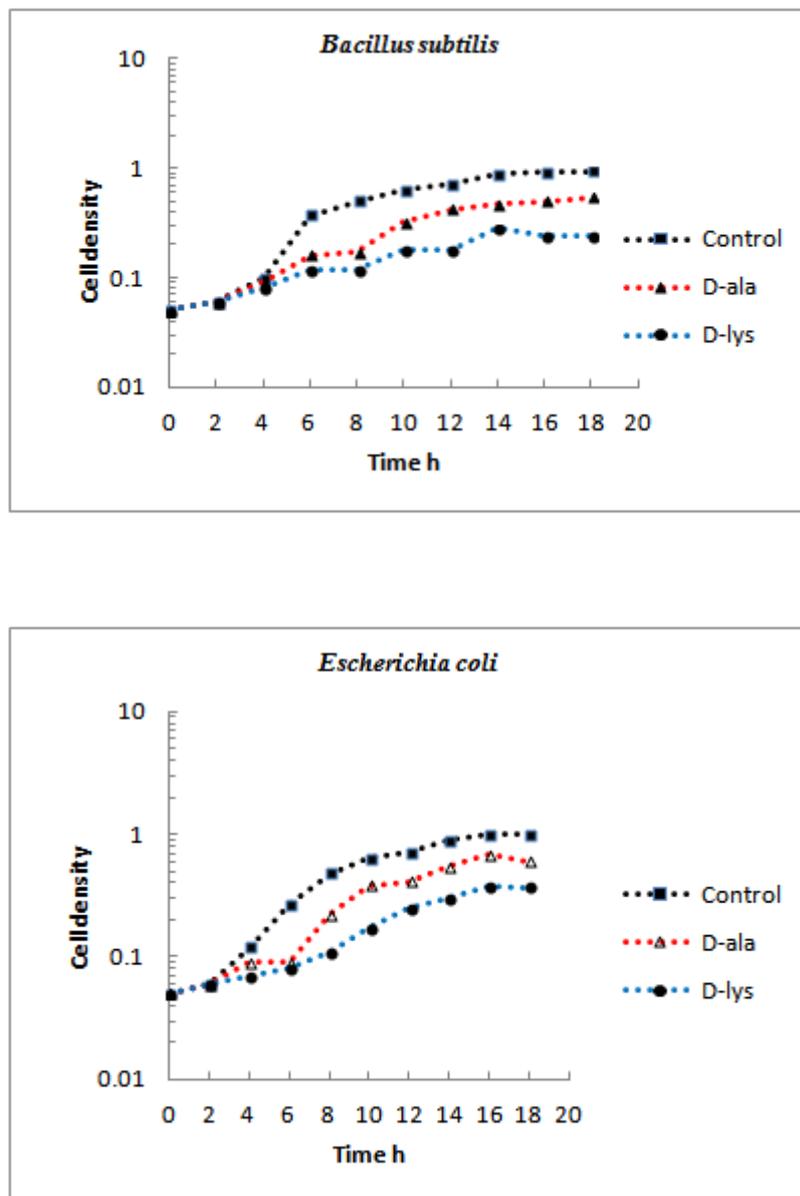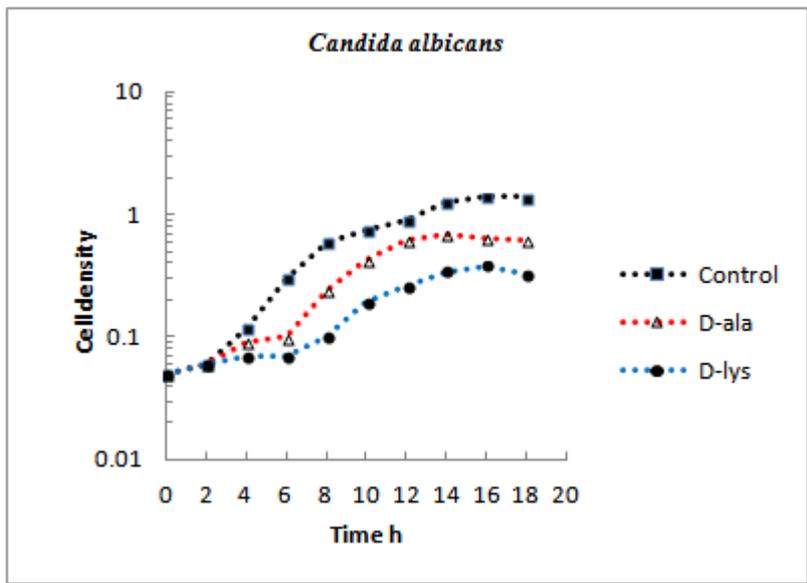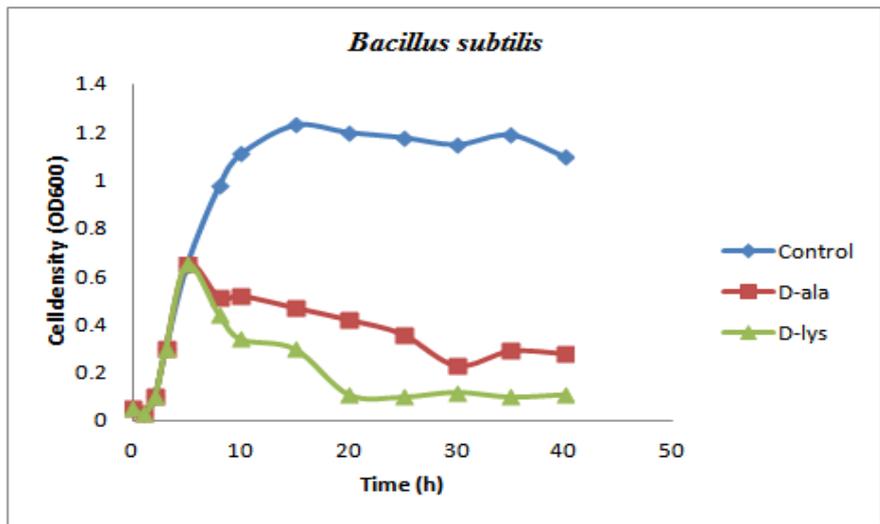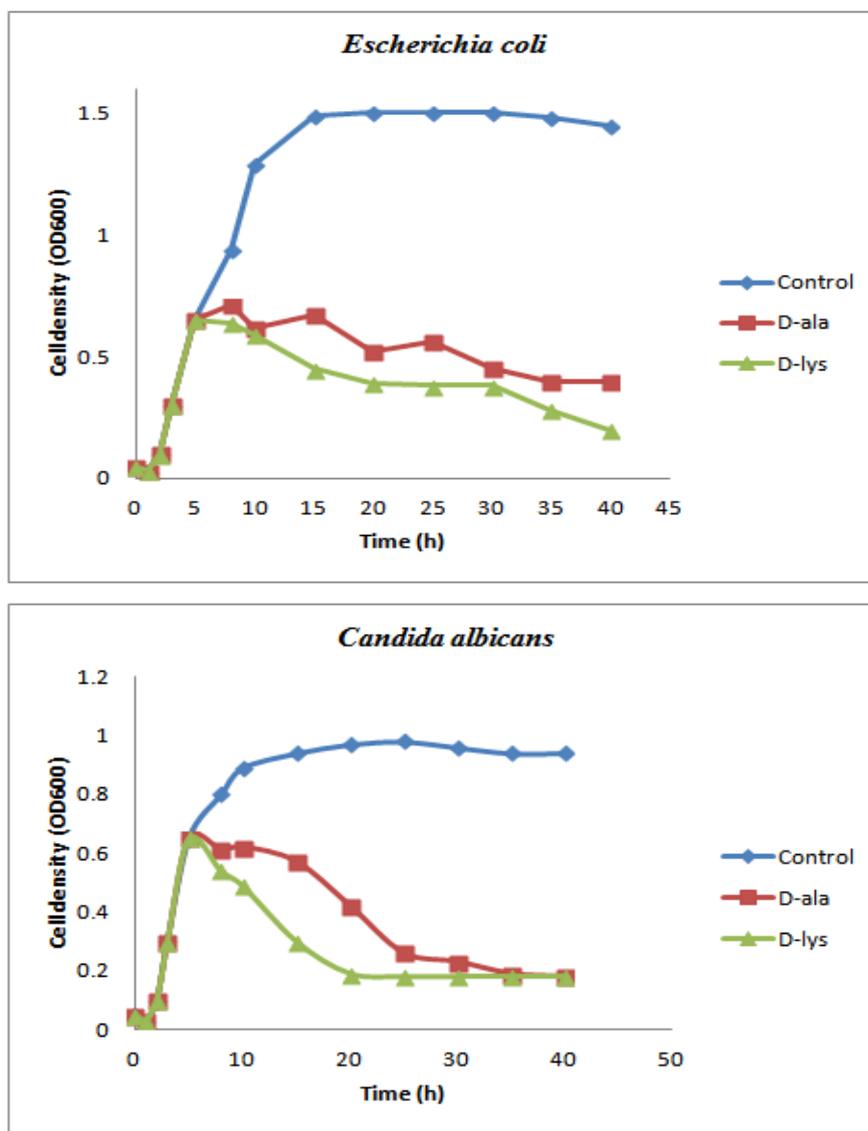
Figure 3. The effects of the addition of sub-MIC concentrations of either D-lys or D-ala to microbial cultures after completion of the lag phase (5h)

Escherichia coli

Candida albicans

The purpose of the present study was to investigate the antibacterial activities of several D-amino acids in different bacterial species representing both Gram negative and Gram positive bacteria. To our knowledge, there has been limited number of reports looking at the growth regulatory effects of D-amino acids in bacteria. Our results indicate that certain D-amino acids, such as D-ala and D-lys, have higher antibacterial activities compared to other D-amino acids, such as D-ser and D-pro (Table 1), against the target bacteria. The observed discrepancy in the their antibacterial activity is most likely related to the difference in cell wall architecture between Gram positive and Gram negative bacteria.

Recently, reported cases of oral candidiasis have noticeably increased[19-21]. Although numerous species of *Candida* encompass part of the oral microflora, their appearance may, conditionally, cause opportunistic infections among immune-compromised hosts[22, 23]. Recent emergence of *Candida* species with reduced susceptibility to antifungal treatment, and the increasing

concerns regarding the safety of chemical preservatives have prompted researchers to investigate antifungal agents from natural resources. The evaluation of antifungal activity of D-amino acids has never been reported in literature. In this study, the effects of the examined D-amino acids on the growth profiles of three candidal species was determined based on continuous monitoring of changes in the optical density of *Candida* growth over time. D-lys and D-ala were found to exhibit considerable degree of antifungal activity towards different species of *Candida*, relative to other tested amino acids.

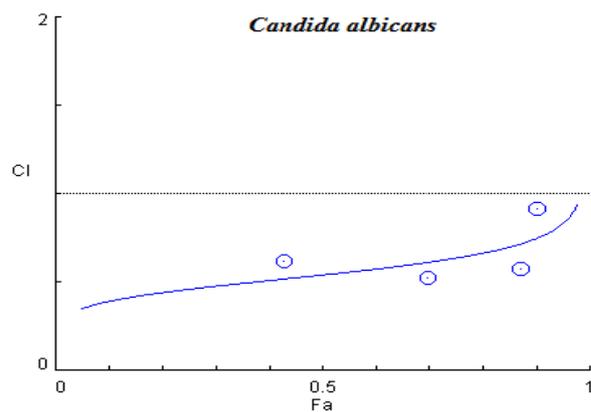**Figure 4A. Chou Talalay curves illustrating the synergistic effects of D-ala**
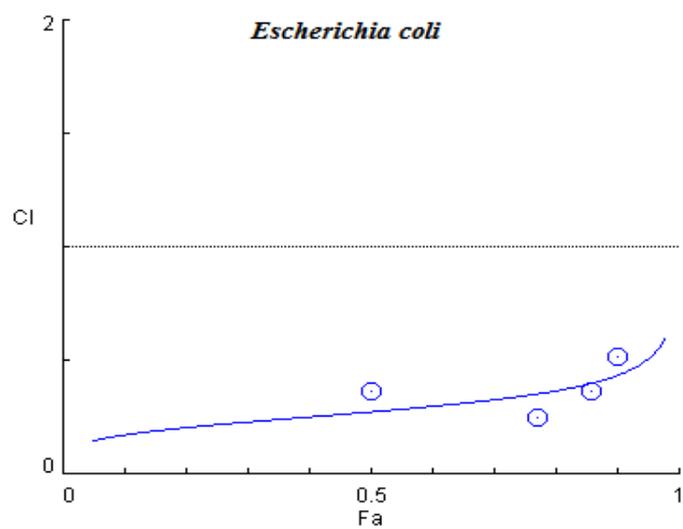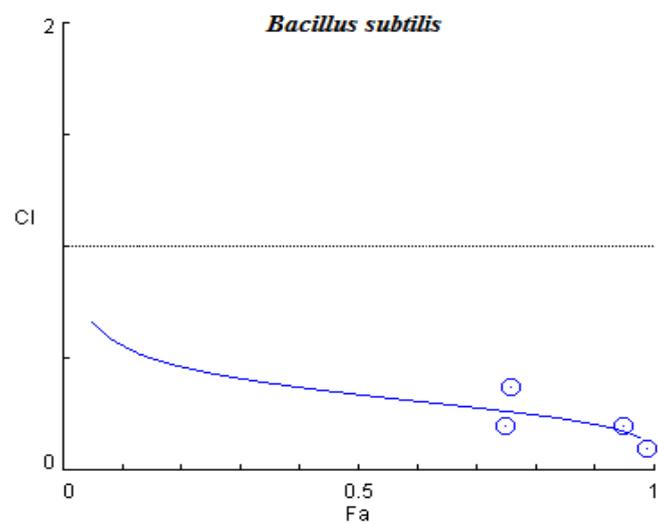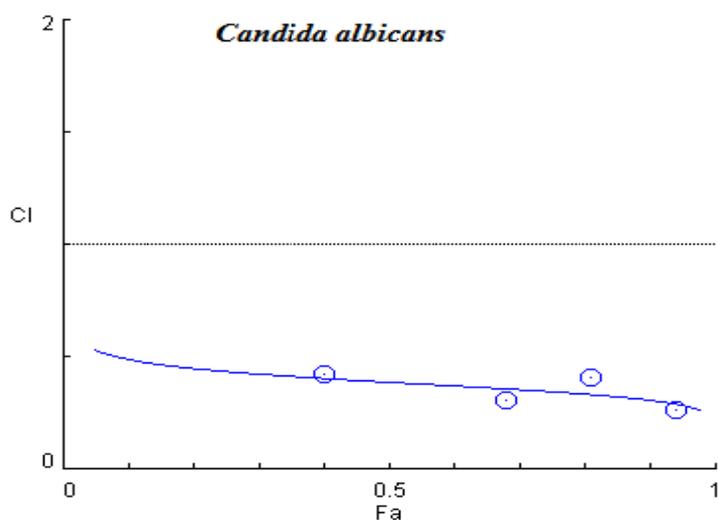
Candida albicans
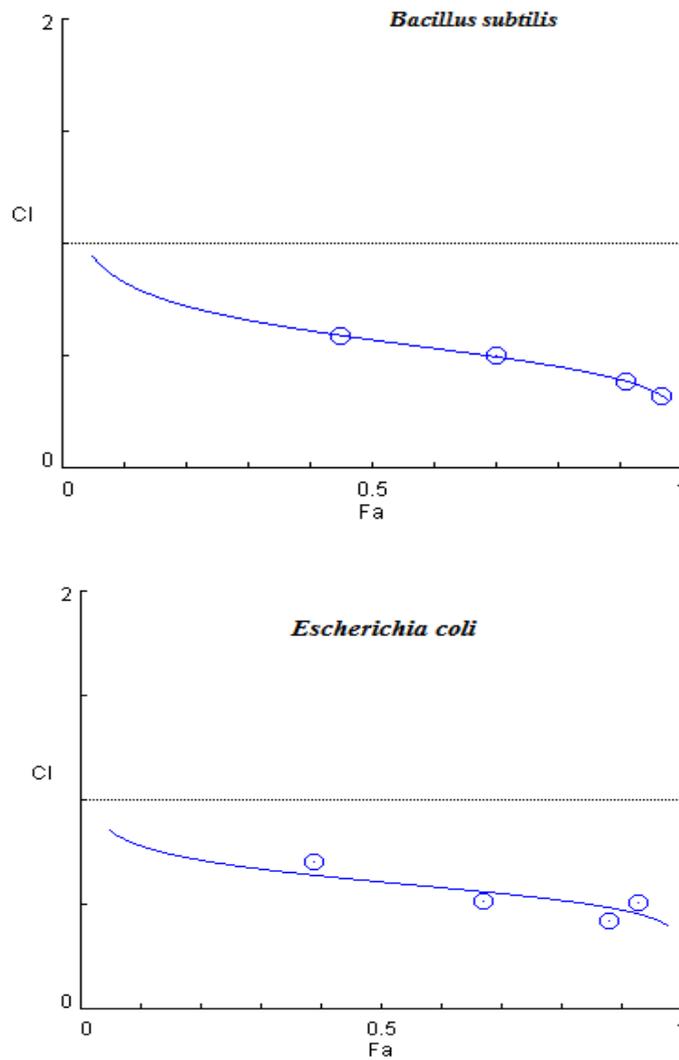
Figure 4B. Chou Talalay curves illustrating the synergistic effects of D-lys

Bacillus subtilis

Escherichia coli

Supportive to their inhibitory role, growth kinetic experiments revealed suppressed growth profiles at sub-MIC concentrations of D-lys and D-ala against all tested microorganisms (Fig 2). Nonetheless, the kinetic profiles of both D-amino acids exhibited typical sigmodial curves with distinguishable lag and logarithmic growth phases, as demonstrated in Figure 2. Our findings are consistent with previous studies, which indicated that sub-MIC levels of antimicrobial reagents reduce the growth rates and increase the lag phase of microbial growth (24-26). The extended lag phases robustly suggest that D-lys and D-ala have successfully suppressed the cells and exerted fungistatic/bacteroistatic effects on the examined species.

It is known that the efficacy of several antimicrobial agents can be improved by synergism. To investigate the potential for synergistic toxicity, a checkerboard assay was utilized. Multiple concentrations of D-ala or D-lys, in combination with Ampicillin or Amphotericin B, were investigated for the presence of synergy, additive effect, or antagonism against the target microorganisms. Our findings indicate that there was a dramatic reduction in the D-amino acids' MIC values as the MICs of both D-amino acids were reduced by approximately 4-fold. The results of pairing Ampicillin or Amphotericin B with either D-ala or D-lys were confirmed in a more specific manner through the utilization of the CompuSyn software program (version 3.0.1, ComboSyn, Paramus, NJ). Our data analysis confirmed that there was synergy at all tested combinations of Ampicillin or Amphotericin B with D-ala or D-lys.

CONCLUSIONS

In conclusion, this is the first report in literature that provides an evidence for a wide spectrum antimicrobial activity exhibited by D-lys and D-ala. Our results might initiate a wide range of possibilities for future formulations of combinational remedies against microbial growth. With the emergence of microbial resistance to several antibacterial reagents, such remedies, with significantly modest concentrations of each individual compound, might aid treatment and prevention of microbial growth in several applications, such as agriculture, food-borne pathogens, surgical equipments and hospital surfaces.

ACKNOWLEDGEMENTS

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التآزر التفاعلي والفاعلية المرتبطة ببعض نظائر الأحماض الأمينية المتوافرة طبيعياً ضد أنواع بكتيرية وفطرية ذات أهمية سريرية

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ملخص

يتم تصنيع واستخدام الأحماض الأمينيه بشكل أساسي على شكل النظير L في جميع ممالك الحياة الثلاثة، لكن البكتيريا تنتج النظير D من أحماض أمينية متنوعة والتي تدخل في توليف وربط شبكة البيبتيدوغلايكان.

أفادت العديد من الدراسات بأن النظير D من أحماض أمينية معينة مرتبط بأنشطة مضادة للميكروبات الفاعلة ضد الاشريكيه القولونيه. و قد أجريت هذه الدراسة للتحقيق في الأنشطة المضادة للميكروبات والفطريات المرتبطة ببعض الأحماض الأمينية (النظير D) والتي تشمل: الألانين، اللايسين، السيرين والبرولين. تشير النتائج التي توصلت إليها الدراسة إلى أنه من بين مجموعة الأحماض الأمينية التي تم فحصها فإن النظير D من اللايسين هو الأكثر فاعلية ضد البكتيريا والفطريات يتبعه النظير D من الألانين، بينما يفتقر كل من النظير D من السيرين والبرولين إلى أي فاعلية مؤثرة ضد البكتيريا والفطريات. أظهرت البكتيريا الموجبة الجرام حساسية أكبر للأنشطة المضادة للميكروبات المرتبطة بنظائر الأحماض الأمينية مقارنة البكتيريا السالبة الجرام. بالإضافة إلى ذلك، كشفت دراسات النمو الحركي أن النظير D من اللايسين والنظير D من الألانين تسببا بتأخير مراحل النمو مما يدل على أن نظائر الأحماض الأمينية أثرت بنجاح على قدرة الكائنات الحية الدقيقة على استخدام العناصر الغذائية بكفاءة وعطلت الوظائف البيولوجية الطبيعية. بالإضافة إلى ذلك فإن التآزر في الفاعلية كان واضحاً بين النظير D من اللايسين أو النظير D من الألانين عندما اقترنا بمضاد حيوي فاعل مثل الأمبيسيلين أو مضاد فطريات فاعل مثل الأمفوتيراسين. إن مثل هذه النتائج قد تشير إلى إمكانية استخدام نظائر الأحماض الأمينية المتوافرة طبيعياً للعلاج والوقاية من نمو الميكروبات في الغذاء أو في التطبيقات الزراعية.

الكلمات الدالة: الألانين−D، اللايسين−D، الأنشطة المضادة للميكروبات والفطريات، التآزر في الفاعلية.