

Optimization of culture conditions for antibacterial substance production from newly isolated *Brevibacillus laterosporus* EA62

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ABSTRACT

Objectives: In the present study, it was reported the effects of some nutritional (amino acid, carbon, nitrogen and metal sources) and physical factors (pH and temperature) on antibacterial substance activity of *Brevibacillus laterosporus* EA62.

Methods: The agar well diffusion assay was performed to evaluate the antibacterial activity of the substance. The antibacterial activity of the new substance was examined against four pathogenic bacteria under different nutritional and physical conditions.

Results: The best antibacterial activity was obtained in modified medium consists of the 5% glucose, 0.1% tryptone, 0.05% MgSO₄ + CaCO₃ and 0.5% glutamic acid. For physical parameters, maximal activity was observed after 72 h when incubated at 37°C, pH 7.0.

Conclusions: This study indicates that *Brevibacillus laterosporus* EA62 could be an important source of antibacterial substances under this medium optimization.

Keywords: This study indicates that *Brevibacillus laterosporus* EA62 could be an important source of antibacterial substances under this medium optimization.

Antibiotics are chemicals which kill or inhibit the growth of bacteria or fungi, and can be used to treat infections by these organisms. Antibiotics are the one of the most important commercially exploited secondary metabolites produced by bacteria, fungi and *Streptomyces* and employed in a wide range. In recent years, soil bacteria have been investigated in terms of antibiotic production potential. The soil is a natural source for microorganisms and their antimicrobial products [1]. Soil bacteria and fungi have played a significant and an important role in antimicrobial product discovery. The selection of natural antimicrobial products from soil microorganisms is considered as an alternative method for the disease control and plant

protection. Considerable research is being done in order to find new antimicrobial producing bacteria isolated from soil and their active substances [2, 3]. Several classes of antimicrobial substances that inhibit the growth of fungi and bacteria in vitro assays have been identified in the last decades [4].

Brevibacillus is omnipresent in agricultural soils, and it can secrete structurally diverse secondary metabolites with broad antibiotic spectra. Some of these metabolites, such as chitinase and gramicidin S, have been extensively studied, and numerous *Brevibacillus* species, which have the potential as antimicrobial agents, have become research hotspots in the recent years [5]. *Brevibacillus laterosporus* can pro-

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duce different virulence factors: parasporal crystalline [6], extracellular protease [7] and lipopeptide antibiotics [8]. Additionally, *B. laterosporus* also secretes short sequence peptides with broad antibiotic spectra, such as loloatin A [9]; however, these metabolites have not been extensively studied.

The number of multi-drug resistant bacterial strains has increased, partly due to the misuse of antibiotics, resulting in serious health challenges in hospital settings. Thus, the discovery of new potential antibacterial substance producing microorganisms has become an important goal. Many species, such as *Streptomyces*, *Bacillus* and *Penicillium* have been studied continuously for their ability to produce antibiotics [10]. Environmental factors such as pH, temperature and medium composition can influence the production of antagonistic substances from bacteria. Several reports have been discussed regarding antibacterial components, its optimizing by altering several physical factors and medium composition [11-13]. In the present study, it has been optimized nutritional and physical parameters to produce for antibacterial substance of *B. laterosporus* EA62.

METHODS

Bacterial Strains and Growth Conditions

B. laterosporus EA62 which was isolated and identified by 16S rRNA gene analysis in the previous study, was used in the current study [14]. Basal growth medium was consisted of 15 g/L glucose, 1 g/L yeast extract, 0.5 g/L MgSO₄, 3 g/L CaCO₃ and 0.5 g/L (NH₄)₂HPO₄ at pH 7.0. The overnight cultures adjusted 10⁸ CFU/ml were inoculated at 10% in 150 mL of basal media and incubated at 37 °C at 150 rpm for 72 h [14]. After incubation, samples were filtered through a 0.22 µm filter, and the filtrate as antibacterial substance was subsequently used to determine the effects of nutritional and physical parameters on antibacterial substance activity against some pathogenic Gram-negative *Yersinia enterocolitica* (ATCC 9610) and *Salmonella typhimurium* (ATCC 14028) the Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) by using agar well diffusion assay [15].

Effects of Different Nutritional and Physical Pa-

rameters on Production of Antibacterial Substance

Carbon sources including sucrose, maltose, potato starch and wheat bran (1.5% w/v), nitrogen sources including organic (corn steep liquor, tryptone and skim milk at 0.1% w/v) and inorganic sources ((NH₄)₂NO₃ and (NH₄)₂SO₃ at 0.05% w/v) were evaluated for their effect of antibacterial substance activity in basal medium. In addition, amino acids including alanine, phenylalanine, valine, tyrosine, lysine, histidine, cystine, arginine, glutamic acid (0.5% w/v) were tested to obtain the best amino acid. The culture medium was supplemented with metal ions such as CaCl₂, FeSO₄, LiSO₄, NaCl, CaCO₃, MgSO₄, and MnSO₄ (0.05% w/v) in basal medium.

Antibacterial substance (grown in basal medium for 72 h) was exposed to various temperatures. Sterile filtered antibacterial substances were incubated for 15-30 min at 30, 40, 45, 50 and 55 °C at the optimal pH of 7.0 and then immediately cooled in ice water. A control was maintained by incubating antibacterial substances at 37 °C. For optimizing pH, different pH was estimated by adjusting pH of antibacterial substances to the 4.0 to 9.0. After incubation for 1 h and before plating for antibacterial activity, pH treated samples were neutralized pH 6.5-7 to determine the antibacterial substance activity.

Statistical Analysis

For statistical analysis, the standard deviation for each experimental result and student's t-test were calculated using Microsoft Excel. All of the assays were carried out in triplicate. The bars correspond to standard deviation [16].

RESULTS

In this study, it was carried out to determine the antibacterial substance activity of *B. laterosporus* EA62 by assessing the effect of nutritional and physical conditions. The isolate was inoculated in basal medium and incubated at 37°C for 72 h; the filtered supernatant as antibacterial substance was used to determine the antibacterial activity against *Y. enterocolitica*, *S. typhimurium*, *S. aureus* and *E. faecalis* under various nutritional and physical conditions. The basal medium was optimized with different carbon and nitrogen sources, metal ions and amino acids.

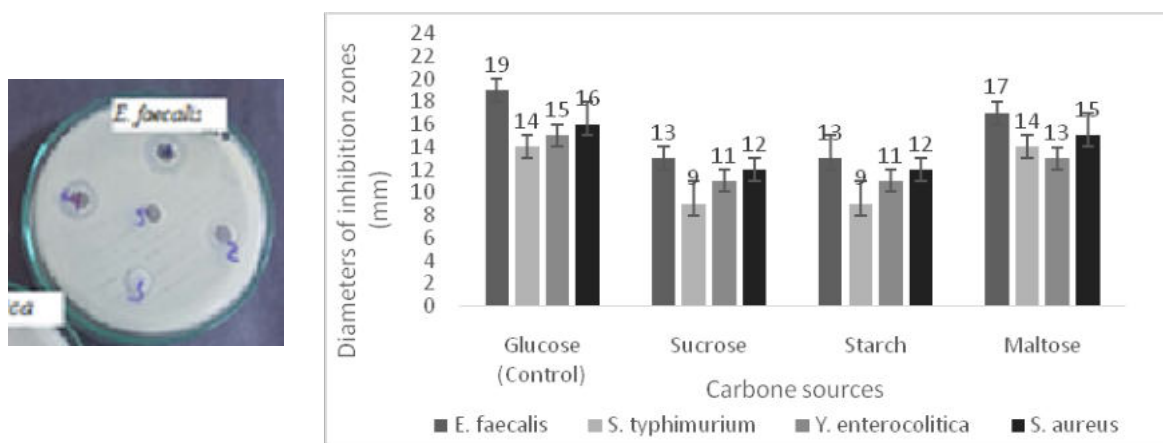


Fig. 1. Effect of different carbon sources on the production of antibacterial substance against pathogens. 1-Glucose, 2-Sucrose, 3-Starch, 4-Maltose, 5-Wheat bran on petri dish.

Among the different carbon and nitrogen sources studied, addition of glucose (Fig.1) and tryptone (Fig. 2) to the basal medium showed highest antibacterial activity against *E. faecalis*. Our data showed that glucose (19 mm) followed maltose (17 mm) against *E. faecalis* and glucose (16 mm) followed maltose (15 mm) against *S. aureus* enhanced the antimicrobial activity. No antibacterial activity was observed using wheat bran (Fig. 1). The production of antibacterial substance was repressed by wheat bran.

Among nitrogen sources, higher antibacterial activity was achieved when tryptone (20 mm) and yeast extract (19 mm) against *E. faecalis* used as a nitrogen source followed tryptone (17 mm) and yeast extract (17 mm) against *S. typhimurium*. In this study, yeast extract and tryptone alone appeared more efficient at the activity of the antibacterial substance and antibacterial activity was not observed in the presence of skim

milk as organic nitrogen source.

Some amino acids were tested in the basal medium to determined inhibitory effects of the antibacterial substance in this study. Glutamic acid has the most effective amino acid on antibacterial substance activity against all bacteria tested, while valine and arginine has the least, phenylalanine and cysteine resulted in no antibacterial substance activity (Fig. 3). Maximum zone of inhibition in basal medium containing glutamic acid was observed against *S. aureus* (19 mm) followed by lysine (17 mm) and alanine (17 mm) against *E. faecalis*.

In this study, a small effect was only observed for MgSO₄ and MgSO₄+CaCO₃ combination among the metal ions, no antibacterial activity was obtained in the presence of the others (Fig. 4). Results showed that a synergistic effect of MgSO₄ and CaCO₃ on the antibacterial substance activity occurred and antibacter-

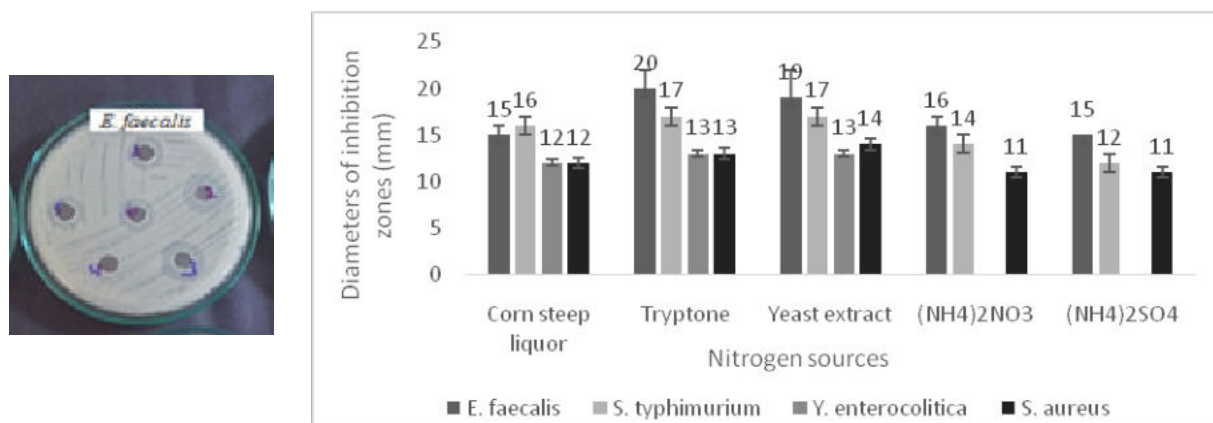


Fig. 2. Effect of different nitrogen sources on the production of antibacterial substance against pathogens. 1-Corn steep Liquor, 2-Tryptone, 3-Yeast extract, 4-Skim milk, 5-(NH₄)₂NO₃, 6-(NH₄)₂SO₃ on petri dish.

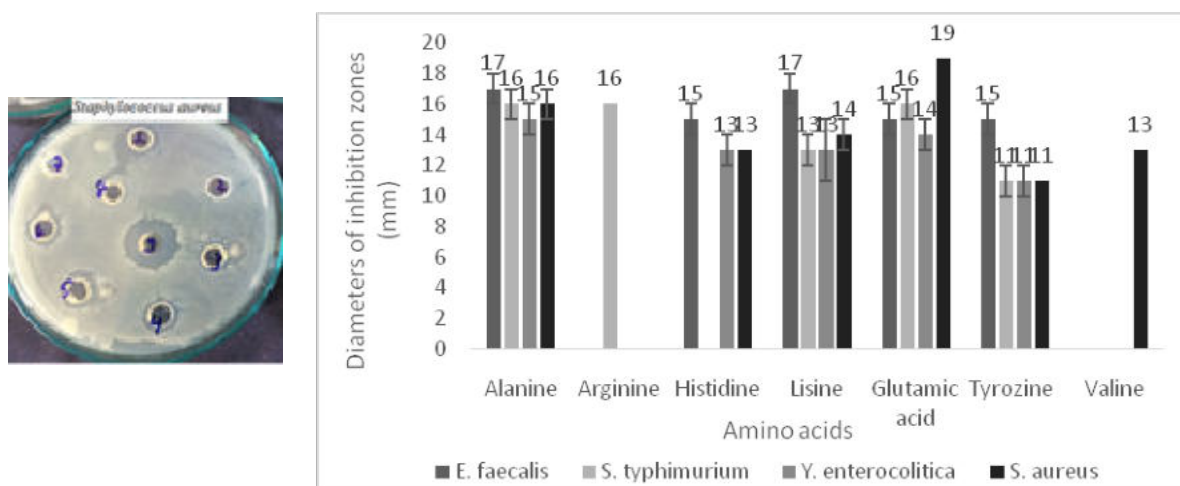


Fig. 3. Effect of different amino acid sources on the production of antibacterial substance against pathogens. 1-Alanine, 2-Phenylalanine, 3-Valine, 4-Tyrosine, 5-Lysine, 6-Histidine, 7-Cystine, 8-Arginine, 9-Glutamic acid on petri dish.

ial activity was considerably decreased when metal ion sources alone were used. Maximal inhibitory zones were determined in presence of MgSO4 (15 mm) against *E. faecalis* and combination with CaCO3 (19 mm) was increased the antibacterial activity against all tested pathogens while CaCO3 alone had no effect.

The effect of different temperature and pH on antibacterial substance activity have been outlined in Figure 5. Treatment of antibacterial substance at 30, 37, 40, 50, 55°C did not show significant from the control. The maximum antibacterial substance activity was obtained at 37 °C as control against *Y. enterocolitica* (17 mm), although 40 °C was also effective at promoting antibacterial activity, as the observed inhibition zone was 15 mm against *S. typhimurium*.

Generally, the maximum activity was obtained at an initial pH of 7.0. At lower and higher pH values were reduced the antibacterial substance activity. The

highest zone of inhibition was obtained for *Y. enterocolitica* (17 mm) at pH 7.0 and the lowest *E. faecalis* and *Y. enterocolitica* (11 mm) at pH 4.0, *E. faecalis* and *Y. enterocolitica* (10 mm) at pH 9.0. No zone of inhibition was recorded below pH 4.0 and above pH 9.0.

Modified medium determined for each bacterial strains was found different. But the best results for each parameters were selected to determine modified medium. According to these results, the maximum production of antibacterial substance was obtained in-optimized medium (consisted of 1.5% glucose, 0.1% of tryptone, 0.05% of MgSO4 +CaCO3 and 0.5% glutamic acid at pH 7.0).

The production of antimicrobial substance in the modified medium showed a slight increase compared to each test bacteria according to the control medium (Table 1). An increase of 17% for *S. typhimurium*, 5%

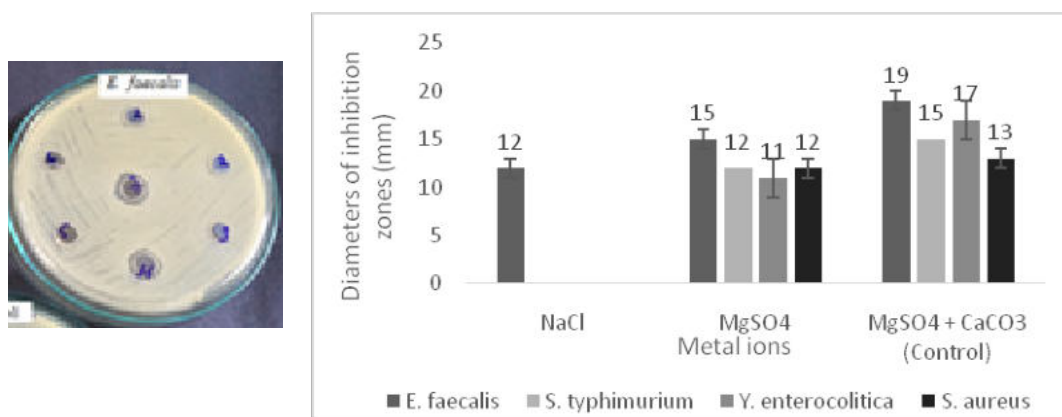


Fig. 4. Effect of different metal ions on the production of antibacterial substance against pathogens. 1-CaCl2, 2-FeSO4, 3-LiSO4, 4-NaCl, 5-KCl, 6-MnSO4, 7-MgSO4 on petri dish.

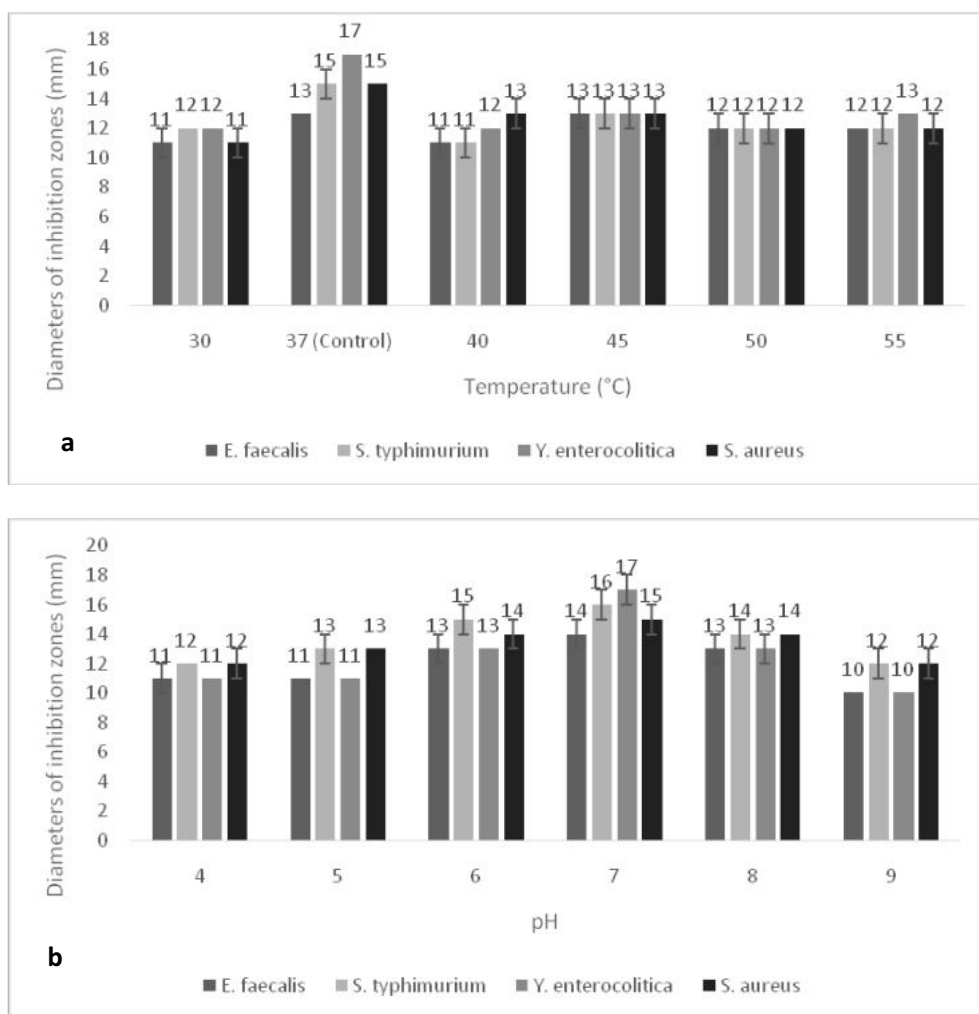


Fig. 5. Effect of temperatures (a) and pH (b) on antibacterial substance activity against pathogens.

for *Y. enterocolitica*, 6% for *S. aureus*, and 7% for *E. faecalis* was found.

DISCUSSION

Prior knowledge and experience in developing a

suitable basal medium play a significant role in the further media optimization [17]. The optimization of various physical and nutritional growth parameters caused an increase in the antibacterial substance activity; the important physical factors that determine the bioprocess are temperature, pH, agitation, inoculum size and inoculum age [18]. In a previous study,

Table 1. Comparison of antibacterial substance activity in modified and basal medium against bacteria tested

Bacterial Strains	Inhibition zones in control medium (mm)	Inhibition zones in modified medium (mm)
<i>Salmonella typhimurium</i>	17 ± 2	20 ± 1
<i>Yersinia enterocolitica</i>	17 ± 1	18 ± 2
<i>Staphylococcus aureus</i>	15 ± 2	16 ± 1
<i>Enterococcus faecalis</i>	13 ± 1	14 ± 2

Data are given as mean ± standard deviation

it was observed that the antibacterial substance showed inhibitory activity against the test pathogens (Gram-negative *Y. enterocolitica* (ATCC 9610) and *S. typhimurium* (ATCC 14028) the Gram-positive bacteria, *S. aureus* (ATCC 25923) and *E. faecalis* (ATCC 29212). Maximum zone of inhibition was observed against *S. aureus* (19 mm) followed by *Y. enterocolitica* (17 mm) [14]. Therefore, in this study, the effects of nutritional and physical parameters were investigated for the production of antibacterial substance from *B. laterosporus* EA62.

Different carbon sources, such as maltose [19], dextrose [20], arabinose [21], fructose [22], and mannitol [23] have been reported to be suitable for the activity of antibiotic substances in different microorganisms. In this study, among the different carbon sources studied, addition of glucose to the basal medium showed highest antibacterial activity against all bacteria tested. In addition, the antagonistic activity of bacteriocins an antimicrobial substance was also increased when glucose was added to the medium in a previous studies [24, 25]. Some studies have reported that organic or inorganic nitrogen sources influence enzyme activity. It was previously reported that antimicrobial activity was considerably decreased when nitrogen sources alone were used in the fermentation media [22]. In present study, higher antibacterial substance activity was achieved when tryptone and yeast extract against all bacteria tested used as a nitrogen source. Similar results were observed for antibiotic activity in batch cultures of *Bacillus laterosporus* ST-1 when grown in the presence of yeast extract in glucose broth [26]. In addition, inhibitory effect of antibacterial substance was only observed for MgSO₄ and MgSO₄ + CaCO₃ combination among the metal ions, no antibacterial activity was obtained in the presence of the others. The addition of MgSO₄ increased iturin A activity by *Bacillus amyloliquefaciens* B128 [27, 28], indicating that that rare earth metals and trace metals, notably manganese, zinc and iron, may trigger the activity of various secondary metabolic pathways. The use of amino acids as nitrogen sources can inhibit the synthesis of secondary metabolites [29]. Therefore, some amino acids were tested in the basal medium to determined inhibitory effects of the antibacterial substance in this study and glutamic acid has found as the most effective amino acid on antibacterial substance activity against all bacteria tested while

phenylalanine and cysteine showed no inhibitory effects on antibacterial substance production.

The effect of different temperature and pH on antibacterial substance activity was also determined and maximum antibacterial substance activity was found at 37 °C and pH 7.0 against all bacteria tested. A similar result for temperature was previously reported, but the antibiotic showed maximum activity at pH 8.0 [30]. Although the maximum antibiotic activity occurred at a low temperature (30 °C) and at pH 7.0 [26], antibiotic activity was achieved at temperatures as high as 40 °C and at pH 7.0 [31].

CONCLUSION

In summary, this is a novel antibacterial substance from the soil microorganism, *Brevibacillus laterosporus* EA62, and it is active against some important gram negative and positive pathogens. The effects of nutritional and physical parameters on antibacterial substance activity were examined. The antibacterial substance produced by EA62 may be potential applications for the pharmaceutical industry and could be a promising candidate for infection diseases after purified and characterized in the near future.

Authors' Contribution

Study Conception: ED, AA, AUA; Study Design: ED, AA, AUA; Supervision: ED; Funding: ED; Materials: ED, AUA; Data Collection and/or Processing: ED, AA, AUA; Statistical Analysis and/or Data Interpretation: ED, AA, AUA; Literature Review: ED, AA; Manuscript Preparation: ED, AA and Critical Review: ED.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

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