Research Square

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Antimicrobial and synergistic activity of the drug association of *Allium sativum* and antibiotics against Group B *Streptococcus*

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Abstract

Maternal colonization by Group B *Streptococcus* during pregnancy increases the risk of neonatal infection due to vertical transmission from mother to fetus before or during labor. The aims of this study were to evaluate the antimicrobial activity of SP80 (obtained from RGE) and its synergism associated with the antibiotic against strains of *Streptococcus agalactiae*. The broth microdilution was used to determine the antimicrobial activity of SP80 and to establish the MIC of SP80 (2.40 mg/mL). By using the disk diffusion method, fifty-five clinical isolates of *S. agalactiae* and 1 ATCC were tested against the association of SP80 with antibiotic PEN and AMP, respectively, for synergistic assessment. The association of SP80 + PEN showed that the mean of the inhibition halos decreased, but it was not significant, with $p=0.07$. In contrast, the association of SP80 + AMP caused the mean inhibition halos to increase with a $p<0.001$, a significant result. SP80 has antimicrobial activity against *S. agalactiae* Gram-positive bacteria, and the association with the antibiotic AMP showed a synergistic effect, which did not occur when in association with PEN.

Introduction

Streptococcus agalactiae or Group B *Streptococcus* (GBS) are bacteria that inhabit the human gastrointestinal and vaginal tracts and can develop into invasive infections in the newborn during the first weeks of life [1].

GBS disease in newborns can be classified according to the time of onset of infection. Early-Onset Disease (EOD) occurs in 12 to 48 hours after birth or up to the first 7 days and is generally due to mother-to-child transmission during childbirth, and it can cause severe problems, such as meningitis, pneumonia, and sepsis. Late-Onset Disease (LOD) occurs between a week to a few months after birth and it can cause meningitis and the contamination source may be in the infant's home environment [2].

In 1996, prophylactic recommendations such as the use of antibiotics during labor were implemented to prevent GBS infections in newborns by the American College of Obstetricians and Gynecologists (ACOG) [3] and Centers for Disease Control and Prevention (CDC) [4] and then in 1997 by the American Academy of Pediatrics (AAP) [5]. According to the Consensus Reviews, the guidelines were revised in 2002 and 2010 and universal recommendations were issued for the collection of vaginal secretions from all pregnant women between 35 to 37 weeks of gestation [6]. In 2018, the responsibility and management for updating the guidelines were transferred from the CDC to ACOG and AAP [7].

Currently, as part of routine prenatal care, all pregnancy, between 36 to 38 weeks of gestation, are screened for GBS. A culture from the vagina and rectum is done to optimize the identification of those who should receive appropriate intrapartum antibiotic prophylaxis (IAP) [8, 9].

The use of antibiotics during pregnancy was not seen to be beneficial, but at the beginning of labor, it is [10]. The use of 5.000.000 IU (intravenous) penicillin G (PEN) is recommended, maintaining 2.500.000 IU

every 4 hours, until the moment of delivery; or starting with 2 g (intravenous) ampicillin (AMP) first and then administering an additional 1 g every 4 hours, until the moment of childbirth [10]. In case of allergic pregnant women, it is recommended 500 mg (intravenous) erythromycin every 6 hours, or 900 mg clindamycin every 8 hours; if the pregnant woman is GBS resistant, 1 g vancomycin is administered intravenously every 12 hours However, this prophylaxis does not prevent LOD [11, 12].

Although prophylactic measures are already well established by conventional medicine, the use of medicinal plants in disease prevention is quite common in developing countries like Brazil [13]. When researching the use of garlic for the treatment of GBS infection in traditional medicine, we were able to find some published articles describing positive results [14, 15].

Garlic (*Allium sativum* L.), part of the family Liliaceae, is one of the most researched medicinal plants in the world and has been used for centuries in cooking and traditional medicine [16]. In Brazil, this plant belongs to a list comprising 71 plants of interest to the *SUS* (acronym for *Sistema Único de Saúde*, i.e. Brazil's Universal Health System) issued by the Ministry of Health's National Program of Medicinal Plants and Herbal [13].

This plant species has been used as a remedy for several diseases, such as antioxidant [17], antitumoral [18], anti-inflammatory [19], immunomodulatory [20], antiviral [21], antimicrobial [22, 23], and cardiovascular protective [24] actions, as well as promoting beneficial effects in diabetic [25] and obese patients [26].

This study aimed to assess the antimicrobial activity of *A. sativum* L. and its association with antibiotics penicillin G and ampicillin against *S. agalactiae* strains.

Material And Methods

Plant material

Garlic bulbs from *A. sativum* L. (Liliaceae), a purple variety, were purchased in a local market in Campos Altos, State of Minas Gerais, Brazil; *Fazenda Tri "S"*, altitude of 1.132 m, in November 2017. For confirmation of the plant's botanical identity, samples of the bulbs were cultivated and the whole plant was subsequently provided to experts in Botany. A voucher specimen, (n ° 502077), was deposited at the *Herbarium Maria Eneyda P. Kaufmann Fidalgo*, São Paulo/Brazil where species identity was confirmed by Dr. Domingos Sávio Rodrigues. All study/experimental protocols involving plant materials were conducted in accordance with institutional, national, and international guidelines and legislation.

Raw Garlic Extract (RGE) and SP80 (Sep-Pak 80%)

SP80, obtained by purifying the RGE, on a Sep-Pak® C18 cartridge column, was carried out as previously described by Torres et al. [27].

Bioassays

2.3.1 Minimum Inhibitory Concentration (MIC): The SP80, reconstituted in ultrapure water, was subjected to a liquid growth inhibition test against *S. agalactiae* (ATCC®12386™) strain. The assay was performed in 96-well microplates. For that, 20 µL of SP80 was applied to each well at a serial dilution of two-fold microtiter broth dilution and added to 80 µL of culture medium TSB containing the microorganism in the logarithmic growth phase for a final concentration of 10^3 cells/mL. After 24 hours of incubation at 35 ± 2 ºC under constant agitation, the absorbance at 595 nm was measured on a Victor 3 - 1420 (Perkin Elmer®) instrument. The MIC was considered the lowest concentration that completely inhibits the growth of the microorganism. The tests were performed in triplicate and the average of 3 readings was considered [28].

2.3.2 Disk Diffusion Assay: SP80 was carried out by disc diffusion test and the concentration used was established from the MIC assay results for *S. agalactiae* (ATCC® 12386™). For antimicrobial tests were used *S. agalactiae* strain (ATCC® 12386™) and fifty-five non-duplicate clinical isolates of *S. agalactiae* from positive vaginal and rectal cultures, kindly supplied by Salomão and Zoppi available in its library. These clinical isolates were confirmed by using the CAMP (*Christie, Atkins, Munch-Petersen*) test [29].

For this purpose, ready-made Petri dishes were used with the Mueller-Hinton agar (MHA) with 5% sheep blood culture medium (150 mm), supplied by Probac, and *S. agalactiae* strains (previously prepared in saline), using alginate swabs, at a concentration of 1.5 x 10^8 CFU/mL, which corresponds to 0.5 in the MacFarland nephelometric scale. The strains of *S. agalactiae* were subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: (1) PEN (10 IU); (2) AMP (10 mcg); (3) PEN (10 IU) with 15 µL SP80; and (4) AMP (10 mcg) with 15 µL SP80. The plates, prepared as duplicates, were incubated at 35 ± 2 °C for 24 hours. The diameter of the clear zone around the disc was measured and expressed in millimeters [30-32].

Statistical Analysis

During planning, the sample size was (n) calculated based on the Analysis of Variance (ANOVA). A significance level of 5% (α) and test power of 80% (1- β) was adopted with a standard deviation of 5 units and a difference of 2 units, therefore found n = 56 samples.

The results obtained were subjected to statistical analysis by using the IBM SPSS software, version 13.0, and were considered significant ($p<0.05$). The non-parametric test used was Wilcoxon; whereas for paired data, *T-test.*

Results

The MIC of SP80 was determined, as triplicates, by employing the microdilution method. The mean and standard deviation of the results obtained was 2.40 ± 0.42 mg/mL. This concentration was used in the disk diffusion tests.

A total of 56 *S. agalactiae* strains, one standard strain (ATCC® 12386™), and 55 clinical isolates were tested against PEN, AMP, and SP80 in association with the respective antibiotics using the disk diffusion test (Figure 1a). The results are shown as mean ± standard deviation as can be seen in the graphical (Figure 1b).

The mean results obtained were compared between the antibiotics (PEN and AMP) both isolated and associated with SP80, respectively. The statistical analysis showed that the mean for AMP, when associated with SP80, increased as compared to AMP alone (*p<0.001*), a significant increase. Otherwise, the mean for PEN, when associated with SP80, decreased as compared to PEN alone (*p=0.07*), a non-significant decrease (Table 1).

Table 1. Comparison of antibiotics, both isolated and in association with SP80 against *S. agalactiae* (median: minimum – maximum).

AMP	**28.46 (23.00 - 40.00)**	***p<0.001**
SP80 + AMP	**29.04 (24.00 - 40.00)**	
PEN	**25.88 (18.00 - 40.00)**	***p=0.07**
SP80 + PEN	**25.53 (16.00 - 39.50)**	

**T-test. Significant (p<0.05).*

The inhibition halos (mm) were compared between the antibiotic combinations (SP80 + AMP and SP80 + PEN), respectively, and the statistical analysis showed that the results were significant (*p<0.001*). The association of SP80 + AMP showed a greater mean inhibition zone (mm) when compared to the mean value obtained for the association of SP80 + PEN for *S. agalactiae* (Table 2).

Table 2. Comparing antibiotics, both isolated and in association with SP80, against *S. agalactiae* (mean: minimum - maximum).

SP80 + AMP	29.04 (24.00 - 40.00)	*p<0.001
SP80 + PEN	25.53 (16.00 - 39.50)	

Wilcoxon test. Significant (p<0.05).

According to the CLSI (2015), when the inhibition halo formed by PEN and AMP is ≥ 24 mm, the strain is sensitive to the antibiotic involved. We compared the inhibition halos obtained with isolated antibiotics, and the result showed us that the strains tested are more resistant to PEN as compared to AMP (Table 3).

Table 3. Comparison of the inhibition halo (mm) of the antibiotics PEN and AMP (isolated) against *S. agalactiae*.

	N	Inhibition halo ≥ 24 mm	Inhibition halo 24 mm	
PEN	56	31 strains	25 strains	*p<0.05
AMP	56	54 strains	2 strains	*p<0.1

Inhibition halo ≥ 24 mm: sensitive;
Inhibition halo 24 mm: resistant;
Wilcoxon test. Significant (p<0.05).

Two strains of *S. agalactiae* that were resistant to the antibiotic AMP alone when tested against the association of SP80 + AMP became sensitive, as shown in figure 2.

Discussion

Garlic is one of the most researched medicinal plants in the world and has been used in traditional medicine for many centuries for the treatment of numerous diseases [33, 34]. According to Majewski [16], garlic's properties result from the combination of several biologically active substances with antimicrobial properties responsible for its healing effect that aroused our interest in studying their antimicrobial activities. To that end, we evaluated their susceptibility and drug interaction when associated with the antibiotic PEN and AMP, both of which belong to the class of β-lactams, against clinical isolates of *S. agalactiae.*

PEN and AMP were used as they are antibiotics of first choice used in the IAP of pregnant women [35]. In addition, no studies were found that evaluated the interaction of these antibiotics with garlic or the prospect of finding ways to enhance the action of these antibiotics, which could aid in the prophylaxis

and/or treatment of this infection. It is worth noting that our study is the first one of its kind to be published in the literature to date.

The complete phytochemical analyses of SP80 were carried out by Torres and collaborators [27] to isolate and identify the bioactive compounds presented in the garlic using Reverse-phase ultrafast liquid chromatography with UV spectroscopy (RP-UFLC-UV), Mass spectrometry (LC-MS) and Nuclear magnetic resonance (NMR). The compounds identified in their study were y-glutamyl-S-allyl-cysteine, y-glutamyl-phenylalanine, and (E) and (Z) -ajoenes. Therefore, the current study aimed to evaluate the synergistic activity of the association of SP80 with antibiotics PEN and AMP.

The SP80 was used instead of the isolated fractions as mentioned above, because according to Majewski [16], garlic's properties result from the combination of several biologically active substances with antimicrobial properties.

The methods most used for assessing antimicrobial activity are disk diffusion and broth microdilution [36-40]. The disk diffusion method was used because it is a quantitative method that also enables a synergistic assessment between SP80, and the antibiotics used in our study.

The MIC of SP80, obtained by purifying the RGE, was determined by the broth microdilution, in triplicate. The MIC used in our tests was the lowest value that completely inhibited microbial growth and exhibited a bacteriostatic action [34]. As our work is pioneering, different extraction methods could lead to distinct biological responses. For this reason, in our study, the MIC established for SP80 was 2.40 mg/mL. This concentration was used in the disk diffusion tests.

When determining the MIC value by using the microdilution method, it was demonstrated that this garlic fraction has antimicrobial activity against *S. agalactiae*. This is an important finding that corroborates all other studies described in the literature regarding this plant's antimicrobial activity [36, 41-44].

The association of SP80 with AMP was more efficient than the association of SP80 with PEN when compared to antibiotics alone, respectively. These results suggest that SP80 + AMP can act synergistically to inhibit the growth of GBS.

According to the CLSI [30], inhibition halos ≥ 24 mm formed by PEN and AMP demonstrate that the strain tested against these antibiotics, respectively, are sensitive. We compared the antimicrobial effect of PEN and AMP, isolated, respectively, against *S. agalactiae* strains, and of the 56 strains tested, 25 strains were resistant to PEN and another 2 were resistant to AMP, thus demonstrating that the strains tested are more resistant to PEN than they are to AMP (Table 3).

There is a dearth of information regarding the mechanism behind reduced PEN susceptibility in GBS. However, Chu *et al.* [45] believe that modification of PEN-blinding proteins is a possible explanation for the reduced PEN susceptibility seen with their GBS isolates. Nevertheless, further molecular work is needed for confirmation.

Another important finding was that the two strains of *S. agalactiae* that were resistant to the antibiotic AMP alone when tested against the association of SP80 + AMP became sensitive.

Hayes *et al*. [44] carried out checkerboard and time-kill assays *in vitro* for determining the synergistic activity of erythromycin and nisin against clinical isolates of Group B *Streptococcus* against invasive and colonizing GBS strains. Their results suggest that erythromycin and nisin can act synergistically to inhibit the growth of GBS. This study differs from ours in the antibiotics used and the methodology.

Garlic has a variety of bioactive compounds, including organosulfur compounds, saponins, phenolic compounds, and polysaccharides. The main ones are organosulfur compounds, especially allicin [46, 47]. According to Choo *et al.* [48], garlic's antibiotic activity is mainly attributable to allicin, which acts on the destruction and inhibition of Gram-positive and negative bacteria.

In the work conducted by Torres et al. [27], the authors showed that other compounds present in garlic, in addition to allicin, have antimicrobial activity and can be used against strains of *S. agalactiae*, it is enough to know how they act to inhibit these bacteria.

From this research, the SP80 from *A. sativum* L. has *in vitro* antimicrobial activity against the bacteria *S. agalactiae*, with a MIC of 2.40 mg/mL; its association with the antibiotic AMP showed a synergistic effect, which did not occur when in association with PEN.

Based on our results, we believe that further studies should be carried out to pave the way for the development of translational research, given that this drug interaction in humans can prove beneficial in the therapy for *S. agalactiae* infections.

Conclusions

The current study reveals, *in vitro*, synergy between SP80 (a combination of several biologically active substances with antimicrobial properties) and AMP against clinical GBS strains and indicates the potential of antimicrobial combination therapy for use in treating clinical GBS infections.

Declarations

Competing Interests

The authors declare no competing interests.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics declarations

The article contains no data concerning studies involving human subjects or inclusion of identifiable human data or clinical trials; thus, no ethical approval was required.

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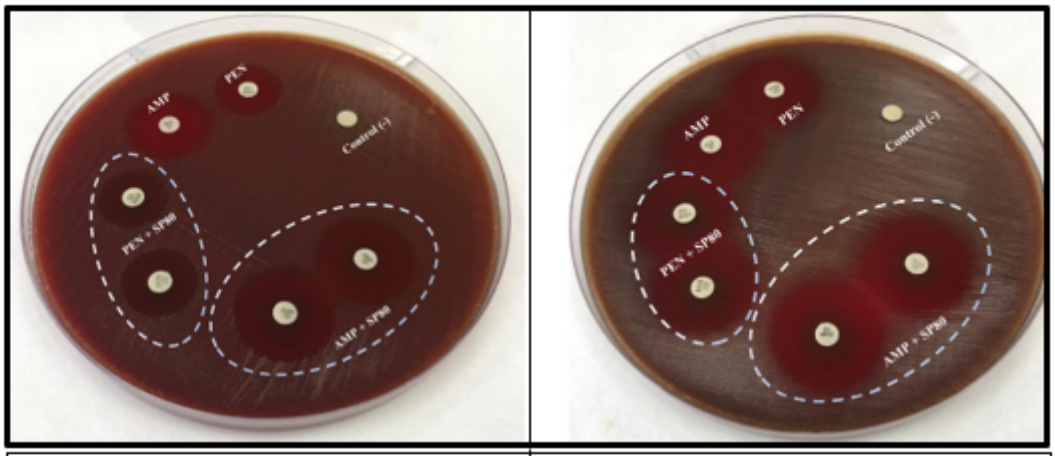
Figures

(a)



S. agalactiae (clinical isolated n.7) | *S. agalactiae* (ATCC® 12386™)

(b)

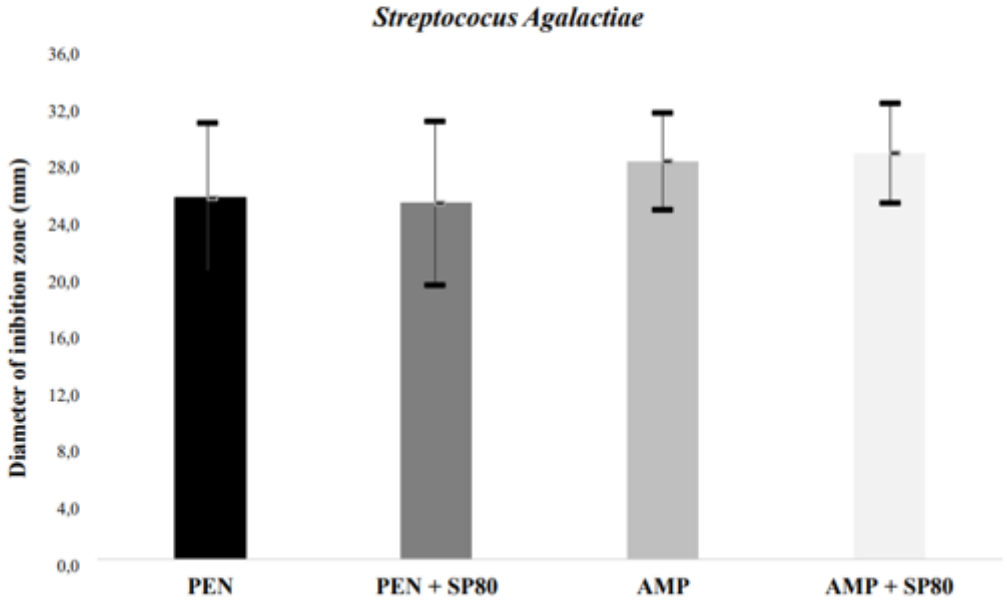


Figure 1

Antimicrobial activity of SP80 associated with penicillin G and ampicillin against *S. agalactiae*. (a) Picture of the experiment: Mueller-Hinton agar (MHA) with 5% sheep blood plate seeded with *S. agalactiae*, incubated at 35 ± 2 °C for 24 hours. The diameter of the clear zone around the disc was measured and expressed in millimeters (mm). (b) Result of inhibition halo the strains of *S. agalactiae* were subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: PEN (10 IU); AMP (10 mcg); PEN (10 IU) with 15 µL SP80; and AMP (10 mcg) with 15 µL SP80. The MIC established for SP80 was 2.40 mg/mL. The data represent mean±SD (n = 56).

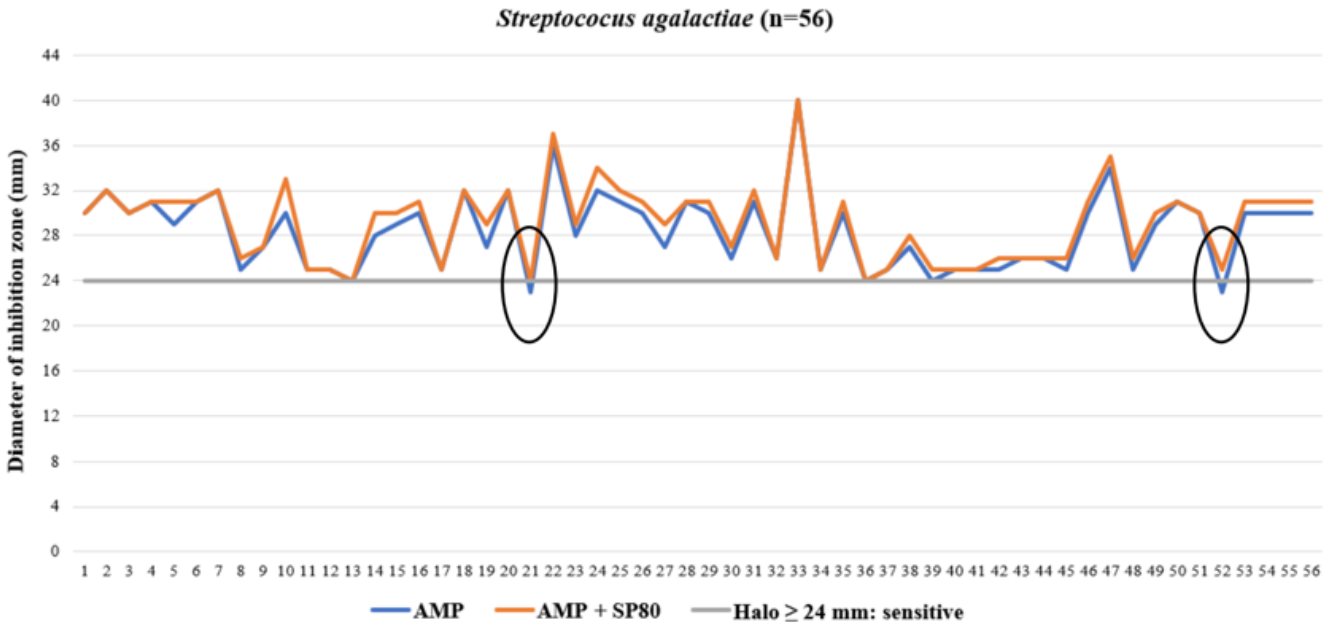


Figure 2

Compared the antimicrobial AMP and AMP + SP80, respectively, against *S. agalactiae* strains. The association of SP80 + AMP became sensitive two strains were resistant.