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# Effect of Sub-Inhibitory Doses of Gentamicin, Amoxicilin, Tsh And Amoxicillin Clavulonic on Biofilm Forming Capacity of B.Coagulans In Probiotic Prolife

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***Abstract:****Environmental conditions and processed foods which inhibit growth of probiotic bacteria and antimicrobial substances may cause alterations in human microbiota, which can lead to various diseases. Probiotics are live microorganisms that recover indiviual health and are usefull in alleviating post-antibiotic treatment syndromes. Probiotic bacteria can be ingested by consuming fermented food products, as well as probiotic treatment. Majority of the market based probiotics contain lactic acid baceria such as lactobacilli, streptococci, and bifidobacteria, due to them being a crucial component of the gastrointestinal microbiota. Bacterial strain of B. coagulans MTCC 5260, obtained from the commercially available probiotic Prolife was used to test the effect of subinhibitory doses of antibiotics amoxicillin, amoxicillin clavulonic, gentamicin and TSH on biofilm forming capacity of the bacterium, by the modification of TCP method. The study has proven that the antibiotics amoxicillin and amoxicillin clavulonic are the optimal choice to be used together with aforementioned probiotic.*

***Key Word****:**probiotics; antibiotics; Lactobacilli; biofilm; MIC*

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## I. Introduction

Complex microbiota residing within the gastrointestinal tract is effective in preventing various diseases. Any alteration in the composition of the normal gut microbiota may cause susceptibility to disease and reduce the efficiency of food digestion [1]. Alterations may be caused due to environmental conditions, processed foods that prevent probiotic bacterial growth and antibacterial substances [2]. Probiotics are living microorganisms that improve individual health and are effective in alleviating post-antibiotic treatment syndromes [3]. The introduction of probiotics into the organism can occur via consummation of fermented food products or the establishment of probiotic treatment [1]. Most probiotics on the market are based on lactic acid bacteria [LAB] such as *lactobacilli*, *streptococci*, and *bifidobacteria*, which are recognized as crucial components of the gastrointestinal microbiota [3]. Biofilms are a consortium of bacterial communities adhered together via a sticky extracellular matrix, which also allows for the adherence to different types of surfaces. Probiotic bacteria use biofilm attachment, specifically to the mucosa layer of the intestine, as a survival strategy against the harsh environment within the gastrointestinal tract [5].

Although there have been several improvements in gastrointestinal infection treatment, the number of antibiotic-resistant pathogens has increased. Thus, there is a major concern about how to treat such infections [6, 7]. There is a recent large emphasis on the therapeutical use of probiotic microorganisms in order to treat gastrointestinal disease and in combination with antibiotics in treatment of different medical conditions. The goal of this treatment is to increase the number of probiotic bacteria and reestablish normal gut microbiota. *Lactobacillus* and *Bifidobacterium* are often implemented due to their resistance towards gastric acid, bile salts, and pancreatic enzymes. Additionally, they are capable of adhering to the intestinal mucosa and can effectively colonize the intestinal tract [8, 9]. *B. coagulans* is a regularly implemented therapeutic as it is a transient colonizing probiotic bacterium that frequents the intestines. After oral administration, it arrives in the stomach as a spore and the acidic pH causes absorption of water, which instigates germination. In the duodenum, the spores germinate and rapidly multiply [10].

Likewise, antibiotics function as inter-microbial signals at minimal inhibitory concentrations. Sub-inhibitory concentrations are found to interfere with the cell regulation system of bacteria including quorum sensing regulators. Quorum sensing is a communication pathway between cells mediated by signaling molecules that trigger the expression of a defined set of genes. These genes usually encode biofilm formation processes, cell motility and antibiotic production [1].

The aim of this paper is to determine the effect of sub-inhibitory doses of antibiotics on the biofilm forming capacity of *B. coagulans.*

## II. Material and Methods

1.Bacterial strain

The used bacterial strain included *B. coagulans*MTCC 5260 from the commercially available probiotic Prolife. The bacterial strain of *B. coagulans*MTCC 5260 was revived upon which it was grown on Muller Hinton agar overnight at 37°C. The bacterial density was adjusted to 0.5 M McFarland standard.

2.Antibiotics

The tested antibiotics included: AmoxicilinClavulonic: Panklav (500 mg + 125 mg) (HEMOFARM), Amoxicillin: 500 mg (HEMOFARM), and TSH: 400 mg sulfamethoxazole + 80 mg trimethoprim, (ROCHE).

3. MIC determination

Minimal inhibitory concentration (MIC) for all tested antibiotics was determined using the broth dilution method [14]. The tested antibiotic concentrations are presented in Table 1.

4. Biofilm formation in the presence of sub-inhibitory doses of antibiotics

**Table no 1**: 23 antibiotic concentrations of each tested antibiotic

| Antibiotic concentrations (μ/ml) |
|---|
| 3072 |
| 1536 |
| 768 |
| 384 |
| 192 |
| 96 |
| 48 |
| 24 |
| 12 |
| 6 |
| 3 |
| 1.5 |
| $7.5 \times 10^{-1}$ |
| $3.75 \times 10^{-1}$ |
| $1.875 \times 10^{-1}$ |
| $9.375 \times 10^{-2}$ |
| $4.6875 \times 10^{-2}$ |
| $2.34375 \times 10^{-2}$ |
| $1.171875 \times 10^{-2}$ |
| $5.85938 \times 10^{-3}$ |
| $2.92969 \times 10^{-3}$ |
| $1.46484 \times 10^{-3}$ |

Table 1 depicts 23 subinhibitory concentrations of each of the tested antibiotics.

Effect of subinhibitory doses of antibiotics of *B. coagulans.*

The effect of various antibiotic subinhibitory dosages on *B. coagulans*biofilm formation was evaluated using a modification of the TCP method [2, 16]. *B. coagulans*, with a density of 0.5 McFarland standard, were grown in TSB in the presence of decreasing concentrations of antibiotics (amoxicillin clavulonic, amoxicillin and TSH) below their MIC. Upon 24 hours of incubation at 37°C, the plates were washed and stained with 0.1% crystal violet. Additionally, 96% ethanol was applied as a solvent for crystal violet prior to spectrophotometric reading at 595 nm. TSB containing a verified antibiotic concentration was implemented as a negative control and TSB with *B. coagulans*was utilized as a positive control. Biofilm formation in the presence and absence of antibiotics was evaluated.

**Statistical analysis**

Data was analyzed using IBM SPSS version 20 (SPSS Inc., Chicago, IL). One-wayanova test and Tukey Kramer test was used to ascertain the significance of differences between biofilm forming capacity of each of antibiotics.Figure 1 illustrates the effect of subinhibitory concentrations of antibiotics on biofilm formation by *B. coagulans.* As can be seen from the figure, the biofilm forming capacities differ in antibiotics.

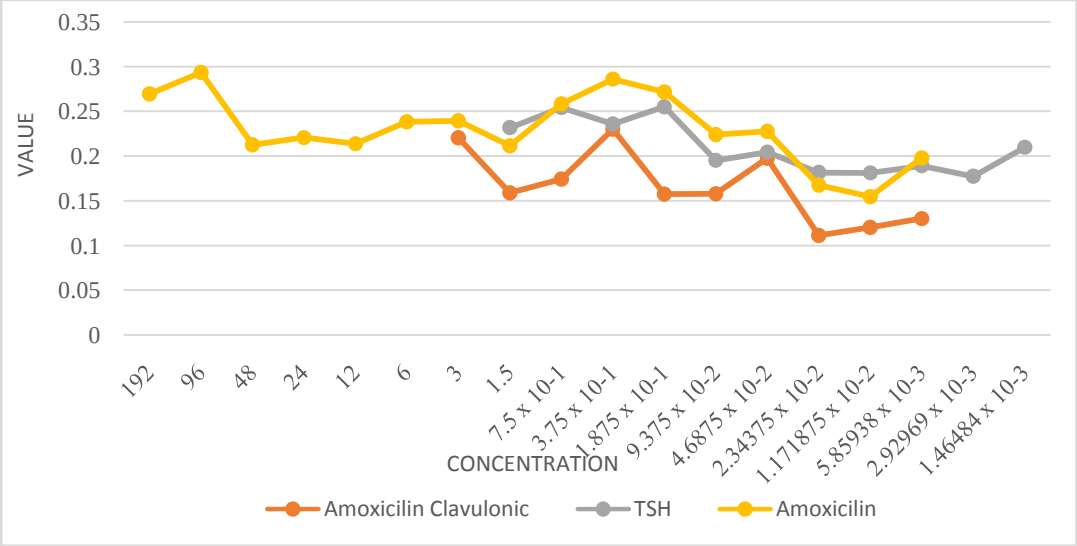


**Figure no 1**: The effect of sub-inhibitory concentrations on biofilm formation by *B. coagulans*

**Table no 2**: Numbers of biofilms formed, and the categories to which they belong.

| **Biofilm Category** | **Number of Biofilm Formations** | **Percentage** |
|---|---|---|
| Non-Adherent | 6 | 16.21% |
| Weakly Adherent | 31 | 83.78% |
| Moderately Adherent | 0 | 0.00% |
| Strongly Adherent | 0 | 0.00% |
| Total | 37 | 100.00% |

Table 2 presents the numbers of biofilms formed, and the consequent categories to which they belong. Biggest number of biofilms formed were weakly adherent, with 83.78%, followed by non-adherent, with 16.21%. There were no strongly adherent biofilms formed.

**Table no 3**: Summary of statistical data obtained for every antibiotic used.

| **Summary of Data obtained** | | | | |
|---|---|---|---|---|
| | ***Antibiotics*** | | | |
| | TSH | Amoxicilin | AmoxicilinClavulonic | Total |
| Number of samples | 11 | 16 | 10 | 37 |
| ∑X | 2.3142 | 3.6861 | 1.6571 | 7.6573 |
| Mean | 0.2104 | 0.2304 | 0.1657 | 0.2070 |
| $\sum X^2$ | 0.4953 | 0.8722 | 0.2893 | 1.6568 |
| Std.Dev. | 0.0291 | 0.0391 | 0.0404 | 0.0448 |

Table 3 present the statistical data obtained for every antibiotic used.

**Table no 4**: The results obtained by One-way anova test.

| **Results Details obtained** | | | | |
|---|---|---|---|---|
| ***Source*** | ***Sum of Squares*** | ***Degrees of Freedom*** | ***Mean Square*** | |
| Between-Antibiotics | 0.0259 | 2 | 0.0130 | *F* = 9.5429 |
| Within-Antibiotics | 0.0462 | 34 | 0.0014 | P = 0.0005 |
| Total | 0.0721 | 36 | | |

Table 4 presents the results that are obtained by One-way anova test.

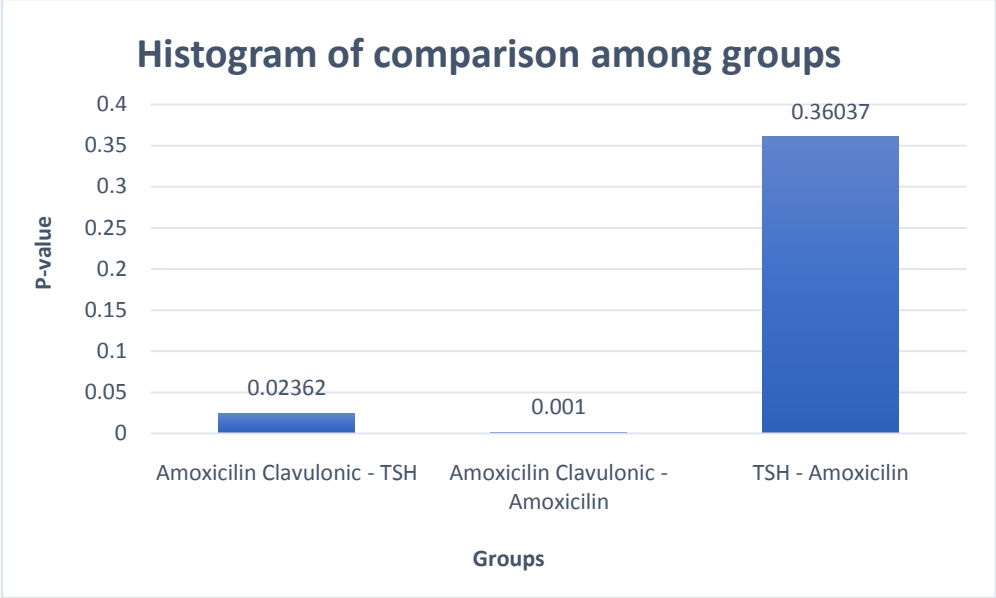


**Figure no 2**: Histogram of comparison among groups

Figure 2 presents a histogram in which the groups and P values between each tested group are presented. The difference between biofilm forming capacity of different antibiotics was observed, and the statistically significant results are obtained between TSH – AmoxicilinClavulonic and AmoxicilinClavulonic – Amoxiclin.

Since one-way anova test determined that there is statistical difference among mean biofilm forming capacity for different antibiotics, Tukey Kramer test was used to determine if the comparison of biofilm forming capacity of two antibiotics is significant.

**Table no 5**: The results of Tukey Kramer test

| Comparison | Tukey p-value | Significant/Insignificant |
|---|---|---|
| AmoxicilinClavulonic - TSH | 0.02362 | Significant |
| AmoxicilinClavulonic - Amoxicilin | 0.00100 | Significant |
| TSH - Amoxicilin | 0.36037 | Insignificant |

*p<0.05 statistically significant

Table 5 presents comparison of groups of antibiotics used in this research.

The significant difference was observed in comparing TSH and amoxicillin clavulonic, with a p value of 0.02362 as presented. Statistical difference was also observed between Amoxicilinclavulonic and amoxicillin with a p value of 0.00100.

## III. Result

**Effect of antibiotic Amoxicilin-clavulonic on biofilm forming capacity**

**Table no 6**: Effects of different sub-inhibitory concentrations of amoxiclin-clavulonic on biofilm forming capacity of *B. coagulans*

| **Concentration (µg/ml)** | **Mean OD** | **Biofilm Category** |
|---|---|---|
| Mean Negative | 0.102 | Non adherent |
| Mean Positive | 0.04775 | Non adherent |
| 3 | 0.22033 | Weakly adherent |
| 1.5 | 0.15875 | Weakly adherent |

| | | |
|---|---|---|
| 7.5 x $10^{-1}$ | 0.17425 | Weakly adherent |
| 1.875 x $10^{-1}$ | 0.23 | Weakly adherent |
| 4.6875 x $10^{-2}$ | 0.15725 | Weakly adherent |
| 2.34375 x $10^{-2}$ | 0.15775 | Weakly adherent |
| 1.171875 x $10^{-2}$ | 0.1975 | Weakly adherent |
| 5.85938 x $10^{-3}$ | 0.111166 | Non adherent |
| 2.92969 x $10^{-3}$ | 0.120083 | Non adherent |
| 1.46484 x $10^{-3}$ | 0.13 | Non adherent |

MIC was observed at a concentration of 12 µg/ml. The positive control indicates non adherent biofilms formation of *B.coagulans* in the absence of the antibiotic. At concentrations ranging from 3 to 1.171875 x $10^{-2}$µg/ml,weakly adherent biofilms were formed until it reached non adherent at a concentration of 5.85938 x $10^{-3}$ µg/ml.

**Effect of antibiotic TSH on biofilm forming capacity**

**Table no 7**: Effects of different sub-inhibitory concentrations of TSH on biofilm forming capacity of *B. coagulans*

| **Concentration (µg/ml)** | **Mean OD** | **Biofilm Category** |
|---|---|---|
| Negative Control | 0.127 | Non adherent |
| Positive Control | 0.242 | Weakly adherent |
| 1.5 | 0.231583 | Weakly adherent |
| 7.5 x $10^{-1}$ | 0.254 | Weakly adherent |
| 3.75 x $10^{-1}$ | 0.235583 | Weakly adherent |
| 1.875 x $10^{-1}$ | 0.255 | Weakly adherent |
| 9.375 x $10^{-2}$ | 0.195 | Weakly adherent |
| 4.6875 x $10^{-2}$ | 0.20433 | Weakly adherent |
| 2.34375 x $10^{-2}$ | 0.181666 | Weakly adherent |
| 1.171875 x $10^{-2}$ | 0.1810833 | Weakly adherent |
| 5.85938 x $10^{-3}$ | 0.189 | Weakly adherent |
| 2.92969 x $10^{-3}$ | 0.17725 | Weakly adherent |
| 1.46484 x $10^{-3}$ | 0.20966 | Weakly adherent |

The MIC was determined at 3072 µg/ml concentration. The positive control indicates weak biofilm formation in the absence of the antibiotic. At concentrations ranging from 1.5to 1.46484 x $10^{-3}$µg/ml, aweak biofilm was formed.

**Effect of antibiotic Amoxicillin on biofilm forming capacity**

**Table no 8**: Effects of different sub-inhibitory concentrations of amoxicillin on biofilm forming capacity of *B. coagulans*

| **Concentration (µg/ml)** | **Mean OD** | **Biofilm Category** |
|---|---|---|
| Mean negative | 0.15825 | Non adherent |
| Mean positive | 0.34575 | Weakly adherent |
| 192 | 0.2693333 | Weakly adherent |
| 96 | 0.29325 | Weakly adherent |
| 48 | 0.212666 | Weakly adherent |
| 24 | 0.2205833 | Weakly adherent |
| 12 | 0.21375 | Weakly adherent |
| 6 | 0.23833 | Weakly adherent |
| 3 | 0.23933 | Weakly adherent |
| 1.5 | 0.2115 | Weakly adherent |
| 7.5 x $10^{-1}$ | 0.258333 | Weakly adherent |
| 1.875 x $10^{-1}$ | 0.28591666 | Weakly adherent |
| 9.375 x $10^{-2}$ | 0.271333 | Weakly adherent |
| 4.6875 x $10^{-2}$ | 0.224 | Weakly adherent |
| 2.92969 x $10^{-3}$ | 0.22758333 | Weakly adherent |
| 1.171875 x $10^{-2}$ | 0.167666 | Non adherent |
| 5.85938 x $10^{-3}$ | 0.154666 | Non adherent |

| | | |
|---|---|---|
| $2.92969 \times 10^{-3}$ | 0.1978333 | Non adherent |

For amoxicillin MIC was determined at the concentration of 384 μg/ml. The positive control indicates weak biofilm formation in the absence of the antibiotic. At concentrations below MIC, ranging from 192 to $2.92969 \times 10^{-3}$ μg/ml, a weak biofilm was formed. A non-adherent biofilm was formed in the range from $1.171875 \times 10^{-2}$ to $2.92969 \times 10^{-3}$ μg/ml.

## IV. Discussion

Human microbial flora is an extremly important part of human immune system as it aids the organism to fight against various diseases and pathogens. It is self renawable however can be disrupted by usage of antibiotics [3]. Antibiotics are used to treat different inflammations and medical conditions that commonly occur in the digestive system. Besides inhibiting growth of pathogenic microorganisms, antibiotics also decrease the number of probiotic bacteria usually found in the gut [6, 9]. In order to reestablish the normal microbial flora in the gut, probiotics are used. Generally, during treatments with antibiotics, probiotics are consumed as well to maintain balance [1, 2, 8]. This research was conducted in order to analyze effect of antibiotics, that are commonly prescribed for inflammation of gastrointestinal tract, on *B. coagulans*, a probiotic microorganism. By diluting the antibiotics to various concentrations, the formation of biofilm by *B. coagulans* was measured.

The biofilm formation by *B. coagulans* was tested in the presence of amoxicillin-clavulonic, amoxicillin and TSH. At the sub-inhibitory concentrations of amoxicillin-clavulonic, the antibiotics served as a signaling molecule that triggered an increase in biofilms formation by B.coagulans to weakly adherent. The lower concentration of antibiotic did not trigger an increase in biofilm formation as it was non adherent, as can be seen in table 6. Upon emergence of the resistant bacteria to amoxicillin by production og beta-lactamase, addition of clavulanic acid has solved the problem. It is a irreversible inhibitor of intracellular and extracellular beta-lactamase. Alone it has only weak antibacterial activity, except *Legionella spp.*, but increases susceptibility of a Gram-positive and Gram-negative bacteria to amoxicillin [21]. Drago et al, reported decrease of biofilm formation of *S. Pneumoniae* by amoxicilli-clavulanic [22].

MIC of TSH was determined as 3072 μg/ml and the sub-inhibitory concentrations of this antibiotic did not provide a signal for the B.coagulans to increase or decrease biofilm formation, as represented in table 7. TSH has been successful in treating gram-positive and gram-negative bacteria in infectionsof gastrointestinal,genitourinary and respiratory tract [17].

With the usage of amoxicillin (table 8), the biofilm formation was weak at the concentrations below the MIC. Eventually, it decreased to non adherent as the antibiotic concentration, as a signaling molecule, decreased. This antibiotic is a member of beta-lactam group that have beta-lactam ring. This ring is essential for antibacterial activity, since this antibiotic inhibits cell-wall synthesis [18]. Amoxicillin is reported as effectively absorbed antibiotic that remains in the intestinal tract in trace amounts. Due to its mode of action, less diarrhea occurence is reported in patients treated with amoxicillin than in ones being treated with ampicillin as another beta-lactame drug [19]. Kaplan et al, stated that subminhibitory concentration of four different beta-lactam antibiotics increase biofilm formation in certain strains of *S.aureus* [20]. It was stated that patterns of induced biofilm formation depend on strain and antibiotic [20].

Using one-way anova and Tukey Kramer tests, statistical signifance was determined for every antibiotic used. Table 3 present summary of statistical data obtained for every antibiotic used, and table 4 presented results from One-way anova. As can be seen in figure 2, which presents a histogram of the p values for each tested group, statistically significant results are obtained between TSH– AmoxicilinClavulonic and Amoxicilin – AmoxicilinClavulonic. Table 5 presents comparison of groups of antibiotics used in this research, obtained by Tukey-Kramer. The p value for comparing TSH and Amoxicillin-Clavulonic was 0.02362, and the p value comparing Amoxicilin and AmoxicilinClavulonic was 0.00100, as presented in table 5. The figure 1 presents effect of sub-inhibitory doses of different antibiotic concentrations on biofilm formation by *B. coagulans.*

According to these results, it can be concluded that among tested antibiotics, amoxicillin and amoxicilllin-clavulonic are a optimal choice to be used together with probiotic bacteria B.coagulans in treating various gastrointestinal conditions. What is more, there is a statistically significant difference in usage of TSH and amoxicilin clavulonic as well as amoxicillin and amoxicillin clavulonic combinations.

The limitations of this study are the usage of only three antibiotic substances and one strain of probiotic bacteria. This research can be further improved by testing biofilm forming capacity of different probiotic strains of bacteria in presence of different antibiotic substances. What is more, by testing various antibiotic substances and probiotic bacteria it would be easier to understand interaction between sub-inhibitory doses of antibiotics with probiotic bacteria which would eventually provide advantages in treating various medical conditions.

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