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Effect of metals on antibiotic sensitivity, growth, and biofilm-forming capacity of *B. subtilis subsp. spizizenii*

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***Abstract – B. subtilis* is normally considered a soil organism, it can be also found in the animal and human gastrointestinal tract. *Bacillus subtilis subsp. spizizenii* is a type of *Bacillus subtilis* complex. It shares up to 99% of homology with *B. subtilis CU1*, which can be represented as a probiotic strain. Metal compounds found in soil or used in agriculture can easily enter the food chain and end up in our gut. Gram-positive bacteria (e.g. *Bacillus spp*.) have good adsorptive capacity for metals due to high peptidoglycan and teichoic acid content in cell walls. There is some evidence that certain metals inside the intestine play an important role in influencing growth and functionality of specific probiotic strains. Some of them have inhibitory, while others have an activating effect on bacteria. This study revealed that metal compounds increased antibiotic susceptibility of *B. subtilis subsp. spizizenii*. Higher concentrations of metal solutions inhibited growth of tested bacteria. Culture did not show affinity to form biofilms before or after addition of metal solutions.**

Keywords – antibiotic susceptibility, biofilms, MIC, metals.

1. Introduction

Various bacteria reside in the gut or arrive there by food consumption. A microbiome is the overall collection of the genetic material of all microorganisms that live on or inside our body or collection of the genetic material of microorganisms in a particular environment (e.g., in your gut). Bacteria within our gut have an important role in digesting food, modulating the immune system, providing protection against harmful microbes, and more. Multiple factors including genotype, antibiotics, mode of delivery, dietary habits, lifestyle, social interactions and environmental factors shape the gut microbiota to make everyone's microbiome unique [1, 2, 3]. Metal compounds can cause alterations in the composition of the gut microbiota. Usually, decrease in richness as well as the diversity of gut microbiota, is observed after exposure to metals [4, 5]. Gram-positive bacteria (e.g. Bacillus spp.) have good adsorptive capacity for metals due to high peptidoglycan and teichoic acid content in cell walls, in contrast to Gram-negative bacteria [6]. The phylum Firmicutes found in colon is mostly composed of gram-positive species, such as Clostridium and Bacillus. There is some evidence that certain metals inside the intestine play an important role in influencing growth and functionality of specific probiotic strains. Some of them have inhibitory, while others have an activating effect on bacteria. It has been concluded that many effects of metals are strain-specific [7].

Bacillus subtilis is a gram positive and catalase positive rods. It is spore-forming bacteria. Although normally considered a soil organism, it is also found in the animal and human gastrointestinal tract [8]. *Bacillus subtilis subsp. spizizenii* is a type of *Bacillus subtilis* complex. It shares up to 99% of homology with *Bacillus subtilis CU1*, which can be represented as a probiotic strain that can have specific outcomes on the immune system of the elderly [9, 10]. Probiotics are commensal bacteria in the gut that have a health beneficial effect on the host organism. However, there are still a few unresolved questions regarding the safety of certain *Bacillus strains*, which is the main reason for their still limited application as probiotics [11, 12].

Biofilms are communities of bacteria joined together by a sticky extracellular matrix. This extracellular matrix is also responsible for adherent biofilms to various surfaces. Probiotic bacteria in the gut also use biofilm attachment to bind to the mucosa layer of the intestine. Biofilm attachment improves their survival rate. Specifically, biofilms provide protection against antibiotics and enzymes [13, 14, 15].
Antibiotics are antimicrobial agents active against bacteria. Their mode of action can be bactericidal or bacteriostatic. Application of antibiotics influences intestinal microbiota. It affects growth, diversity and antibiotic resistance of bacteria. Since *Bacillus subtilis* are partially considered as probiotic bacteria, normally found in the human gastrointestinal tract, this study will show their antibiotic susceptibility in the presence of metal compounds that can end up in our gut via food intake [16, 17].
In this paper, the effect of metal compounds on biofilm forming capacity, bacterial growth, and changes in antibiotic sensitivity is examined. It is assumed that metal compounds would increase antibiotic sensitivity and suppress growth.

2. Methods

1. Cultivation of *B. subtilis subsp. spizizenii* strain

Bacillus subtilis subsp. spizizenii (ATCC 6633) was cultivated on solid and liquid media (trypticase soy broth (TSB) broth, TSB agar). After overnight incubation at 37 C, the turbidity of bacterial density is adjusted to 0.5 McFarland standard, as such was used for further tests.

2. Determination of antibiotic susceptibility before the addition of metal supplements

Bacteria is previously cultivated on TSB agar. Susceptibility to fifteen types of antibiotics will be performed using the standard Kirby-Bauer disk diffusion method [18]. Antibiotics (Liofilchem) are listed in Table 1 below.

3. Microbroth dilution method

Microbroth dilution method will be used to determine the minimal dose of metal supplement necessary to inhibit the growth of bacteria (minimum inhibitory concentration - MIC). It is accomplished through the standardized broth microdilution assay procedure [19, 20]. 96-well microtiter plates were used. The metal salts were aseptically diluted in TSB broth in the following w/V solutions: 1%, 0.5%, 0.25%, 0.12%, 0.06%, 0.03%, 0.015%, 0.007%, 0.003%, 0.0018%, 0.0009%. The 96-well plate contained 100 ul of different concentrations of metal solutions ($CuSO_4$, $ZnSO_4$ x $7H_2O$, $Fe(NO_3)_3$, and Mg), 100 ul TSB broth, and 20 ul of *B. subtilis subsp. spizizenii* (0.5 McFarland standard). This test was done in triplets. The

purpose was to determine the exact concentration of each metal that inhibits bacterial growth. After overnight incubation at 37 C visible growth of bacteria is recorded and MICs have been determined.

4. Determination of biofilm forming capacity

This test determines how different concentrations of $CuSO_4$ - copper (II) sulfate pentahydrate (Sigma-Aldrich), $ZnSO_4$ x $7H_2O$ - zinc sulfate heptahydrate (Sigma-Aldrich), $Fe(NO_3)_3$ - iron (III) nitrate (Fisher Scientific), and magnesium complex (Twinlab - dietary supplement from local pharmacy) will facilitate the biofilm formation. This test will be performed using TCP method. The 96-well plate contained different concentrations of metals, TSB medium, and 20 ul of *B. subtilis subsp. spizizenii* (0.5 McFarland standard). The inoculated plate should be covered with a lid and incubated for 24 h at 37 C. After incubation the content of the plates is discarded and washed. Crystal violet assay is used as a method of indirect biofilm quantification. Each microtiter-plate well is stained with 120 ul of 0.1% crystal violet and set aside for 10 minutes. Microliter-plate is decanted again and washed with distilled water. The test is done in triplets [21, 22].

5. Determination of antibiotic susceptibility after addition of metals

Susceptibility to fifteen types of antibiotics (Table 1) after addition of metal solutions will be performed using the Kirby-Bauer disk diffusion method [18].

Table 1. List of fifteen antibiotic discs used for antibiotic susceptibility testing.

Name of antibiotic	Micrograms	Abbreviation
Cefoxitin	30	FOX30
Gentamicin	10	CN10
Oxacillin	1	OX1
Amoxicillin	10	AML10
Ceftazidime + clavulanic acid	40	CAL40
Ciprofloxacin	5	CIP5
Streptomycin	10	S10
Vancomycin	30	VA30
Erythromycin	15	E15
Ceftazidime	10	CAZ10
Amoxicillin-clavulanic acid	30	AUG30
Azithromycin	15	AZM15
Kanamycin	30	K30
Tetracycline	30	TE30
Ampicillin	2	AMP2

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3. Results

After testing the effect of metal compounds on growth, antibiotic susceptibility, and biofilm forming capacity, the following results were obtained.

Table 2 shows results obtained after performing antibiotic susceptibility test for *B. subtilis subsp. spizizenii.* It compares diameters of the inhibition zone (in millimeters), before and after addition of four different metal compounds.

Table 2. Antibiotic susceptibility test for *B. subtilis subsp. spizizenii*. Diameter of the zone of inhibition is in millimeters. (* - partially bactericidal)

	B. spizizenii	***B. spizizenii* + Mg**	***B. spizizenii* + $ZnSO_4$ x $7H_2O$**	***B. spizizenii* + $Fe(NO_3)_3$**	***B. spizizenii* + $CuSO_4$**
FOX30	25	28	27	28	24
CN10	20	22	21	20	21
OX1	15	18	18	17	15
AML10	9	12*	15*	14*	11
CAL40	0	0	0	0	0
CIP5	29	32	32	32	35
S10	19	20	18	19	19
VA30	18	21	20	20	20
E15	21	25	28	24	25
CAZ10	0	0	6	8	0
AGU30	21	23	25	24	22
AZM15	20	22	21	23	22
K30	22	24	24	23	24
TE30	26	31	29	30	27
AMP2	0	0	0	0	0

Since diameters of inhibition zones for fifteen antibiotics were measured manually, Figure 1 visualizes sizes of diameters and possible manual errors during the measurement process.

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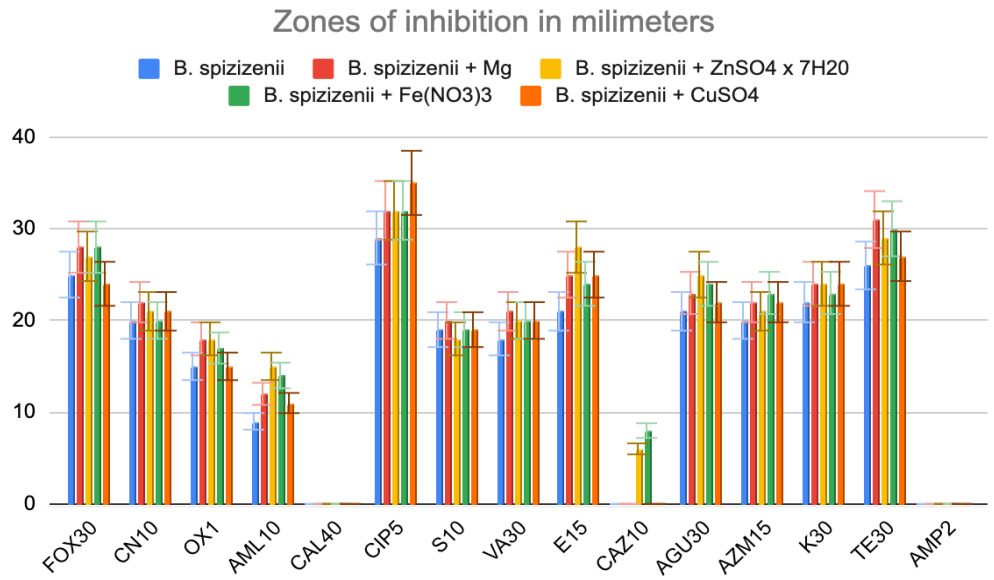

Figure 1. Antibiotic susceptibility to fifteen antibiotics measured by zone of inhibition (in millimeters).

Results of microbroth dilution tests are presented in Table 3 and Figure 2. Table 3 shows how different concentrations of metal (w/V) solutions affect growth of *B. subtilis subsp. spizizenii*, while minimum inhibitory concentrations of metals are summarized in Figure 2.

Table 3. Growth of *B. subtilis subsp. spizizenii* under different concentrations of metal solutions

w/V solution	Mg	$Fe(NO_3)_3$	$CuSO_4$	$ZnSO_4$ x $7H_20$
1%	No growth	No growth	No growth	No growth
0.5%	No growth	No growth	No growth	No growth
0.25%	No growth	Growth	No growth	No growth
0.12%	No growth	Growth	No growth	No growth
0.06%	Growth	Growth	No growth	No growth
0.03%	Growth	Growth	Growth	No growth
0.015%	Growth	Growth	Growth	Growth
0.007%	Growth	Growth	Growth	Growth
0.003%	Growth	Growth	Growth	Growth
0.0018%	Growth	Growth	Growth	Growth
0.0009%	Growth	Growth	Growth	Growth

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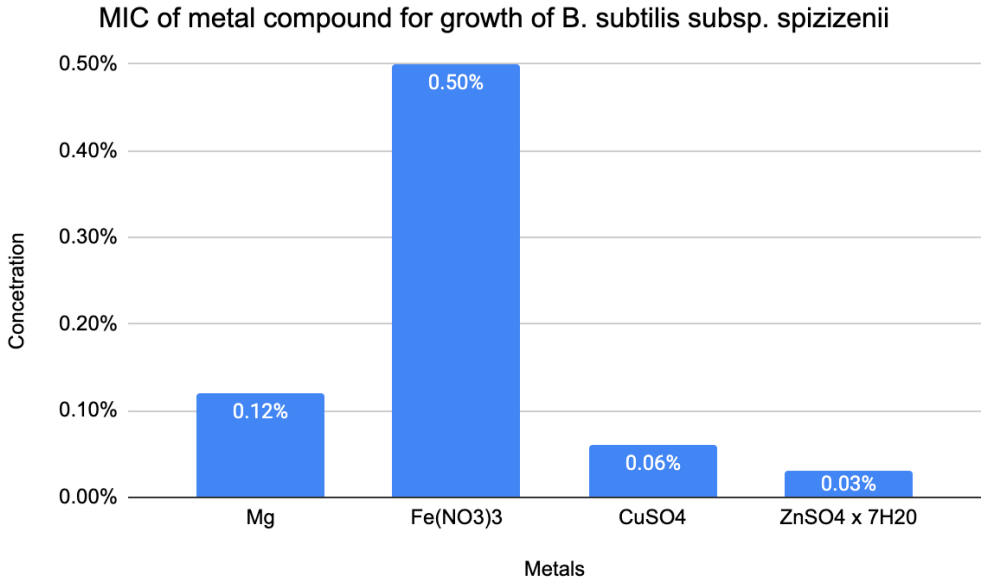

Figure 2. Minimum inhibitory concentration (MIC) of metal compound for growth of *B. subtilis subsp. spizizenii*

B. subtilis subsp. spizizenii did not show affinity to form biofilms before (visible to the naked eye) or after addition of metal solutions at any concentration (w/V). The limitation of this study might be that the optical density of each microplate was not measured using ELISA reader.

4. Discussion

In order to test the antibiotic sensitivity and growth, for this particular experiment, different metals had been taken to test this effect. In this particular experiment, one of the metals that had been used was zinc sulfate, a specific solid that can have a colorless crystalline structure. In a historical approach, it is known that zinc could be found in soil where different plants are harvested, but in different areas there is something known as solid deficiency, where plants cannot develop properly and grow because of the lack of zinc. And in order for this to be corrected, people have experimented and found out that in order to correct this deficiency, zinc sulfate can be added to the soil in order to have the proper growth of different crops. Because these metals are used in order to grow crops, this may have a different effect when the crops are consumed as a food source. [23, 24, 25].

Copper (II) sulfate pentahydrate is most commonly described as an inorganic compound that could be found in copper in the form of salt. It is highly soluble in water. This type of salt has a usage as an additive in order to recover pentose sugars from the fronts of palm oils. It had been used as well to prove specific antimicrobial properties when working with specific types of bacteria, but most importantly here with *Bacillus subtilis* [26, 27]. Copper (II) sulfate is used as fungicide in agriculture, as an additive for fertilizers and food [28].

Iron (III) nitrate, or in other words ferric nitrate, is a type of metal that can be used in many fields. This type of compound can be used to treat different sludges and wastewaters, it can be used to remove nitrogen from different plants and it can also be used in analytical chemistry [29, 30].
All three of metals aforementioned, zinc sulfate, copper (II) sulfate pentahydrate, and iron (III) nitrate, can be found in soil or are used in agriculture. In that way they can get into the food chain and enter the human gut.

One of the most abundant minerals that are important for different metabolic processes in the human body is magnesium. It can be found in over 300 enzymes as a cofactor and it regulates different biochemical reactions that are processed in the human body. Usually, magnesium is provided as a type of dietary supplement, people consume it in order for their body to function properly, and different amounts of these minerals are given to people based on various factors [31]. For example, magnesium citrate helps with constipation, it acts as laxative, while magnesium aspartate is important for digestion of macronutrients [32].

B. subtilis subsp. spizizenii showed visible growth at 0.06% (w/V) magnesium solution (Table 3). Dietary supplement was used as a source of magnesium. No significant changes were recorded in antibiotic susceptibility tests in presence of Mg, except with amoxicillin. Addition of Mg solution slightly changed property of *B. subtilis subsp. spizizenii*. According to obtained results amoxicillin was partially bactericidal (a few colonies appeared within the inhibition zone) for tested bateria, in the presence of magnesium.

Susceptibility to fifteen types of antibiotics (Table 1), before and after addition of metal solutions, will be performed using Kirby-Bauer disk diffusion method. This test showed that *B. subtilis subsp. spizizenii* is completely resistant to ampicillin (AMP2), as well as to ceftazidime+clavulanic acid (CAL40).
Antibiotic sensitivity of *B. subtilis subsp. spizizenii* did not significantly change for the following antibiotics: gentamicin (CN10), streptomicin (S10), vancomycin (VA30), azithromycin (AZM15), kanamycin (K30), cefoxitin (FOX30), oxacillin (OX1), ciprofloxacin (CIP5). Change in diameter was less or equal to 3 mm. Note that diameters were measured manually, and manual errors (gross errors) should be taken into account.

Difference in diameter of zone inhibition of erythromycin (E15) with addition of zinc sulfate heptahydrate and without metal solution is 7 mm. There was an increase in diameter size of the inhibition zone for tetracycline (TE30) and amoxicillin (AML10) in presence of magnesium, zinc sulfate heptahydrate, and iron (III) nitrate solutions, compared to diameters of inhibition zones before addition of metal compounds. Besides that, a few colonies of bacteria were observed within amoxicillin zones of inhibition. Amoxicillin was partially bactericidal for *B. subtilis subsp. spizizenii*, in presence of magnesium, zinc sulfate heptahydrate, and iron (III) nitrate solutions.

B. subtilis subsp. spizizenii without presence of metal solutions was resistant to ceftazidime (CAZ10). With addition of zinc sulfate heptahydrate, and iron (III) nitrate solutions, zones of inhibition were 6 and 8, respectively.

Since *B. subtilis subsp. spizizenii* shares the biochemical similarities with *Bacillus subtilis subsp. subtilis* results for these two strains can be compared. There is up to 58 to 68% is the DNA relatedness between these two bacteria [10, 33, 34]. According to Silman *et al*. vancomycin showed great bactericidal effect for *B. subtilis* in general [35]. Our data shows that zones of inhibition obtained by vancomycin (VA30) are ~20 mm, while the largest zones of inhibition were recorded in presence of ciprofloxacin (CIP5) ranging from 32-35 mm in diameter (Figure 1). Sim *et al.* obtained similar results about CIP5 and TE30, where zones of inhibition were 32 and 31, respectively [36].

Bacterial growth was registered for all four metal compounds at different concentrations (Table 3).
No bacterial growth was registered for Mg at the concentrations 0.1%, 0.5%, 0.25%, 0.12%, while bacterial growth occured at all other tested w/v solutions (Table 3). *B. subtilis spizizenii* growth occurred at all other w/v solutions of iron (III) nitrate except at the concentrations 0.1% and 0.5%. Growth of bacteria in the presence of copper (II) sulfate w/v solution occurred at concentrations 0.03-0.0009%. The lowest growth rate was observed in the presence of zinc sulfate heptahydrate solution, bacterial growth occurred only on concentrations 0.015-0.0009% (Table 3). The lowest concentration of chemical (drug, antimicrobial) that inhibits visible growth of microorganism (in this case bacteria) in overnight culture is known as minimum inhibitory concentration (MIC) [37].

After overnight incubation at 37 C MICs were recorded (Figure 2). Obtained MICs of metal solutions that inhibit growth of *B. subtilis subsp. spizizenii* are: magnesium 0.12%, iron (III) nitrate 0.50%, 0.06% copper (II) sulfate, 0.03% zinc sulphate heptahydrate. Considering that, growth of tested bacteria is slightly inhibited by iron (III) nitrate solution (bacteria is growing in presence of metal solution whose concentration is <0.50%), while it is tolerating much lower concentrations of zinc sulfate heptahydrate solution (<0.03%).

For this experiment laboratory strain of *B. subtilis subsp. spizizenii* was used. This strain did not form biofilms at all. According to other studies, during domestication of laboratory strains of *B. subtilis* accumulation of mutation can occur which can lead to their inability to form well-structured biofilms. Compared to the laboratory strains, undomesticated strains of *B. subtilis* usually form rich and strong biofilms [38, 39].

5. Conclusion

B. subtilis complex is normally found in soil, however it is also found in the human gut as harmless bacteria. Further research is needed for its wider application on the probiotic market due to safety concerns. Metal traces can be found in soil, wastewaters, products used in agriculture, fungicides, etc. as

such they can easily enter our food chain and end up in the human gut. This study investigated how specific metal compounds influence growth, antibiotic susceptibility, and biofilm forming capacity of *B. subtilis subsp. spizizenii.*

Based on the results that have been retrieved, we can conclude that higher concentrations of metal solutions inhibited growth of tested bacteria, while it showed good tolerance to majority of lower concentrations of metals. Generally, culture showed increased sensitivity against antibiotics after addition of metal solutions. *B. subtilis subsp spizizenii* used in this experiment was laboratory strain and was not able to form biofilms. No influence of metals was recorded there. Overall, application of these metals showed antimicrobial affinity, and can be used for further research to reveal benefits and effects in the domain of Microbiology.

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