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EFFECT OF Mg, Zn, Ca, AND Fe SUPPLEMENTS ON GROWTH, PROBIOTIC POTENTIAL, AND BIOFILM-FORMING CAPACITY OF *LACTOBACILLUS* BACTERIA

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ABSTRACT

The gut microbiome is a complete set of microorganisms found in the gastrointestinal (GI) tract. *Lactobacillus spp*. are beneficial bacteria that are normally found in the gut. They are used as a type of probiotic in order to create a homeostasis in the human microflora. One of the challenges for these bacteria is the ability to adapt to different environments in order to survive and function properly. The main research question of this study was to see whether specific dietary supplements (calcium citrate, iron(II)fumarate, zinc, magnesium oxide) change bacterial properties of *Lactobacillus reuteri (L. reuteri)* (DSM 17938) and *Lactobacillus rhamnosus (L. rhamnosus)* GG (ATCC 53103). Presence of dietary supplements affected antibiotic sensitivity of tested bacteria. Results of biochemical testing minimally changed. MICs of tested supplements have been determined as well, where *L. reuteri* tolerated higher concentrations of supplements compared to *L. rhamnosus*. Probiotic efficiency was also tested through bile and acid tolerance assays. Obtained results showed that presence of dietary supplements did not alter probiotic efficiency. According to other studies *L. reuteri* and *L. rhamnosus* form biofilm structures in the human gut, however they showed very low affinity to form biofilms before and after treatment with dietary supplements when tested in vitro. Previous studies showed that *L. reuteri* could be used in a treatment for autistic-spectrum disorders. According to results from this study, patients with ASD should avoid zinc in the form of capsules (as dietary supplements) from their diets, since it inhibits growth *of L. reuteri.* In general, tested dietary supplements, except zinc, did not affect bacterial properties on a large scale.

Keywords: *Lactobacillus reuteri; Lactobacillus rhamnosus;biofilms; antibiotic sensiitivity; microbiome*

INTRODUCTION

Probiotics can have a vast effect on the improvement of the microbial balance in the human body. The most important effect where the microbial balance can be improved is in the gastrointestinal (GI) tract. The most popular types of bacteria that are used in probiotic therapy are *Bifidobacterium* species, and *Lactobacillus* (Williams, 2010). When describing the microbiome, it is important to note that it is a very diverse group of microorganisms that can live in a particular environment. These microorganisms are the key element in the establishment of different organisms, including human organisms as well. In the human organism, their primary location is located in the human gut. They have a symbiotic relationship in which each of these organisms benefits from each other and continue living and growing. These beneficial microorganisms eventually adapt to their environment and build stable homeostasis in a specific organism. If for some reason the homeostasis is imbalanced and some change occurred in the environment, the symbiotic relationship can be altered and diseases can occur (Schwabe & Jobin, 2013; Ursell *et al*, 2012). It is important to note that each microorganism has a function in the immune system. Diets that influence the different metabolic, taxonomic, and genetic functions of our microbiota are the ones that are high-sugar and fat diets, because they can influence the growth of specific chronic diseases and can cause allergies, obesity, and type 2 diabetes (Tilg & Moschen, 2015). The microbiome is overly exposed to a vast amount of antibiotics. These antibiotics can alter the microbiome, and expose it to obtain immediate effects that influence human health (Francino, 2016).

Lactobacillus bacteria is a type of anaerobic gram-positive bacteria that has no ability to form spores. Either in a form of coccobacilli or rods, these bacteria have different characteristics that are mainly applied in fermentation. These types of bacteria can adjust to different habitats, one of them being in animal or man mucosal membranes, in spoiled food, manure, and different plant materials. 261 species make up the genus of *Lactobacillus* (Duar *et al*, 2017; Hammes & Vogel, 1995; Zheng *et al*, *2020*). *Lactobacillus* is the most widely used type of probiotic to create homeostasis in the human microflora. The advantage of this bacteria is that it has a mechanism that appears in the anti-adhesion factor products (hydrogen peroxide for example) which are lethal for different kinds of pathogens that are entering the host body (Reid & Burton, 2002; Williams, 2010).

Probiotic *Lactobacillus* bacteria have been proven to have an effect when treating a disease called gastroenteritis, which is caused by pathogenic bacteria and retroviruses (Reid & Burton, 2002). Apart from the already mentioned probiotic functions, they have health effects and flavor-forming properties, and they are crucial bacteria in pharmaceutical and food applications. One of the challenges for these bacteria is the ability to adapt to different environments to survive and function properly (Yang *et al*, 2017).

Lactobacillus reuteri (L. reuteri) is known for its different functions in food fermentation. Fermentation is one of the key elements for certain bacterial growth because it can have major beneficial effects on the GI tract. *L. reuteri* has been found on the skin, in breast milk, and in the GI tract as one of the most important locations. (Duar *et al*, 2017; Mu *et al*, 2018).

This bacterium can tolerate and survive enzyme-enriched and low pH environments, it can compete against pathogenic microorganisms, for host-probiotic interactions it can adhere to the epithelium, and it is safe to consume. Studies have shown that when *L. reuteri* formed biofilms, the immunological advantages and health benefits had still been retained. These studies could be taken into consideration when trying to use biofilm formations from this species in the future probiotic production and selection strategies (Jones & Versalovic, 2009; Mu *et al*, 2018).

There had been studies in which there had been a correlation between treating autism spectrum disorder (ASD) using *L. reuteri.* For treating ASD, patients were assigned to consume *L.reuteri* probiotics in order to test their effectiveness on this disease. This pilot study had the goal to provide all the relevant information and evidence that there is an effect in these treatments. In the end, the results were successful and it was proven that there was a social-behavioral improvement in ASD patients after consumption of oral *L. reuteri* (Kong *et al*, 2020).

L. rhamnosus was isolated for the first time from the human intestinal flora. The effects of this bacteria have shown that it is effective in treating certain infectious diseases. One of them is infantile retrovirus diarrhea, it can have effects on preventing diseases among infants and it has the modulatory function of different immune responses (Salminen *et al*, 2002; Salminen *et al*, 2004).

One of the benefits of this bacteria is its ability to resist and survive in the highly concentrated acidic and basic environment that is provided within our bodies. It can colonize GI walls. This bacterium has the benefit of protecting the human digestive tract from harmful microorganisms such as *Candida albicans* (Corcoran *et al*, 2005; Flach *et al*, 2018; Verdenelli *et al*, 2009).

People that are showing different Gastrointestinal symptoms (GIS) and altered gut microbiota correlate with ASD. Food, customized pills, and probiotics are usually used in order to balance dysbiosis in the human body. *L. rhamnosus* have a beneficial effect when it comes to treating severe ASD symptoms. It reduces depression and anxiety, and it can induce regions of the gamma-aminobutyric acid (GABA) that is found in mice (Abdellatif *et al*, 2020).

Magnesium is one of the most important dietary supplements for muscle contraction, nervous system regulation, protein formation, and it is involved in molecular processes for DNA, RNA, and protein synthesis. In the gut, magnesium

deficiency influences the bifidobacteria. Changes in the gut microbiota can lead to systemic inflammation. One study had been performed on mice, where a certain group of mice was tested to see whether they would have a significant change if they were fed with a magnesium deficiency diet and with a normal diet. Results showed that the mice that were fed with a magnesium deficiency diet, had lower gut bifidobacteria content. They had lower mRNA content that is usually used as a barrier in the ileum. Magnesium can play a very important role in the gut microbiome (Pachikian *et al*, 2010). Studies have shown that Magnesium has a significant effect on the thermotolerance of probiotic lactobacilli. More specifically, it has been shown that it has a major effect on *Lactobacillus rhamnosus*. It is proven that *Lactobacillus* bacteria can increase their survivability resistance against increased temperature levels when intaking approximately 10-50 mmol 1^{-1} of magnesium (Yang *et al*, 2017). Stimulating and enhancing the intake of magnesium as a dietary supplement has proven that it is capable of increasing the production of lactic acid by *Lactobacillus rhamnosus* (Lew, 2014).

Zinc deficiency can cause major problems in pregnancies and it can lead to some elevated inflammatory levels. It can lead to depression, cognitive impairments in animal and human models and can cause mental lethargy. Pregnant women can be prone to low zinc levels, and more studies are done in order to testify the correlation between zinc and the microbiome. One study had been done on pregnant mice, where they studied whether the gut-brain interactions are going to signal triggers by the microbiome, caused by the lower levels of zinc. The mice had been assigned with different diets that contained different levels of zinc. One group was given a diet that contained low levels of zinc, the second group had an average level of Zn, while the last group had higher levels of zinc. They had been tested with this sort of diet for 8 weeks. Mice that had a zinc-rich diet were at an advantage because their microbiome, inflammatory cytokine levels, and gut pathology were partially rescued. One of the conclusions that can be made is that this type of deficiency can cause major problems to the body. (Foligné *et al*, 2020; Sauer & Grabrucker, 2019).

Maintaining a healthy calcium-based diet is another factor that influences homeostasis. Over 30% of calcium ingestion is absorbed in the small intestine. Calcium intakes are almost always absorbed in the upper intestine. One study had been done on mice to showcase the effects of calcium that have a probiotic-like effect on the gut microbiota. The test was done on obese mice where they wanted to see if there would be a transfer of the slimming effect that mice have to the obese category. One of the reasons why this experiment was conducted was because of the major health concerns that are found between obese people and their increased energy intake. The final result from this study was that the gut microbiota was modulated by calcium intake. A host cross-talk effect was established and a healthier metabolic profile was obtained in obese mice (Chaplin *et al*, 2016; Peacock, 2010). Calcium phosphates are effective against *Salmonella* infections. Protection can be provided for the human gastrointestinal tract with the help of calcium phosphate, due to their ability to reinforce *Lactobacillus* bacteria. These bacteria had been shown to have a weakness against *Salmonella* bacteria because there had been a rapid increase in the bile salts and acid concentrations in the intestine. Calcium phosphate was used to determine the growth of this bacteria and to see their effectiveness on bile salt and fatty acid concentrations in the intestinal lumen. This supplement had provided a less aggressive environment and showed that there is a stimulated growth in *Lactobacillus* bacteria. The outcome of this experiment is that the cytotoxicity and the fatty acid and bile salts are decreased with the addition of calcium phosphate, which can enhance the effects of bacteria to sustain all the effects that can be caused in the gut (Bovee-Oudenhoven *et al*, 1999).

Studies have shown that there is a correlation between the gut microbiome and iron when treating intestinal inflammatory diseases. Antibiotics, anti-inflammatory drugs, sugars, processed foods, and some other factors can disrupt the microbiome balance, leading to unwanted health disorders. Iron as a dietary supplement can stimulate homeostasis and make up for the disorders that were caused by other environmental factors (Yilmaz & Li, 2018). As already mentioned, lactic bacteria have a lower survival rate in very acidic environments. One scientific study had been done to test the effect of iron oxide as support against the very acidic pH values in the GI tract (Novin *et al*, 2021).

The main objective of this research was to observe and test the growth, probiotic potential, and biofilm-forming capacity of *L. reuteri and L. rhamnosus* – before and after the addition of supplements. Obtained results might have a positive or negative effect on *Lactobacillus spp.* This would lead to a conclusion in terms of modifying diets to avoid the negative effect of supplements on probiotic bacteria.

MATERIAL AND METHODS

Cultivation of bacteria and preparation of solutions

Lactobacillus rhamnosus (*L. rhamnosus)* GG (ATCC 53103) from Waya and *Lactobacillus reuteri* (*L. reuteri*) (DSM 17938) from BioGaia Protectis, were bought in the local pharmacy. Both of these types of bacteria were taken in a liquid state. Four types of media were used to cultivate the bacteria: trypticase soy broth (TSB), Mueller Hinton (MH) broth, De Man, Rogosa and Sharpe (MRS) agar, and MH agar. The bacterial strains were cultivated and incubated overnight, at the temperature of 37°C. After the incubation step, the turbidity of bacterial density was adjusted to 0.5 McFarland standards to perform more tests. The dietary supplements that were used in this experiment are zinc, magnesium oxide, calcium citrate, and iron (II) fumarate, from Twinlab manufacturer. All are bought in the form of capsules in the local pharmacy. These were aseptically diluted in MH broth to 0.1% w/V solutions, and as such used for further tests.

Biochemical Tests and Antibiotic Susceptibility test Before and After the Addition of Dietary Supplements

The biochemical tests that were performed were: Bile esculin agar, Iron agar, Urease test, Sulfur indole motility media, Simmon's citrate agar, Oxidase, Mannitol Salt agar, and catalase. The results were labeled after overnight incubation at 37°C (Kralj *et al*, 2004; Nair & Surendran, 2005). Both strains that were used have been cultivated on TSB agar. For the experiment, a variety of fifteen types of antibiotics (Liofilchem) were used and they followed the Kirby-Baurer disk diffusion method (Biemer, 1973).

Microbroth Dilution Method

This method requires a minimal amount of the metal compounds that can be taken in the form of a dietary supplement that can inhibit bacterial growth (minimum inhibitory concentration - MIC). This is performed by using the specified microdilution assay procedure (Wiegand *et al*, 2008; Kavanagh *et al*, 2019). In MH broth, the dietary supplements were aseptically diluted, and the w/V solutions were the following: 1%, 0.5%, 0.25%, 0.12%, 0.06%, 0.03%, 0.015%, 0.007%, 0.003%, 0.0018%, 0.0009%. Different metal concentrations were in the 96-well plate, and the overall amount in these plates was 100 µl. The metal concentrations were in specific forms of dietary supplements (magnesium oxide, zinc, calcium citrate, iron (II) fumarate) in the 100 µl of MH broth. Separately, 190 µl of saline solution was prepared with 10 µl of bacteria (1:20 concentration). The test was performed in triples. These experiments were used to determine the exact concentrations of the specific dietary supplements used for inhibiting bacterial growth. The bacteria were incubated overnight at 37°C, and there were visual results recorded where the MICs have been determined.

Testing Bacterial Growth by Using a Spectrophotometer

After the determination of MICs was accomplished, the bacterial growth was recorded. It was done by using a spectrophotometer. In each cuvette, 900 µl of MH broth was added as well as 100 µl of bacteria. The absorbance was recorded at wavelengths 600 nm (Akinduti *et al*, 2019).

Determination of Biofilm-Forming Capacity

Using the TCP method, the test was performed in triplets. For this method, the 96-well plate was used, containing a variety of metal concentrations, TSB broth medium, and 20 µl of the strains that were tested (0.5 McFarland standard). A lid should be placed onto the inoculated plate for 24 hours, at 37°C. Once the incubation was achieved, the plates that were used were discarded and washed. To visualize the indirect biofilm quantification, a Crystal violet assay was performed. Each microtiter plate contained 120 µl of 0.1% crystal violet, which was left to stain for 10 minutes. After this was achieved, the microtiter plate was once again decanted and washed with distilled water (O'Toole, 2011; Stepanovic *et al*, 2007). 200 ml of 96% ethanol was then inserted, and the incubation lasted for 10 minutes. Once the incubation step was completed, the content was then transferred onto a new 96-well plate using an ELISA plate reader, the optical density (OD) was measured at 595 nm. The average and standard deviation were able to be determined because of the sufficient absorbance values that were determined, where the extent of biofilm formation was measured as well. Once all the bacterial strains were tested, there were four categories to which each specific strain could

belong, which were the following: strong biofilm formers, moderate, weak, and non-adherent.

Probiotic Efficiency: Bile and Acid Tolerance Assays

L. reuteri and *L. rhamnosus* were characterized for their probiotic efficiency. This characterization was tested with the acid and bile tolerance assays. To determine the bile tolerance was determined with a methodology by Shehata *et al.* in which for preparing MRS broth which contained bile, it had to have 0.3 g (w/v) of bile salt. The aliquots of 100 µl of MRS broth cultures that were left overnight were inoculated onto the 96-well plate with the 100 µl of MRS broth which contained bile. For the negative control, 200 µl of MRS broth which contained bile was included as well. After each hour of incubation (from 0-3 hours respectively) at 37°C in a CO_2 incubator, by using an ELISA reader, the absorbance at 625 nm was measured. This experiment was done in triplicates as well (Garrote *et al*, 2005; Shehata et al., 2016; Jacobsen et al., 1999). Low pH tolerance was verified according to a protocol by Millette et al. (2008), in which there were slight moderations. In this case, 1 ml of MRS broth cultures that were left overnight were inoculated into 9 ml in sterile MRS broth that was adjusted to specific pH values (1, 2, 3) with 1 M HCL, and a pH meter. Using a spectrophotometer, the initial absorbance levels that were measured were set at 600 nm. Once this was performed, the concentrations were incubated at 37°C in CO_2 for 3 hours. The absorbance was measured again after three hours in hourly intervals (0 h, 1 h, 2 h, 3 h). This experiment was conducted as well in triplicate (Millette *et al*, 2008; Yang *et al*, 2018).

RESULTS AND DISCUSSION

After the research about the effect of dietary supplements on Lactobacillus was conducted, an experiment was performed, and obtained results are explained below. For this experiment *Lactobacillus reuteri (L. reuteri)* (DSM 17938) and *Lactobacillus rhamnosus* (*L. rhamnosus)* GG (ATCC 53103) were subjected to tests in presence of four dietary supplements from the local pharmacy: calcium citrate, magnesium, zinc, and ferrous fumarate (iron).

Table 1 represents the antibiotic susceptibility test for *L. reuteri* before and after the addition of dietary supplements (zinc, calcium citrate, magnesium oxide, iron (II) fumarate). Numbers in the table below represent the diameter of the zone of inhibition (in millimeters). The susceptibility has been tested on fifteen different disks. In the *L. reuteri* category without the presence of ditary supplements, four antibiotic discs had the most abundant zone of inhibition. The disks that had the most abundant zone of inhibition was E15, which had the diameter zone of 46 mm, followed by AZM15 with a zone of 44 mm, CN10 with 34 mm, and the last would be CIP5 with 30 mm. Other antibiotic discs, such as FOX30, S10, AUG30, and K30, showed smaller zones of inhibition, while *L. reuteri* was resistant to the rest of the tested antibiotics. With the addition of supplements, zones of inhibition of certain antibiotic discs increased while others decreased. The interesting part is that *L. reuteri* did not grow at all at MRS agar in the presence of zinc. Therefore, antibiotics susceptibility results could not be recorded. It can be concluded that zinc successfully inhibits the growth of *L. reuteri.*

Table 1 Antibiotic susceptibility test for *L. reuteri* before and after addition of dietary supplements. Numbers in the table below represent the diameter of the zone of inhibition (in millimeters).

	L. reuteri	L. reuteri + Calcium citrate	L. reuteri + MgO	L. reuteri + Zn	L. reuteri + iron (II) fumarate
FOX30	12	28	0	no bacterial growth	0
CN10	34	20	25	no bacterial growth	22
OX1	0	0	0	no bacterial growth	0
AML10	0	20	24	no bacterial growth	20
CAL40	0	0	0	no bacterial growth	0
CIP5	30	15	0	no bacterial growth	0
S10	27	20	21	no bacterial growth	17
VA30	0	0	0	no bacterial growth	0
E15	46	40	35	no bacterial growth	39
CAZ10	0	0	0	no bacterial growth	13
AUG30	27	15	32	no bacterial growth	33
AZM15	44	35	34	no bacterial growth	33
K30	24	25	17	no bacterial growth	23
TE30	0	0	18	no bacterial growth	15
AMP2	0	0	0	no bacterial growth	0

Table 2 represents the antibiotic susceptibility test for *L. rhamnosus* before and after the addition of dietary supplements. Before addition of dietary supplemets, there were three different disks on which the inhibitory zones were shown the most, and those were E15 with 41 mm, AZM15 with 31 mm, and AUG30 with 29 mm. Other disks that had zones of inhibition, but not as much as the first three were on disks CN10, AML10, CIP5, S10, K30, and TE30. *L. rhamnosus* was resistant to the rest of the tested antibiotics. After the addition of supplements, most of the tested antibiotics had increased inhibition zone diameter.

Table 2 Antibiotic susceptibility test for *L. rhamnosus* before and after addition of dietary supplements. Numbers in the table below represent the diameter of the zone of inhibition (in millimeters).

	L. rhamnosus	*L. rhamnosus* + Calcium citrate	*L. rhamnosus* + MgO	*L. rhamnosus* + Zn	*L. rhamnosus* + iron (II) fumarate
FOX30	0	0	0	0	0
CN10	13	17	13	23	16
OX1	0	0	0	0	0
AML10	16	15	22	17	19
CAL40	0	0	0	0	0
CIP5	27	31	31	34	28
S10	8	12	10	0	9
VA30	0	0	0	0	0
E15	41	41	45	40	45
CAZ10	0	0	0	0	0
AUG30	29	34	30	32	34
AZM15	31	42	44	41	39
K30	8	10	9	0	9
TE30	28	41	39	35	34
AMP2	0	0	0	0	0

Lactobacillus reuteri and *Lactobacillus rhamnosus* underwent biochemical testing, before and after the addition of dietary supplements. In Tables 3 and 4 the results from testing *L. reuteri* and *L. rhamnosus* were made in order to observe their behavior on different media before and after the addition of supplements.

L. reuteri and *L. rhamnosus* had the same results when tested on different media before the supplement treatment. The urease test was negative, and it should indicate that the bacteria are not capable of hydrolyzing urea. Simmon's Citrate Agar was used to determine if these bacteria had more than one carbon source. The result was negative, meaning that there are more carbon sources in this type of bacteria rather than just gathering it from citrate. Bile esculin agar was positive for both bacteria, which indicates that these bacteria can hydrolyze esculin in the presence of bile. For the Triple Sugar Iron Agar (TSI), when combined with these bacteria, both had a yellow butt and slant, which is an indicator for Glucose and lactose and/or sucrose fermentation. These bacteria have the capability to reduce sulfur and ferment carbohydrates.

Sulfur Indole Motility (SIM) media is capable of testing different media factors of bacteria, or in other words, the ability to reduce sulfur, produce indole and observe bacterial motility. For both sulfur and indole, the results were negative, indicating that reduction of sulfur and production of indole did not occur, but the motility of these bacteria was positive.

Mannitol Salt Agar (MSA) is a type of media that tests the ability of the bacteria if they can withstand a very high salt concentration environment and if they are capable of fermenting sugar mannitol. For both bacteria, their results were negative, leading to the conclusion that they are not capable of fermenting mannitol. The oxidase test was proven negative for both bacteria as well because they do not possess a cytochrome oxidase system. For the catalase test, it was negative for both bacteria, meaning that these bacteria were not able to break down hydrogen peroxide.

The following results were obtained for *L. reuteri* in presence of dietary supplements (Table 3). For the Urease test, Simon's Citrate Agar, and TSI agar the bacteria were negative when combined with every dietary supplement respectively. The results did not show any changes when compared to the bacterial culture without the addition of dietary supplements.

Bile esculin Agar had different effects once it was combined with the dietary supplements. The results were positive for the *L. reuteri* to hydrolyze esculin in the presence of bile with magnesium oxide, calcium citrate, and iron (II) fumarate. In combination with Zn, the outcome of the bacteria changed, meaning that it was unable to hydrolyze esculin in the presence of bile.

Dietary supplements affected the SIM media. Supplements such as magnesium oxide, and iron (II) fumarate, did not have any effect on the characteristics of the results compared to Zn, and calcium citrate, which changed the bacterial motility. Motility was present in *L. reuteri* without the addition of dietary supplements, and with the addition of magnesium oxide and iron (II) fumarate supplements respectively, while the mobility was not present with Zn and calcium citrate respectively as well.

Mannitol Salt Agar, as well as Oxidase and catalase tests, when combined with the specific dietary supplements, did not show a difference in the results for *L. reuteri.*

Table 3 Biochemical properties of *L. reuteri* before and after the addition of dietary supplements

Type of biochemical test	*L. reuteri*	*L. reuteri* + MgO	*L. reuteri* + Zn	*L. reuteri* + Calcium citrate	*L. reuteri* + iron (II) fumarate
Urea test	-	-	-	-	-
Simmon's Citrate Agar	-	-	-	-	-
Bile esculin Agar	+	+	-	+	+
Triple Sugar Iron Agar (TSI)	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation
Sulfur Indole Motility Media (SIM)	H2S - Indole - motility +	H2S - Indole - motility +	H2S - Indole - motility -	H2S - Indole - motility -	H2S - Indole - motility +
Mannitol Salt Agar (MSA)	-	-	-	-	-
Oxidase test	-	-	-	-	-
Catalase test	-	-	-	-	-

Table 4 represents the behaviour of *L. rhamnosus* bacteria before and after it was combined with the dietary supplements (calcium citrate, magnesium oxide, zinc, and iron (II) fumarate).

For the Urease test, Simon's Citrate Agar, and TSI agar, the results did not show any changes when compared to the results for *L. rhamnosus* without the addition of dietary supplements.

For Bile Esculin agar, the results remained the same for the *L. rhamnosus* to hydrolyze esculin in the presence of bile with magnesium oxide, iron (II) fumarate, and Zn. In combination with calcium citrate, the result changed, meaning that it was unable to hydrolyze esculin in the presence of bile.

Dietary supplements did not have an effect on the SIM media with *L. rhamnosus*, compared to *L. reuteri*. The motility of *L. reuteri* (Table 4) was affected with specific dietary supplements, while these supplements did not have an effect and sulfur reduction, indole production, and motility of *L. rhamnosus*. The results remained the same as before the treatment.

MSA media had the most significant change. With the addition of dietary supplements respectively for *L. rhamnosus*, all of them had a positive result on MSA, which indicates the survivability of high salt concentrations and that they were able to ferment sugar mannitol.

For both Oxidase and Catalase tests, the final result did not change with the addition of dietary supplements. *L. rhamnosus* did not possess a cytochrome oxidase system, and they are unable to catalase enzyme which breaks down hydrogen peroxide.

Table 4 Biochemical properties of *L. rhamnosus* before and after the addition of dietary supplements

Type of biochemical test	*L. rhamnosus*	*L. rhamnosus* + Magnesium citrate	*L. rhamnosus* + Zn	*L. rhamnosus* + Calcium citrate	*L. rhamnosus* + iron (II) fumarate
Urea test	-	-	-	-	-
Simmon's Citrate Agar	-	-	-	-	-
Bile esculin Agar	+	+	+	-	+
Triple Sugar Iron Agar (TSI)	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation	yellow butt and slant: Glucose and lactose and/or sucrose fermentation
Sulfur Indole Motility Media (SIM)	H2S - Indole - Motility +	H2S - Indole - motility +	H2S - Indole - motility +	H2S - Indole - motility +	H2S - Indole - motility +
Mannitol Salt Agar (MSA)	-	+	+	+	+
Oxidase test	-	-	-	-	-
Catalase test	-	-	-	-	-

Results of microbroth dilution assay are shown below (Fig. 1 and 2). These results are showing how different concentrations of dietary supplements (w/V) affect the growth of tested Lactobacillus strains (*L. reuteri* and *L. rhamnosus*). Figures 1 and 2 are summarizing the minimum inhibitory concentrations (MIC) of dietary supplements. Minimum inhibitory concentration is the lowest concentration of a chemical that prevents/inhibits the visible growth of a microorganism after overnight incubation (Andrews, 2001). MICs have been recorded after 24 h incubation at 37 C.

MICs of dietary supplement (w/V) solutions that inhibit growth of *L. reuteri* are: calcium citrate 0.25%, zinc 0.12%, magnesium oxide 0.25%, iron (II) fumarate 0.5%, while for *L. rhamnosus* MICs are the following: calcium citrate 0.06%, zinc 0.06%, magnesium oxide 0.5%, iron (II) fumarate 0.06%. Based on this data it is easy to conclude that *L. reuteri* tolerates higher concentrations of dietary supplements compared to *L. rhamnosus.*

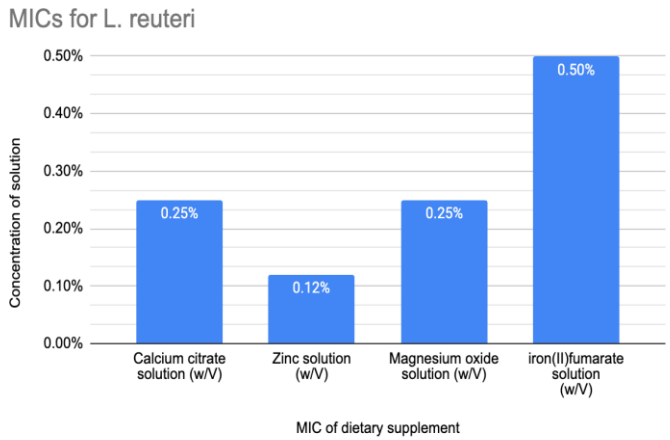

Figure 1 MICs for *L. reuteri* after 24 h incubation.

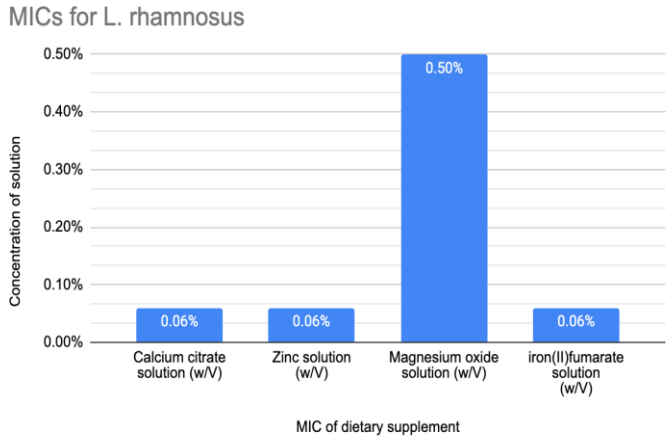

Figure 2 MICs for *L. rhamnosus* after 24 h incubation.

After the microbroth dilution assay was performed, quantification of *Lactobacillus* bacteria was also determined by measuring its absorbance. It was done using a spectrophotometry method and absorbance was measured at a wavelength of 600 nm (Figure 3).

According to Beer's law absorbance is directly proportional to the concentration of the measured substance; higher absorbance - the higher concentration. The number of molecules in a substance affects the proportion of light absorbed (Swinehart, 1962).

The measured substance contained *L. reuteri* or *L. rhamnosus*, with and without dietary supplement. The number of *L. reuteri* bacteria decreased in presence of calcium citrate, Zn, and MgO, while absorbance increased in presence of iron (II) fumarate. The concentration of *L. rhamnosus* increased only in presence of calcium citrate. No absorbance was recorded for *L. reuteri* and *L. rhamnosus* in the presence of MgO. The limitation of this part of the experiment is an old spectrophotometer which may not give super precise results.

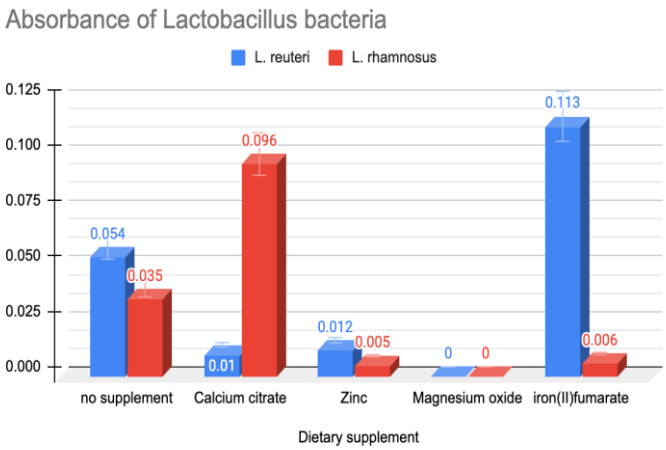

Figure 3 Absorbance of *Lactobacillus* bacteria at 600 nm measured using a spectrophotometer.

Biofilm forming capacity was recorded by using the ELISA instrument and recorded at an absorbance of 595 nm. The cut-off optical density (ODc) for the classification of biofilms was calculated using the standardized formula:

ODc = average OD of negative control + 3x STDEV of negative control.

Biofilm categories were determined using formulas from table 5.

Table 5 Standardized formula for determination of biofilm categories:

Formula	Biofilm formation
OD < ODc	Non-adherent (NA)
ODc < OD ≤ 2ODc	Weak (W)
2ODc < OD ≤ 4ODc	Medium (M)
4ODc < OD	Strong (S)

Most of the biofilms ended up in non-adherent or weak categories. Dietary supplements do not seem to affect the formation of biofilms for these two bacteria: *L. reuteri* and *L. rhamnosus.* Tables are available in the supplementary file.

The pH tolerance of *L. reuteri* and *L. rhmanosus* was tested using 1 ml of overnight MRS broth cultures that were inoculated into 9 ml in sterile MRS broth that was adjusted to specific pH values (1, 2, 3) using 1 M HCL, and a pH meter. Absorbance was measured in hourly intervals (after 0h, 1h, 2h, 3h) at 600 nm using a spectrophotometer.

Table 6 represents the average absorbance values of bacterial cultures (*L. reuteri* and *L. rhamnosus*), with or without the presence of dietary supplements, measured after 0 hours. Tables 7, 8, and 9 represent the same but recorded after 1, 2, and 3 hours, respectively. Absorbance is directly proportional to the concentration of a measured substance.

Based on the rest of the literature it's shown that *L. reuteri* and *L. rhamnosus* can tolerate low pH values and the presence of various enzymes, which makes them a very good probiotic (Mu *et al*, 2018; Salas-Jara *et al*, 2016; Corcoran *et al*, 2005; Flach *et al*, 2018; Verdenelli *et al*, 2009).

According to obtained results in tables 6, 7, 8, and 9, *L. reuteri* and *L. rhamnosus* can grow at the following pH values: 1, 2, and 3, even in the presence of dietary supplements. The lowest absorbance measured was for *L. reuteri* in presence of zinc (0.170) at pH=1, measured after 2 h incubation. It is important to note that *L. reuteri* did not grow at all at MH agar in presence of zinc (Table 2). The highest absorbance measured was 1.950 for *L. rhamnosus* in presence of iron (II) fumarate at pH=1, recorded after 2 h incubation.

The main limitation of this part of the experiment might be an old spectrophotometer, so systematic errors should be taken into account.

Table 6 Acidity test: Absorbance of *L. reuteri* and *L. rhamnosus* cells at pH: 1, 2, and 3 are recorded after 0 hours.

Time: after 0 hours.	pH=1	pH=2	pH=3
Bacteria; bacteria and supplement			
L. reuteri	0.816	0.859	0.958
L. reuteri + MgO	0.947	0.622	0.922
L. reuteri + Zn	0.506	0.432	0.676
L. reuteri + Calcium citrate	0.559	0.507	0.481
L. reuteri + iron(II)fumarate	0.517	0.551	0.672
L. rhamnosus	0.666	0.716	1.179
L. rhamnosus + MgO	1.111	0.746	0.870
L. rhamnosus + Zn	0.983	0.983	1.190
L. rhamnosus + Calcium citrate	0.173	0.582	0.515
L. rhamnosus + iron (II)fumarate	0.763	0.640	1.589

Table 7 Acidity test: Absorbance of *L. reuteri* and *L. rhamnosus* cells at pH:1, 2, and 3 recorded after 1 hour

Time: after 1 hour	pH=1	pH=2	pH=3
Bacteria; bacteria and supplement			
L. reuteri	0.347	0.731	0.907
L. reuteri + MgO	0.500	0.825	0.798
L. reuteri + Zn	0.307	0.490	0.539
L. reuteri + Calcium citrate	0.373	0.496	0.514
L. reuteri + iron (II) fumarate	0.509	0.571	0.624
L. rhamnosus	0.588	0.692	1.793
L. rhamnosus + MgO	0.617	0.922	0.817
L. rhamnosus + Zn	0.425	0.291	1.462
L. rhamnosus + Calcium citrate	0.470	0.188	0.485
L. rhamnosus + iron(II)fumarate	0.255	1.531	0.385

Table 8 Acidity test: Absorbance of *L. reuteri* and *L. rhamnosus* cells at pH:1, 2, and 3 recorded after 2 hours.

Time: after 2 hours.	pH=1	pH=2	pH=3
Bacteria; bacteria and supplement			
L. reuteri	0.324	0.693	0.823
L. reuteri + MgO	0.392	0.792	0.815
L. reuteri + Zn	0.170	0.344	0.545
L. reuteri + Calcium citrate	0.198	0.418	0.442
L. reuteri + iron (II) fumarate	0.377	0.457	0.764
L. rhamnosus	0.538	0.535	0.639
L. rhamnosus + MgO	0.513	0.630	1.128
L. rhamnosus + Zn	0.687	1.455	0.460
L. rhamnosus + Calcium citrate	0.389	0.253	0.488
L. rhamnosus + iron (II) fumarate	1.950	1.430	1.333

Table 9 Acidity test: Absorbance of *L. reuteri* and *L. rhamnosus* cells at pH:1, 2, and 3 recorded after 3 hours.

Time: after 3 hours	pH=1	pH=2	pH=3
Bacteria; bacteria and supplement			
L. reuteri	0.284	0.635	0.910
L. reuteri + MgO	0.370	0.799	1.049
L. reuteri + Zn	0.170	0.283	0.508
L. reuteri + Calcium citrate	0.203	0.563	0.433
L. reuteri + iron(II)fumarate	0.319	0.538	0.697
L. rhamnosus	0.661	0.958	0.292
L. rhamnosus + MgO	0.441	1.286	0.653
L. rhamnosus + Zn	1.107	1.024	1.344
L. rhamnosus + Calcium citrate	0.306	0.576	0.518
L. rhamnosus + iron(II)fumarate	0.155	0.543	1.120

In order to check probiotic efficiency, the Growth of *L. reuteri* and *L. rhamnosus* (before and after the treatment of dietary supplements) was also tested in the presence of bile. It was done by measuring the absorbance of bacterial cells in an MRS broth (Tables 10-17) and absorbance is proportional to the concentration of a measured substance. 100 ul of MRS broth cultures were inoculated into a 96-well plate together with 100 ul of MRS broth containing bile. Negative control was also

included. The absorbance was measured at 625 nm using an ELISA reader. Data was recorded after 0, 1, 2 and 3 hours of incubation at 37 C.

Table 10 Absorbance of *L. reuteri* in presence of bile measured using ELISA reader after 0 h incubation.

Without bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.125	0.930	0.277	0.430	0.280	0.937
0.128	0.971	0.295	0.337	0.265	0.940
0.139	1.015	0.400	0.320	0.287	0.956
AVG = 0.128	AVG = 0.972	AVG = 0.324	AVG = 0.362	AVG = 0.277	AVG = 0.944

With bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.137	1.830	0.279	0.447	0.293	1.415
0.122	1.620	0.309	0.516	0.346	1.118
0.127	1.784	0.237	0.401	0.318	1.253
AVG = 0.129	AVG = 1.745	AVG = 0.275	AVG = 0.455	AVG = 0.317	AVG = 1.262

Table 11 Absorbance of *L. rhamnosus* in presence of bile measured using ELISA reader after 0 h incubation.

Without bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.125	1.269	1.214	1.336	0.318	0.998
0.128	1.243	1.238	1.340	0.306	1.050
0.132	1.224	1.194	1.340	0.308	1.022
AVG = 0.128	AVG = 1.245	AVG = 1.215	AVG = 1.339	AVG = 0.311	AVG = 1.023

With bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.137	1.803	1.721	1.705	0.323	1.492
0.122	1.404	1.506	1.700	0.405	1.437
0.127	1.919	1.715	1.760	0.381	1.392
AVG = 0.129	AVG = 1.709	AVG = 1.647	AVG = 1.722	AVG = 0.369	AVG = 1.440

Table 12 Absorbance of *L. reuteri* in presence of bile measured using ELISA reader after 1 h incubation.

Without bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.130	1.008	0.263	0.373	0.266	1.026
0.127	0.968	0.294	0.342	0.257	1.016
0.135	1.076	0.328	0.334	0.309	1.007
AVG = 0.131	AVG = 1.017	AVG = 0.295	AVG = 0.350	AVG = 0.277	AVG = 1.016

With bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.129	1.392	0.403	0.382	0.286	1.178
0.122	1.477	0.434	0.319	0.307	1.134
0.130	1.402	0.342	0.329	0.318	1.078
AVG = 0.127	AVG = 1.424	AVG = 0.393	AVG = 0.343	AVG = 0.304	AVG = 1.130

Table 13 Absorbance *of L. rhamnosus* in presence of bile measured using ELISA reader after 1 h incubation.

Without bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.130	1.347	1.246	1.383	0.317	1.069
0.127	1.402	1.306	1.392	0.354	1.133
0.135	1.337	1.300	1.422	0.327	1.104
AVG = 0.131	AVG = 1.362	AVG = 1.284	AVG = 1.399	AVG = 0.333	AVG = 1.102

With bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.129	1.342	1.401	1.310	0.551	1.389
0.122	1.414	1.346	1.380	0.402	1.254
0.130	1.345	1.377	1.400	0.465	1.405
AVG = 0.127	AVG = 1.367	AVG = 1.375	AVG = 1.363	AVG = 0.473	AVG = 1.349

Table 14 Absorbance of *L. reuteri* in presence of bile measured using ELISA reader after 2 h incubation.

Without bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.128	1.091	0.279	0.378	0.306	1.165
0.130	1.055	0.282	0.368	0.298	1.164
0.132	1.205	0.348	0.390	0.333	1.141
AVG = 0.130	AVG = 1.117	AVG = 0.303	AVG = 0.379	AVG = 0.312	AVG = 1.157

With bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.135	1.462	0.215	0.220	0.196	1.177
0.122	1.429	0.207	0.264	0.194	1.007
0.128	1.214	0.232	0.300	0.226	1.059
AVG = 0.128	AVG = 1.368	AVG = 0.218	AVG = 0.261	AVG = 0.205	AVG = 1.081

Table 15 Absorbance of *L. rhamnosus* in presence of bile measured using ELISA reader after 2 h incubation.

Without bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.128	1.518	1.377	1.410	0.412	1.177
0.130	1.469	1.410	1.480	0.414	1.226
0.132	1.511	1.371	1.499	0.410	1.194
AVG = 0.130	AVG = 1.499	AVG = 1.386	AVG = 1.463	AVG = 0.412	AVG = 1.199

With bile

Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.135	1.391	1.418	1.504	0.351	1.273
0.122	1.504	1.560	1.605	0.342	1.206
0.128	1.513	1.454	1.616	0.371	1.300
AVG = 0.128	AVG = 1.469	AVG = 1.477	AVG = 1.575	AVG = 0.355	AVG = 1.260

Table 16 Absorbance of *L. reuteri* in presence of bile measured using ELISA reader after 3 h incubation.

Without bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.130	1.190	0.286	0.352	0.345	1.237
0.127	1.116	0.320	0.420	0.339	1.216
0.132	1.299	0.387	0.434	0.377	1.178
AVG = 0.130	AVG = 1.202	AVG = 0.331	AVG = 0.402	AVG = 0.354	AVG = 1.210

With bile

Negative control	***L. reuteri***	***L. reuteri* + Zn**	***L. reuteri* + iron (II) fumarate**	***L. reuteri* + calcium citrate**	***L. reuteri* + MgO**
0.130	1.275	0.254	0.258	0.238	1.071
0.127	1.234	0.221	0.327	0.232	0.952
0.135	1.119	0.224	0.309	0.268	0.932
AVG = 0.313	AVG = 1.209	AVG = 0.233	AVG = 0.298	AVG = 0.246	AVG = 0.985

Table 17 Absorbance of *L. rhamnosus* in presence of bile measured using ELISA reader after 3 h incubation.

Without bile					
Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.130	1.542	1.417	1.454	0.594	1.277
0.127	1.533	1.483	1.501	0.610	1.340
0.132	1.598	1.482	1.579	0.621	1.304
AVG = 0.130	AVG = 1.552	AVG = 1.461	AVG = 1.511	AVG = 0.608	AVG = 1.307
With bile					
Negative control	***L. rhamnosus***	***L. rhamnosus* + Zn**	***L. rhamnosus* + iron (II) fumarate**	***L. rhamnosus* + calcium citrate**	***L. rhamnosus* + MgO**
0.130	1.482	1.422	1.554	0.337	1.183
0.127	1.560	1.431	1.699	0.420	1.194
0.135	1.400	1.501	1.759	0.346	1.247
AVG = 0.131	AVG = 1.480	AVG = 1.451	AVG = 1.667	AVG = 0.368	AVG = 1.208

According to obtained results, *L. reuteri* and *L. rhamnosus* do tolerate the presence of biles even with the addition of dietary supplements. Absorbance for some of them decreased while for some increased in the presence of bile. Based on average values from tables 10 - 17, the lowest is confirmed for *L. reuteri* with zinc solution, as it was shown during acidity testing. Small systematic error for ELISA reader should be taken into account since values for negative control slightly changed over a 3h period, which should not be the case.

CONCLUSION

Gut microbiome is a complete set of microorganisms found in the intestinal tract. The more the microbiome is enriched with different species it has more benefits for human organisms. Diet can be one of the ways to regulate the behavior of the gut microbiome. *Lactobacillus* has an important role in preventing pathogenic bacteria from infecting the human microbiome in the GI tract. *Lactobacillus spp.* are normally found in the human GI tract and are also marked as probiotics and their consumption is preferable. This paper is focused on *Lactobacillus reuteri* (*L. reuteri*) (DSM 17938) and *Lactobacillus rhamnosus* (*L. rhamnosus)* GG (ATCC 53103). Both of these bacteria are beneficial for the human gut microbiome. Some experimental studies also showed positive effects in treating ASD patients with diets rich in *L. reuteri* and *L. rhamnosus*.

Nowadays many people take dietary supplements and the main purpose of this research is to investigate whether specific dietary supplements (calcium citrate, iron(II)fumarate, zinc, magnesium oxide) inhibit growth or cause any changes in *L. reuteri* and *L. rhamnosus.*

According to obtained results, the presence of dietary supplements affected *L. reuteri* and *L. rhamnosus* differently. Antibiotic sensitivity of *L. reuteri* for some tested antibiotics increased, while for others the zone of inhibition decreased in the presence of dietary supplements. What is important to note is that *L. reuteri* did not grow at all on MRS agar in presence of zinc. *L. rhamnosus* showed mostly increased zones of inhibition in the presence of dietary supplements.

Outcome of most of the biochemical tests performed did not change in the presence of dietary supplements, for both *L. reuteri* and *L. rhamnosus*.

For *L. reuteri* bile esculin agar had different effects once it was combined with the dietary supplements. In combination with zinc, the outcome of the bacteria changed, meaning that it was unable to hydrolyze esculin in the presence of bile. Dietary supplements had an effect on the SIM media. Supplements such as magnesium oxide, and iron(II)fumarate, did not have any effect on the characteristics of the *L. reuteri* compared to Zn, and calcium citrate, which changed the bacterial motility. Motility was present in these bacteria without the addition of dietary supplements, while the mobility was not present with Zn and calcium citrate respectively as well.

L. rhamnosus showed different results for bile esculin agar test in the presence of calcium citrate, meaning that it was unable to hydrolyze esculin in the presence of bile. Dietary supplements did not have an effect on the SIM media with *L. rhamnosus*, compared to *L. reuteri*.

Both, *L. reuteri* and *L. rhamnosus* did grow in presence of different concentrations of dietary supplements. MICs differ among tested supplements, where it is shown that *L. reuteri* tolerates higher concentrations of dietary supplements compared to *L. rhamnosus.*

When it comes to biofilm forming capacity, once classified to categories most of biofilms were non-adherent at all, or weakly adherent. It looks like dietary supplements did not affect this property since positive controls (without dietary supplements) also showed non-adherent or weak biofilms. According to literature, both of tested bacteria do form biofilm structures in the human gut, however it seems they have very low biofilm forming capacity when tested *in vitro*.

L. reuteri and *L. rhamnosus* underwent biles and acid tolerance assays to check their probiotic efficiency. Obtained results showed that they tolerate presence of biles and low pH, even with addition of dietary supplements. Based on average values the lowest absorbance was recorded for *L. reuteri* with zinc solution, as it was shown during acidity testing.

As already mentioned, according to previous studies *L. reuteri* could be used in a treatment for autistic-spectrum disorders. After this research patients with ASD should avoid zinc in the form of capsules (as dietary supplements) from their diets, since it inhibits growth of *L. reuteri.*

Overall, tested dietary supplements did not affect bacterial properties on a large scale, except zinc, that inhibited growth of *L. reuteri*. Further research should be focused on a cellular level in order to see how these and similar supplements behave and what causes the changes.

REFERENCES

Abdellatif, B., McVeigh, C., Bendriss, G., & Chaari, A. (2020). The promising role of probiotics in managing the altered gut in autism spectrum disorders. *International journal of molecular sciences*, *21*(11), 4159. https://doi.org/10.3390/ijms21114159

Akinduti, P. A., Motayo, B., Idowu, O. M., Isibor, P. O., Olasehinde, G. I., Obafemi, Y. D., ... & Adeyemi, G. A. (2019, August). Suitability of spectrophotometric assay for determination of honey microbial inhibition. In *Journal of Physics: Conference Series* (Vol. 1299, No. 1, p. 012131). IOP Publishing. https://doi.org/10.1088/1742-6596/1299/1/012131

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of antimicrobial Chemotherapy*, *48*(suppl_1), 5-16. https://doi.org/10.1093/jac/48.suppl_1.5

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., ... & Bork, P. (2011). Enterotypes of the human gut microbiome. *nature*, *473*(7346), 174-180. https://doi.org/10.1038/nature09944

Arundine, M., & Tymianski, M. (2003). Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell calcium*, *34*(4-5), 325-337. https://doi.org/10.1016/s0143-4160(03)00141-6

Biemer, J. J. (1973). Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. *Annals of Clinical & Laboratory Science*, 3(2), 135-140. https://doi.org/10.1093/ajcp/71.1.131a

Bovee-Oudenhoven, I. M., Wissink, M. L., Wouters, J. T., & Van der Meer, R. (1999). Dietary calcium phosphate stimulates intestinal lactobacilli and decreases the severity of a salmonella infection in rats. *The Journal of nutrition*, *129*(3), 607-612. https://doi.org/10.1093/jn/129.3.607

Chaplin, A., Parra, P., Laraichi, S., Serra, F., & Palou, A. (2016). Calcium supplementation modulates gut microbiota in a prebiotic manner in dietary obese mice. *Molecular nutrition & food research*, *60*(2), 468-480. https://doi.org/10.1002/mnfr.201500480

Chen, H. Y., Cheng, F. C., Pan, H. C., Hsu, J. C., & Wang, M. F. (2014). Magnesium enhances exercise performance via increasing glucose availability in the blood, muscle, and brain during exercise. *Plos one*, *9*(1), e85486. https://doi.org/10.1371/journal.pone.0085486

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and environmental microbiology*, *71*(6), 3060-3067. https://doi.org/10.1128/aem.71.6.3060-3067.2005

De Baaij, J. H., Hoenderop, J. G., & Bindels, R. J. (2015). Magnesium in man: implications for health and disease. *Physiological reviews*. https://doi.org/10.1152/physrev.00012.2014

Doron, S., & Gorbach, S. L. (2006). Probiotics: their role in the treatment and prevention of disease. *Expert review of anti-infective therapy*, *4*(2), 261-275. https://doi.org/10.1586/14787210.4.2.261

Duar, R. M., Lin, X. B., Zheng, J., Martino, M. E., Grenier, T., Pérez-Muñoz, M. E., ... & Walter, J. (2017). Lifestyles in transition: evolution and natural history of the genus Lactobacillus. *FEMS Microbiology Reviews*, *41*(Supp_1), S27-S48. https://doi.org/10.1093/femsre/fux030

Flach, J., van der Waal, M. B., Kardinaal, A. F. M., Schloesser, J., Ruijschop, R. M. A. J., & Claassen, E. (2018). Probiotic research priorities for the healthy adult population: A review on the health benefits of Lactobacillus rhamnosus GG and Bifidobacterium animalis subspecies lactis BB-12. *Cogent Food & Agriculture*, *4*(1), 1452839. https://doi.org/10.1080/23311932.2018.1452839

Flemming, H. C., Neu, T. R., & Wozniak, D. J. (2007). The EPS matrix: the "house of biofilm cells". *Journal of bacteriology*, *189*(22), 7945-7947. https://doi.org/10.1128/jb.00858-07

Francino, M. P. (2016). Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. *Frontiers in microbiology*, *6*, 1543. https://doi.org/10.3389/fmicb.2015.01543

Garrote, G. L., Serradell, M. A., Abraham, A. G., Añon, M. C., Fossati, C. A., & De Antoni, G. L. (2005). Development of an immunochemical method to detect Lactobacillus kefir. *Food and Agricultural Immunology*, *16*(3), 221-233. https://doi.org/10.1080/09540100500244146

Gröber, U., Schmidt, J., & Kisters, K. (2015). Magnesium in prevention and therapy. *Nutrients*, *7*(9), 8199-8226. https://doi.org/10.3390/nu7095388

Foligné, B., George, F., Standaert, A., Garat, A., Poiret, S., Peucelle, V., ... & Daniel, C. (2020). High-dose dietary supplementation with zinc prevents gut inflammation: Investigation of the role of metallothioneins and beyond by transcriptomic and metagenomic studies. *The FASEB Journal*, *34*(9), 12615-12633. https://doi.org/10.1096/fj.202000562rr

Hammes, W. P., & Vogel, R. F. (1995). The genus lactobacillus. In *The genera of lactic acid bacteria* (pp. 19-54). Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-5817-0_3

Holding, A. J., & Collee, J. G. (1971). Chapter I Routine biochemical tests. In *Methods in microbiology* (Vol. 6, pp. 1-32). Academic Press. https://doi.org/10.1016/s0580-9517(08)70573-7

Husarova, V. M., Lakatosova, S., Pivovarciova, A., Babinska, K., Bakos, J., Durdiakova, J., ... & Ostatnikova, D. (2016). Plasma oxytocin in children with autism and its correlations with behavioral parameters in children and parents. *Psychiatry investigation*, *13*(2), 174. https://doi.org/10.4306/pi.2016.13.2.174

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Paerregaard, A., ... & Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of Lactobacillus spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and environmental microbiology*, *65*(11), 4949-4956. https://doi.org/10.1128/aem.65.11.4949-4956.1999

Jones, S. E., & Versalovic, J. (2009). Probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors. *BMC microbiology*, *9*(1), 1-9. https://doi.org/10.1186/1471-2180-9-35

Kavanagh, A., Ramu, S., Gong, Y., Cooper, M. A., & Blaskovich, M. A. (2019). Effects of microplate type and broth additives on microdilution MIC susceptibility assays. *Antimicrobial agents and chemotherapy*, 63(1). https://doi.org/10.1128/aac.01760-18

Kong, X. J., Liu, J., Li, J., Kwong, K., Koh, M., Sukijthamapan, P., ... & Song, Y. (2020). Probiotics and oxytocin nasal spray as neuro-social-behavioral interventions for patients with autism spectrum disorders: A pilot randomized controlled trial protocol. *Pilot and feasibility studies*, *6*(1), 20. https://doi.org/10.1186/s40814-020-0557-8

Kralj, S., van Geel-Schutten, G. H., Van Der Maarel, M. J. E. C., & Dijkhuizen, L. (2004). Biochemical and molecular characterization of Lactobacillus reuteri 121 reuteransucrase. *Microbiology*, *150*(7), 2099-2112. https://doi.org/10.1099/mic.0.27105-0

LeBlanc, J. G., Chain, F., Martín, R., Bermúdez-Humarán, L. G., Courau, S., & Langella, P. (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial cell factories*, *16*(1), 1-10. https://doi.org/10.1186/s12934-017-0691-z

Leonardi, A., Zanoni, S., De Lucia, M., Amaretti, A., Raimondi, S., & Rossi, M. (2013). Zinc uptake by lactic acid bacteria. *International Scholarly Research Notices*, *2013*. https://doi.org/10.5402/2013/312917

Lew, L. C., Choi, S. B., Tan, P. L., & Liong, M. T. (2014). M n2+ and M g2+ synergistically enhanced lactic acid production by L actobacillus rhamnosus FTDC 8313 via affecting different stages of the hexose monophosphate pathway. *Journal of applied microbiology*, *116*(3), 644-653. https://doi.org/10.1111/jam.12399

López, D., Vlamakis, H., & Kolter, R. (2010). Biofilms. *Cold Spring Harbor perspectives in biology*, *2*(7), a000398. https://doi.org/10.1101/cshperspect.a000398

Macfarlane, S., & Dillon, J. F. (2007). Microbial biofilms in the human gastrointestinal tract. *Journal of applied microbiology*, *102*(5), 1187-1196. https://doi.org/10.1111/j.1365-2672.2007.03287.x

Mackay, A. D., Taylor, M. B., Kibbler, C. C., & Hamilton-Miller, J. M. (1999). Lactobacillus endocarditis caused by a probiotic organism. *Clinical Microbiology and infection*, *5*(5), 290-292. https://doi.org/10.1111/j.1469-0691.1999.tb00144.x

MacWilliams, M. P. (2012). Indole test protocol. *American Society for Microbiology, Washington, DC*. https://doi.org/10.21236/ada606526

Marrie, T. J., Nelligan, J., & Costerton, J. W. (1982). A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. *Circulation*, *66*(6), 1339-1341. https://doi.org/10.1161/01.cir.66.6.1339

McDermott, A. J., & Huffnagle, G. B. (2014). The microbiome and regulation of mucosal immunity. *Immunology*, *142*(1), 24-31. https://doi.org/10.1111/imm.12231

Millette, M., Cornut, G., Dupont, C., Shareck, F., Archambault, D., & Lacroix, M. (2008). Capacity of Human Nisin- and Pediocin-Producing Lactic Acid Bacteria To Reduce Intestinal Colonization by Vancomycin-Resistant Enterococci. *Applied and Environmental Microbiology*, 74(7), 1997–2003 https://doi.org/10.1128/aem.02150-07

Miralles, M. C., Flores, J., & Perez-Martinez, G. (1996). Biochemical tests for the selection of Staphylococcus Strains as potential meat starter cultures. *Food Microbiology*, *13*(3), 227-236. https://doi.org/10.1006/fmic.1996.0028

Mottet, C., & Michetti, P. (2005). Probiotics: wanted dead or alive. *Digestive and liver disease*, *37*(1), 3-6. https://doi.org/10.1016/j.dld.2004.09.010

Mu, Q., Tavella, V. J., & Luo, X. M. (2018). Role of Lactobacillus reuteri in human health and diseases. *Frontiers in microbiology*, *9*, 757. https://doi.org/10.3389/fmicb.2018.00757

Mulle, J. G., Sharp, W. G., & Cubells, J. F. (2013). The gut microbiome: a new frontier in autism research. *Current psychiatry reports*, *15*(2), 1-9. https://doi.org/10.1007/s11920-012-0337-0

Näse, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., ... & Meurman, J. H. (2001). Effect of long–term consumption of a probiotic bacterium, Lactobacillus rhamnosus GG, in milk on dental caries and caries risk in children. Caries research, 35(6), 412-420. https://doi.org/10.1159/000047484

Nair, P. S., & Surendran, P. K. (2005). Biochemical characterization of lactic acid bacteria isolated from fish and prawn. https://tspace.library.utoronto.ca/handle/1807/5203

Newcomer, J. W., Farber, N. B., & Olney, J. W. (2000). NMDA receptor function, memory, and brain aging. *Dialogues in clinical neuroscience*, *2*(3), 219. https://doi.org/10.31887/dcns.2000.2.3/jnewcomer

Nielsen, F. H., & Lukaski, H. C. (2006). Update on the relationship between magnesium and exercise. https://pubag.nal.usda.gov/catalog/46884

Novin, D., Seifan, M., Ebrahiminezhad, A., & Berenjian, A. (2021). The effect of iron oxide nanoparticles on Lactobacillus acidophilus growth at pH 4. *Bioprocess and Biosystems Engineering*, *44*(1), 39-45. https://doi.org/10.1007/s00449-020-02417-2

O'Toole G. A. (2011). Microtiter dish biofilm formation assay. *Journal of visualized experiments*: JoVE, (47), 2437. https://doi.org/10.3791/2437

Pachikian, B. D., Neyrinck, A. M., Deldicque, L., De Backer, F. C., Catry, E., Dewulf, E. M., ... & Delzenne, N. M. (2010). Changes in intestinal bifidobacteria levels are associated with the inflammatory response in magnesium-deficient mice. *The Journal of nutrition*, *140*(3), 509-514. https://doi.org/10.3945/jn.109.117374

Peacock, M. (2010). Calcium metabolism in health and disease. *Clinical Journal of the American Society of Nephrology*, *5*(Supplement 1), S23-S30. https://doi.org/10.2215/cjn.05910809

Reid, G., & Burton, J. (2002). Use of Lactobacillus to prevent infection by pathogenic bacteria. *Microbes and infection*, *4*(3), 319-324. https://doi.org/10.1016/s1286-4579(02)01544-7

Roos, S., & Jonsson, H. (2002). A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus componentsThe GenBank accession number for the sequence reported in this paper is AF120104. *Microbiology*, *148*(2), 433-442. https://doi.org/10.1099/00221287-148-2-433

Saginur, R. A. P. H. A. E. L., Clecner, B., Portnoy, J. O. S. E. P. H., & Mendelson, J. A. C. K. (1982). Superoxol (catalase) test for identification of Neisseria gonorrhoeae. *Journal of Clinical Microbiology*, *15*(3), 475-477. https://doi.org/10.1128/jcm.15.3.475-477.1982

Salas-Jara, M. J., Ilabaca, A., Vega, M., & García, A. (2016). Biofilm forming Lactobacillus: new challenges for the development of probiotics. *Microorganisms*, *4*(3), 35. https://doi.org/10.3390/microorganisms4030035

Salminen, M. K., Rautelin, H., Tynkkynen, S., Poussa, T., Saxelin, M., Valtonen, V., & Järvinen, A. (2004). Lactobacillus bacteremia, clinical significance, and patient outcome, with special focus on probiotic L. rhamnosus GG. *Clinical infectious diseases*, *38*(1), 62-69. https://doi.org/10.1086/380455

Salminen, M. K., Tynkkynen, S., Rautelin, H., Saxelin, M., Vaara, M., Ruutu, P., ... & Järvinen, A. (2002). Lactobacillus bacteremia during a rapid increase in probiotic use of Lactobacillus rhamnosus GG in Finland. *Clinical infectious diseases*, *35*(10), 1155-1160. https://doi.org/10.1086/342912

Sauer, A. K., & Grabrucker, A. M. (2019). Zinc deficiency during pregnancy leads to altered microbiome and elevated inflammatory markers in mice. *Frontiers in neuroscience*, *13*, 1295. https://doi.org/10.3389/fnins.2019.01295

Schwabe, R. F., & Jobin, C. (2013). The microbiome and cancer. *Nature Reviews Cancer*, *13*(11), 800-812. https://doi.org/10.1038/nrc3610

Shehata, M. G., El Sohaimy, S. A., El-Sahn, M. A., & Youssef, M. M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Annals of Agricultural Sciences*, 61(1), 65–75. https://doi.org/10.1016/j.aoas.2016.03.001

Skillern, J. K., & Overman, T. L. (1983). Oxidase testing from Kligler's iron agar and triple sugar iron agar slants. *Current Microbiology*, *8*(5), 269-271. https://doi.org/10.1007/bf01577726

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. A*PMIS: acta pathologica, microbiologica, et immunologica Scandinavica*, 115(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

Swinehart, D. F. (1962). The beer-lambert law. *Journal of chemical education*, *39*(7), 333. https://doi.org/10.1021/ed039p333

Tarrand, J. J., & Gröschel, D. H. (1982). Rapid, modified oxidase test for oxidase-variable bacterial isolates. *Journal of Clinical Microbiology*, *16*(4), 772-774. https://doi.org/10.1128/jcm.16.4.772-774.1982

Tilg, H., & Moschen, A. R. (2015). Food, immunity, and the microbiome. *Gastroenterology*, *148*(6), 1107-1119. https://doi.org/10.1053/j.gastro.2014.12.036

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature*, *449*(7164), 804-810. https://doi.org/10.1038/nature06244

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., & Knight, R. (2012). Defining the human microbiome. *Nutrition reviews*, *70*(suppl_1), S38-S44. https://doi.org/10.1111/j.1753-4887.2012.00493.x

Verdenelli, M. C., Ghelfi, F., Silvi, S., Orpianesi, C., Cecchini, C., & Cresci, A. (2009). Probiotic properties of Lactobacillus rhamnosus and Lactobacillus paracasei isolated from human faeces. *European journal of nutrition*, *48*(6), 355-363. https://doi.org/10.1007/s00394-009-0021-2

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, 3(2), 163–175. https://doi.org/10.1038/nprot.2007.521

Williams, N. T. (2010). Probiotics. *American Journal of Health-System Pharmacy*, *67*(6), 449-458. https://doi.org/10.2146/ajhp090168

Yang, Y., Huang, S., Wang, J., Jan, G., Jeantet, R., & Chen, X. D. (2017). Mg2+ improves the thermotolerance of probiotic Lactobacillus rhamnosus GG, Lactobacillus casei Zhang and Lactobacillus plantarum P-8. *Letters in applied microbiology*, *64*(4), 283-288. https://doi.org/10.1111/lam.12716

Yang, E., Fan, L., Yan, J., Jiang, Y., Doucette, C., Fillmore, S., & Walker, B. (2018). Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *Amb Express*, *8*(1), 1-14. https://doi.org/10.1186/s13568-018-0536-0

Yilmaz, B., & Li, H. (2018). Gut microbiota and iron: the crucial actors in health and disease. *Pharmaceuticals*, *11*(4), 98. https://doi.org/10.3390/ph11040098

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., ... & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International journal of systematic and evolutionary microbiology*, *70*(4), 2782-2858. https://doi.org/10.1099/ijsem.0.004107

Zendeboodi, F., Khorshidian, N., Mortazavian, A. M., & da Cruz, A. G. (2020). Probiotic: conceptualization from a new approach. *Current Opinion in Food Science*, *32*, 103-123. https://doi.org/10.1016/j.cofs.2020.03.009