Periodicals of Engineering and Natural Sciences ISSN 2303-4521
Vol. 8, No. 1, March 2020, pp.552-561

Adherence of prebiotic fibers, antioxidants and B complex vitamins on the survival of probiotics

Mirela Musić[1], Amina Aščerić[1], Esma Kurtanović[1], Neira-Una Hrapović[1], Almira Bogdanić[1], Amar Ćosić[1], Altijana Hromić-Jahjefendić[1]
[1] International University of Sarajevo, Department of Genetics and Bioengineering

ABSTRACT

The studies on the relationship between probiotics and prebiotics as well as probiotics and antioxidants have gained significant increase in popularity over the past decade. However, exposure to antibiotics can result in disrupting the human microbiome, causing several related diseases. Additionally, probiotics can lose some of their beneficial effects when in contact with stomach acid which has a pH between 2 and 3. The purpose of this study was to examine the effects of prebiotic fibres and antioxidants and B complex vitamins on the survival of probiotics. When combined with those, a sufficient amount of probiotics survives and carries out their beneficial functions. This study points out the importance of mentioned compounds in maintaining a healthy organism as well as their role in preserving the life of probiotics in our gut.

Keywords: Probiotics, Prebiotics, Antibiotics, B complex vitamins, Gastrointestinal (GI) tract

Corresponding Author:
Asst. Prof. Dr. Altijana Hromic-Jahjefendic
Department of Genetics and Bioengineering
International University of Sarajevo, Sarajevo, Bosnia
Hrasnička cesta 15
E-mail: ahromic@ius.edu.ba

1. Introduction

1.1 Probiotics

These bacteria are naturally created by fermentation in foods. Fermented milk was the first food containing microorganisms [1]. Even though the purposes of probiotics are still under the investigation, some benefits include improvement of the immune system, prevention and treatment of diarrhea, boost of mental health, decrease in blood pressure, weight loss, prevention of food poisoning, and the production and absorption of vitamins, especially B vitamins [2]. In order to be considered as a probiotic, bacteria should have some properties, which include survivability, adhesion to epithelial cells, and antimicrobial activity against pathogenic bacteria. When we talk about survivability, bacteria should have the ability to survive in the stomach and small intestines. However, many of them are not active at pH lower than 4 and it has been found that 99% of them were killed within 30 minutes at pH 3.

1.2 Prebiotics

Prebiotics are defined as a substrate that is selectively used by the host microorganism to produce a health benefit [4]. These substrates are non-digestible fibers, such as apple skin, which have the ability to stimulate growth and activity of healthy bacteria in gut microbiota by passing through the digestive system “unchanged” [4]. However, in the large colon, they become fermented and by that process, they feed beneficial bacteria colonies and help to increase the number of desirable bacteria in our digestive systems. Some of the benefits of prebiotics include enhancement of mineral absorption, potential anti-cancer properties, anti-inflammatory and other immune-assisting effects, encouragement of normal blood sugar levels, boost of bone health and aid in weight loss, and decreases the risk of cardiovascular disease [5]. Researchers have also found that prebiotics can help with an increase of the number of helpful bacteria that live in our gut, which can reduce risk of disease and improve general well-being [6].

1.3 Antioxidants

Antioxidant is a substance that can successfully remove prooxidants by creating products that are not toxic and do not damage the cells. Antioxidants protect the body from prooxidative action in several ways: inhibiting the formation of ROS/RNS (reactive oxygen/reactive nitrogen species), reducing the oxidative ability of prooxidants, chelating with metal ions, inhibiting oxidative enzymes [7]. Thus, the function of the antioxidant is the neutralization of free radicals and the protection of the cells against their toxic activity, which prevents the occurrence and the development of diseases associated with oxidative stress.

Antioxidants are formed in the cell or fed into the body by food or by supplements. They act to prevent the formation of new free radicals in the body, destroy the already created radicals (scavengers) or repair damage to the cells created by their action [8]. According to the method of action of antioxidants, we can separate them into three different groups: primary, secondary and tertiary. Primary antioxidants have a preventive effect of reducing the formation of free radicals (albumin, transferrin). Secondary antioxidants (cleaners) remove the resulting free radicals (superoxide-dismutase, catalase, glutathione peroxidase, glutathione, vitamin C, vitamin E, carotenoids, flavonoids). Tertiary antioxidants (repair enzymes) repair defects or remove biomolecules of the damaged radicals before their accumulation causes new damage (phospholipases, proteases, peptidases, DNA polymerase I etc.) [9]. Antioxidant activity depends on many factors, among which the most important are bioavailability, oxidative potential, reaction speed with free radical, stability, and low reactivity of the resulting derivative antioxidant-free radical [10].

1.4 B complex vitamins

There are eight B-group vitamins (B_1, B_2, B_3, B_5, B_6, B_7, B_9 and B_{12}) and most of them play an important role in crucial energy metabolism reactions and biosynthesis [2], [11], [12]. Therefore, their effect on the growth of probiotics was intensively studied [11], [13], [14].

For most of the brewery strains studied, both vitamin B5 (pantothenic acid), and to a lesser degree, vitamin B3 (niacin) were essential, while only several of them needed vitamin B2 (riboflavin). Vitamin B6 (pyridoxine), B9 (folic acid) and B1 (thiamin) were mostly found to be non-essential [14]. This was in contrast to findings from *Lactobacillus* isolated from the milk, which generally requires vitamin B7 (biotin) and B9. More importantly, this early work hinted that some species and strains could synthesize vitamin B9 even though, neither B9 nor needed precursors were added to the growth medium [14].

Research conducted in the past two decades confirmed and elaborated the early findings from the Fifties. Single stains of *Lactobacillus*, both newly identified or reclassified were tested and their need for vitamins as well as their ability to synthesize them is well documented. It became common knowledge that many individual probiotic strains may require several vitamins from the B group (in a few mg/L range) for normal growth [11], [13], [15], [16]. Needs of these bacteria were evolutionarily conditioned by the abundance of the vitamins in the environments where these bacteria thrive.

Currently, the focus of main research on probiotic bacteria and vitamin B shifted from their need for these vitamins to their ability to produce these important vitamins because such production is seen as an important component of the benefits which are claimed to be delivered (“conferred”) by these bacteria to humans as their host. Many known probiotic strains were found to produce B-group vitamins [11].

1.5 Antibiotics

Antibiotics are powerful drugs that fight infections and can cure diseases when used appropriately. They either prevent microbes from duplicating or kill them. Before bacteria can divide and cause symptoms, the immune system can typically destroy them. White platelets (WBCs) attack harmful bacteria and, even if symptoms still occur, the immune system can usually adapt and fight off the infection. However, if the number of harmful bacteria is highly increased, more so excessive, the immune system usually cannot fight them all. In such cases, antibiotics are very useful.

Penicillin belongs to the group of drugs, all of which have a common fundamental structure. The key structural component of penicillin is the β-lactam ring. It has been the most widely utilized antibiotic for many gram-positive bacterial infections. Penicillin is beneficial in the treatment of bacterial infections caused by susceptible, usually gram-positive organisms [17].

Ampicillin is a β-lactam antibiotic that has been utilized broadly to treat bacterial diseases since 1961 [17]. It can occasionally result in allergic responses that extend in severity from a rash to potentially deadly hypersensitivity. In addition, ampicillin is able to penetrate gram-positive and some gram-negative bacteria. It acts as an inhibitor of the bacterial cell wall synthesis, which ultimately leads to cell lysis – death of the cell [17].

The main purpose of this study was to demonstrate how different prebiotic fibers, antioxidants and vitamins from B complex influence the survival of probiotics at the low pH and in the presence of antibiotics.

2. Materials and methods

All chemicals used for the media preparation and analysis were purchases from Sigma Aldrich. Probiotics supplements used in the study were containing 14 different probiotic strains (Protexin, UK). According to the manufacturer’s specification, each capsule contained 14 strains of probiotics bacteria, corresponding to minimum 2 billion live microorganisms per capsule, i.e., 2x109 colony forming units (CFU) per capsule, equivalent to 10 billion live microorganisms per gram (1x1010 CFU/gram). The composition included four strains of *Bifidobacteria*, seven strains of *Lactobacilli*, and three remaining strains belong to *Bacillus*, *Lactococcus* and *Streptococcus.*

2.1 Media preparation

The simulated gastric fluid (SGF) was prepared as per Rao *et al.* (1989). The pH of the simulated gastric fluid (SGF) was adjusted using a pH meter (CyberScan, USA) and balanced with 1 M HCl to the value of 3, which corresponds to the real pH in the human gut after admission of the food. In 100 mL of sterile MRS broth prepared according to the manufacturer’s protocol, a capsule supplement containing 14 probiotic strains described previously was incubated for 12 hours at 37°C. This stock was used for the further growth and measurement.

2.2 Preparation of a prebiotics, antioxidants and antibiotics

In this study, apple skin and oatmeal extract were used as a prebiotic source. 10 g of each was chopped in smaller pieces using pestle and mortar and dissolved in 10 mL of distilled water. The suspension was slightly warmed and filtered through the filter paper. Filtered suspensions were kept in the fridge protected from light.

Green and mint teas were used as antioxidant source for this study. 15 g of each (3 tea bags) were infused separately in 20 mL of distilled, boiled water and left for 10 minutes at room temperature. 1mL of each was used for the growth of probiotics.

Three different concentrations of antibiotics were made (250, 500, 1000mM). The stock solution of penicillin and ampicillin used were added accordingly to the final desired mM concentration. Millipore filter disc was used for filtration and injection of antibiotic solutions into Eppendorf tubes, which makes them ready to use afterwards.

2.7 Growing bacterial cultures and measuring optical density

After incubation, probiotic strains were inoculated under the laminar flow hood and in close proximity to the Bunsen burner flame, as to ensure no contamination occurs. The tubes contained a medium at pH 3 without any added prebiotics, a medium with 5mL of apple skin extract and with 5mL of oatmeal extract, for the experiment of investigating the impact of prebiotics on the lives of probiotics at acidic medium. In case of antioxidants, 1mL of green tea infusion and 1mL of mint tea infusion were added, separately, to 30 mL medium with pH3.

In tests for effect of antibiotics in addition to prebiotics, each tube contained 5mL of medium, 1mL of probiotics, 1mL of prebiotic and 6μL of antibiotic; with the prebiotic, as well as the type and concentration of antibiotic differing from tube to tube. The same ratio was used for the experiment including antioxidants in mixture with antibiotics, instead of the prebiotics.

Tubes with vitamin B were separated into tubes with vitamin B_6, vitamin B_{12} and B complex. 0.33g of Vitamin B complex capsules (dm, Germany) was added to 10mL of the medium. 2mL of vitamin B_6 and vitamin B_{12}, from ampules, was added to 10mL of the medium, separately.

When the contents are properly pipetted in each tube, they were placed in an incubator at 37 ℃ for 2h. MultiskanTM FC Microplate Photometer (Thermo Fisher Scientific, USA) and 96 well plate is used for the OD_{600} measurements, where each well was filled with 300μL of sample from each tube. The first measurement was done after 2 h and the others were performed in the following manner: after 4h, 6h, 8h, 10h and 24h.

2.8 Statistical analysis

All experiments were performed in triplicates and statistical analysis was performed in GraphPad Prism 8 (GraphPad Spftware, La Jolla, CA, USA). Data are shown as the mean ± SD and significance was determined using ANOVA (two-way) and Tukey’s test. Values of $P<0.05$ were considered statistically significant.

3. Results and discussion

3.1 Effect of prebiotics

In order to estimate the survival of the probiotic culture in acidic pH in the presence of the fibers, the samples with added apple and oatmeal were compared to the ones without prebiotics after 2, 4, 6, 8, 10 and 24h. As shown in Figure 1., the highest growth of bacteria was observed with oatmeal, while as expected, samples without the prebiotic source showed the lowest growth rate. The number of bacteria present in samples with oatmeal was significantly higher than in samples without prebiotics, after every 2 hours. Also, the number of probiotics was significantly higher in the samples with addition of apple than in the samples without prebiotics after 6 and 24 hours of incubation in pH 3.

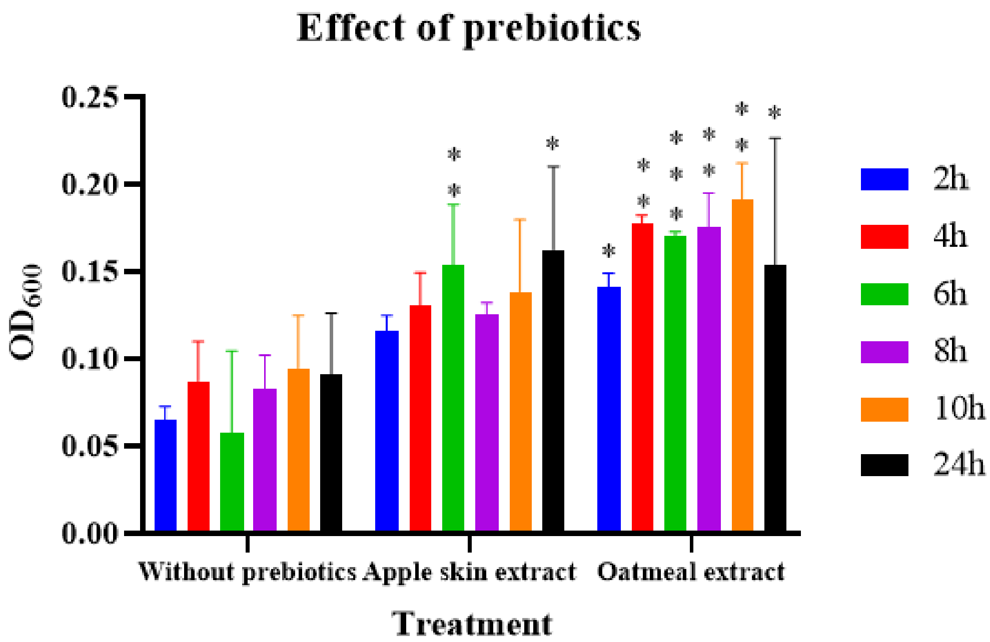

Figure 1: Effect of prebiotic fibers on the growth of probiotics at pH 3, *p<0.05, **p<0.01, ***p<0.001

3.2 Effect of antioxidants

As presented in Figure 2., results show differences between antioxidant effect on bacterial growth at pH 3. Green tea infusion had the highest positive effect on probiotic growth, as it was constantly increased every 2 hours. Mint tea infusion also showed positive effects however, not as noticeable as with the green tea. The number of bacteria after 10 and 24 hours of incubation in acidic medium was significantly higher in the samples with added antioxidants than in the samples without any addition.

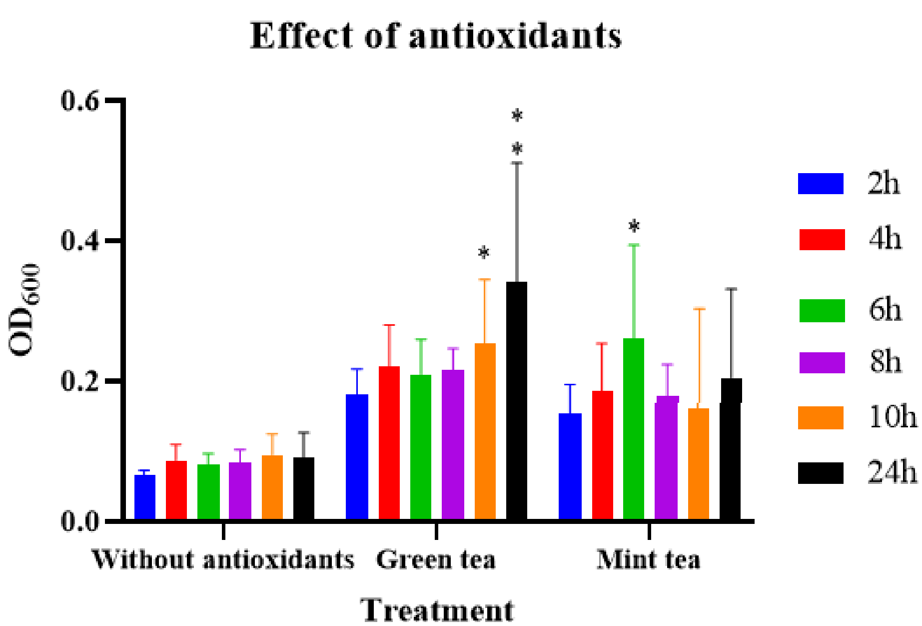

Figure 2: The influence of green tea and mint tea on bacterial growth at pH 3, *p<0.05, **p<0.01

3.3 Effect of vitamin B

Figure 3., shows the effect of vitamin B on the growth of bacteria in acidic medium. The highest growth at pH 3 was observed with B complex added to the medium, and the difference between this treatment and treatment with B_6 and B_{12} was significant. For treatments with B_6 and B_{12}, growth was as low as in the vitamin-free control, and means for all three at pH 3 were not significantly different from 2 to 10 hours of incubation.

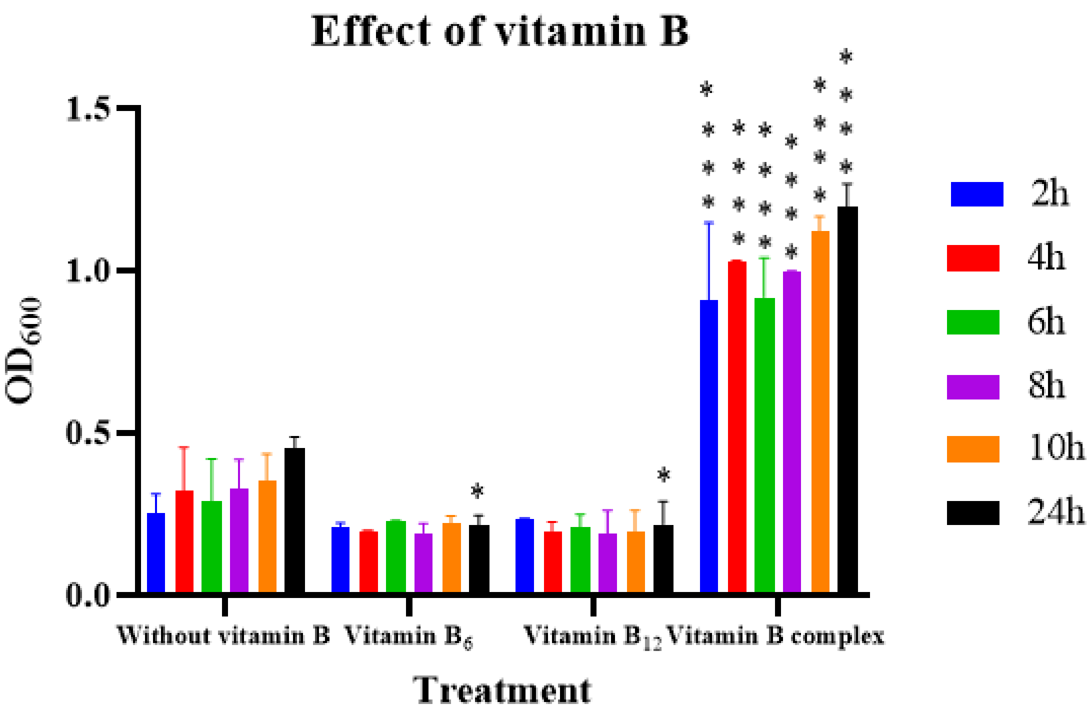

Figure 3: Effect of vitamin B on the growth of probiotics at pH 3. *p<0.05, ****p<0.0001

3.4 Prebiotic effect on the bacterial growth in the presence of antibiotics

Figure 4. shows difference in bacterial concentrations measured in samples with apple skin extract, oatmeal extract and with no prebiotic fiber. All of the samples contained the same amount and type of growth medium and probiotic strains, but different type of antibiotic (ampicillin or penicillin) and their concentrations (250mM, 500mM, 1000mM).

In compliance with the line chart, there was a remarkable difference in the bacterial growth in samples containing prebiotics, when compared to the ones lacking it. Even in such low pH value as pH 3, probiotic strains were able to grow in far greater quantities when they are supplemented with prebiotic fibers. It is especially noteworthy to mention the effect of oatmeal extract on the bacterial growth, which seemed to be significantly more efficient in promoting the growth of probiotics than apple skin extract.

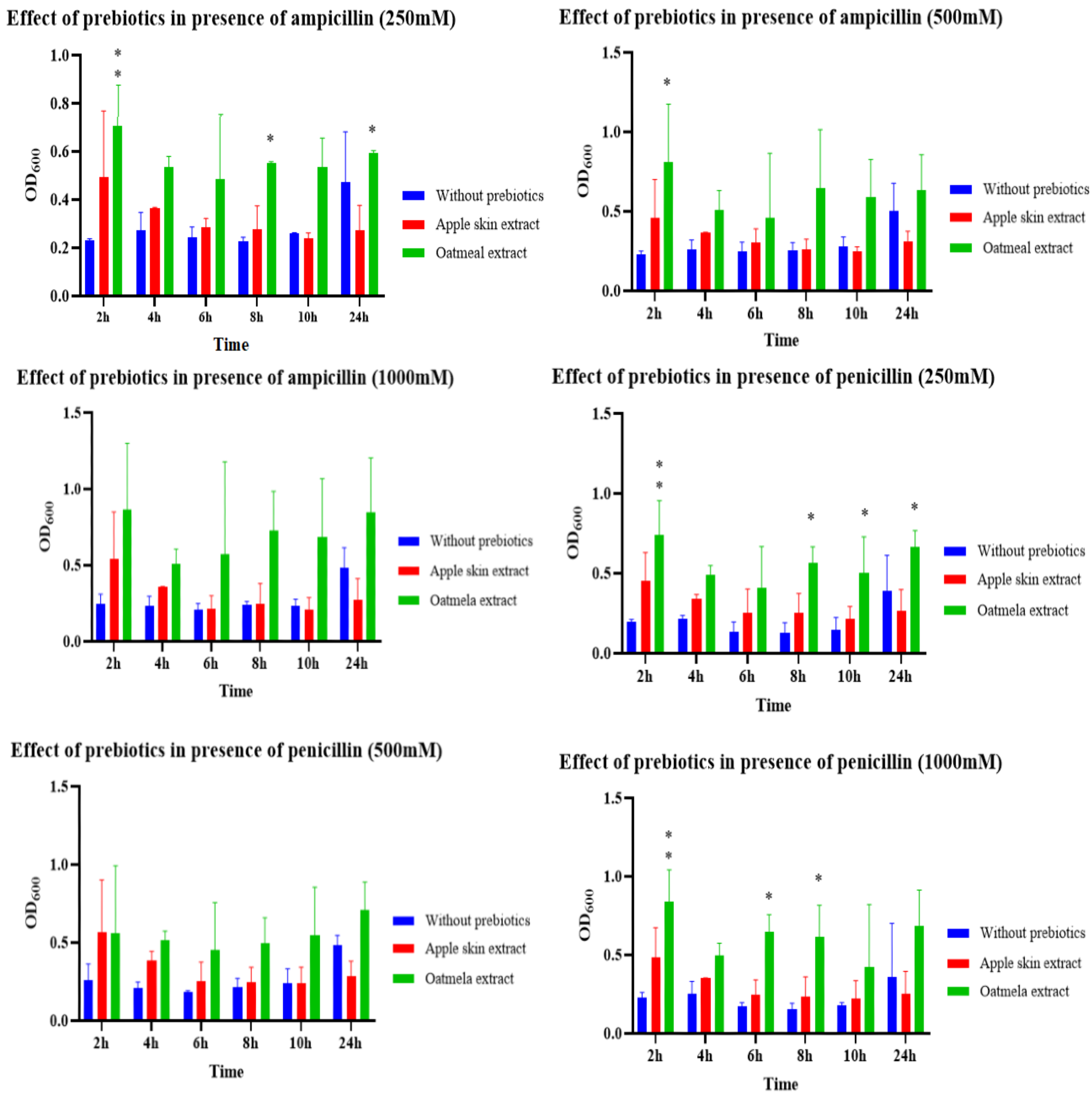

Figure 4: Prebiotic effect on the bacterial growth in the presence of antibiotics at pH 3, *p<0.05, **p<0.01

3.5 Antioxidant effect on bacterial growth in presence of antibiotics

Figure 5. shows that even when antibiotics were present in the concoction of antioxidants, the growth of bacteria was not affected, nonetheless, the bacteria were replicated even more. Green tea showed greater effect on bacterial growth compared to mint tea. We can observe that samples with antioxidants exhibit approximately a two-fold increase of bacteria which supported our hypothesis. Antioxidant protection system is highly sophisticated to protect the cells and organs of the body against reactive oxygen species.

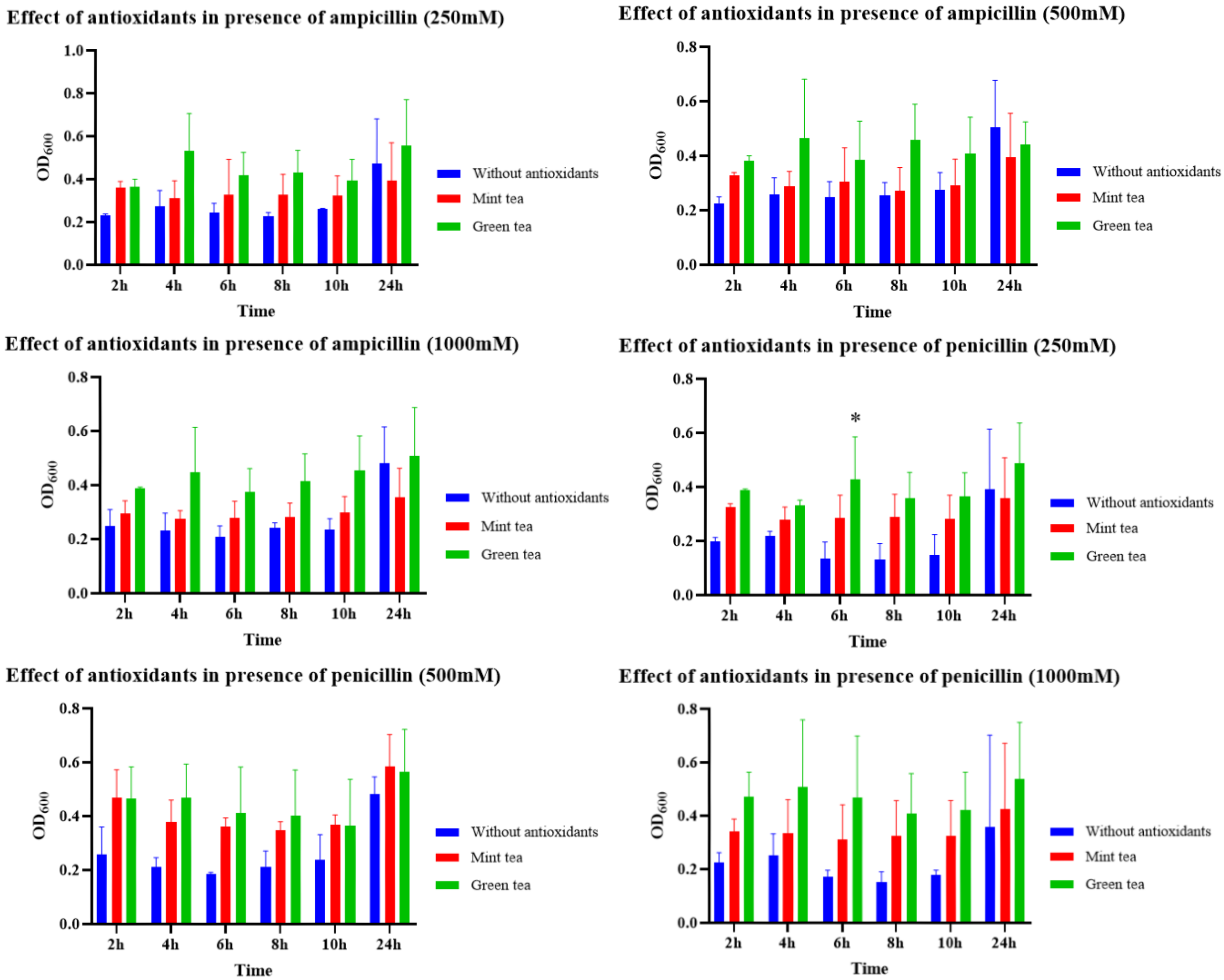

Figure 5: Antioxidant effect on bacterial growth in the presence of antibiotics at pH3, *p<0.05

4. Conclusion

According to previous research of Chung et al. [18], several probiotic strains involved in the generation of anti-inflammatory IL-10 were able to utilize apple pectin as a prebiotic fuel for growth. According to the data obtained in this study, the apple skin extract as a source of prebiotics proved the capacity to enhance the growth of bacteria without the antibiotic, but also to positively impact the concentration of bacteria in samples that contained the antibiotics. In the study of Carlson *et al*. [19], it was demonstrated that oatmeal as a source of prebiotic fiber had the highest production of propionate at 24h and increased the beneficial genus *Collinsella*. This study confirmed the favorable impact of oatmeal on the growth of probiotics in the absence of antibiotics. Oatmeal extract also demonstrated a highly beneficial effect on the growth of the bacteria in both the acidic and neutral environments, being notably more favorable in increasing the concentration of probiotics than the apple skin extract as a prebiotic source. Furthermore, it appears that the probiotic strains used in this study are more susceptible to the bactericidal effects of penicillin, which reduces the concentration of probiotics significantly more than its companion ampicillin, considerably lowering the bacterial growth.

One of the objectives of this experiment was to analyze the effect of antioxidants in addition to an acidic medium together with probiotics, as well as the relationship between these variables in the presence of aggressive antibiotics. It has been shown that, when exposed to pH 3, antioxidants provided the protective effect on probiotics and allowed them to grow over 24 hours. Regarding the comparison between mint and green tea, it can be concluded that green tea is a better protective agent for probiotics. According to previous studies, this examination also confirmed that among all antioxidants analyzed, green tea showed the highest optical density in medium at pH 3 during the entire measurement [20]. After an influx of antibiotics, which have their unique activation time, bacterial growth decreases depending on the concentration and type of the antibiotic used.

Regarding the effect of vitamin B on the growth of bacteria, the highest growth at pH 3 was observed with the B complex added to the medium. For treatments with B_6 and B_{12}, growth appeared as low as in the vitamin-free samples. Study confirmed that the vitamin B complex provided better conditions for culture survival at pH 3 than Vitamin B_6 and B_{12}.

In summary, our results suggest that an addition of prebiotics and a vitamin B complex to probiotics has a positive effect of the survival of those healthy bacteria in our gut, even in the presence of antibiotics. Every future research on probiotics and prebiotics is important for development of symbiotics, which provides even greater benefits than the intake of probiotics alone. Furthermore, different types of supplements of antioxidant compounds may have a positive effect on probiotic growth and protect us from aggressive drugs such as ampicillin and penicillin, even in the media at pH 3, which is the closest to the pH of the gastrointestinal tract.

References

[1] R. Fuller, *Probiotics: The Scientific Basis*, 1st ed. London: Chapman & Hall, 1992.

[2] P. Markowiak and K. Śliżewska, “Effects of Probiotics, Prebiotics, and Synbiotics on Human Health,” *Nutrients*, vol. 9, no. 9, pp. 1201, 2017.

[3] G.R. Gibson, H.M. Probert, J. van Loo, R.A. Rastall, M.B. Roberfroid, “Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics,” *Nutr. Res. Rev*, vol. 17, pp. 259-275, 2004.

[4] D.J. Goossens, M. Russel, A. Thijs, A. van den Bogaard, E. Stobberingh, R. Stockbrügger, “Survival of the probiotic, L. plantarum 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition”, *Digestive and Liver Disease*, vol. 37, no.1, pp. 44-50, 2005.

[5] J. Slavin, “Fiber and prebiotics: mechanisms and health benefits,” *Nutrients*, vol. 5, no. 4, pp. 1417-35, 2013.

[6] A. Florowska, K. Krygier, T. Florowski, E. Dluzewska, "Prebiotics as functional food ingredients preventing diet-related diseases," *Food and Function*, vol. 7, no. 5, pp. 2147-55, 2016.

[7] M. Segundo, L. Magalheas, S. Reis, “Methodological aspects about in vitro evaluation of antioxidant properties,” *Anal Chim Acta.*, vol. 613, no. 1, pp. 1-19, 2008.

[8] P.C. Wootton-Beard, L. Ryan, “Improving public health: The role of antioxidant-rich fruit and vegetable beverages,” *Food Research International*, vol. 44, no. 10, pp. 3135-3148, 2011.

[9] D. Huang, B. Ou, RL. Prior, “The Chemistry behind Antioxidant Capacity Assays,” *J Agric Food Chem.*, vol. 53, no. 6, pp. 1841-56, 2005.

[10] RH. Bisby, R. Brooke, S. Navaratnam, “Effect of antioxidant oxidation potential in the oxygen radical absorption capacity (ORAC) assay,” *Food Chem.*, vol. 108, no. 3, pp. 1007-7, 2008.

[11] J.G. LeClanc, F. Chain, R. Martin, L.G. Bermúdez-Humarán, S. Courau, P. Langella, “Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria,” *Microbial cell factories*, vo. 16, no. 1, pp. 77, 2017.

[12] J.G. LeBlanc, J.E. Laino, M.J. del Valle, V. Vannini, D. van Sinderen, M.O. Taranto, G.F. de Valdez, G.S. de Giori, F. Sesma, “B-group vitamin production by lactic acid bacteria-current knowledge and potential applications,” *J Appl Microbiol.*, vol. 111, no. 6, pp. 1297-309, 2011.

[13] S.A. Hayek, S. Ibrahim, “Current Limitations and Challenges with Lactic Acid Bateria: A Review,” *Food and Nutrition Sciences*, vol. 4, no. 11, pp. 73-87, 2013.

[14] C. Russell, R. Bhandarri, K. Walkert, “Vitamin requirements of thirty-four lactic acid bacteria associated with brewery products,” *J. gen. Microbiol.*, vol. 10, pp. 371-376, 1954.

[15] A. Wegkamo, B. Teusink, W. De Vos, E. Smid, “Development of a minimal growth medium for *Lactobacillus plantarum*,” *Letters in Applied Microbiology*, vol. 50, no. 1, pp. 57-64, 2010.

[16] C. Yao, J. Chou, T. Wang, H. Zhao, B. Zhang, “Pantothenic Acid, Vitamin C, and Biotin Play Important Roles in the Growth of *Lactobacillus helveticus*,” *Front. Microbiol*, vol. 9, pp.1194, 2018.

[17] A. Ashangar, N. Gharib Naseri, “Analysis of Three Penicillin Antibiotics (Ampicillin, Amoxicillin and Cloxacillin) of Several Iranian Pharmaceutical Companies by HPLC,” *E-Journal of Chemistry*, vol. 4, no. 4, 2007.

[18] W.S. Chung, A.W. Walker, P. Louis, J. Vermeiren, D. Bosscher, S.H Duncan, H.J Flint, “Modulation of the human gut microbiota by dietary fibers occurs at the species level,” BMC Biol., vol. 14, no. 3, 2016.

[19] J.L. Carlson, J.M. Erickson, J.M. Hess, T.J. Gould, J.L. Slavin, “Prebiotic dietary fiber and gut health: Comparing the in vitro fermentations of beta-glucan, inulin and xylooligosaccharide,” *Nutrients*, vol. 9, no. 12, pp. 1361, 2017.

[20] D. Najgebauer-Lejko, “Effect of green tea supplementation on the microbiological, antioxidant, and sensory properties of probiotic milks,” Dairy Sci Technol., vol. 94, no. 4, pp. 327-339, 2014.