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Utilization of purple sweet potato synbiotic drink as a source of lactic acid bacteria exopolysaccharides for immunomodulation

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ABSTRACT

Immunomodulators, such as exopolysaccharides (EPS), can be found in products processed through lactic acid bacteria (LAB) fermentation. Purple sweet potatoes have the potential to be used as ingredients for making synbiotic drinks because of the high content of oligosaccharides. This study aimed to determine the effectiveness of purple sweet potato synbiotic drinks in improving the immune system in vivo. The experiment was conducted on male mice (Balb-C, 12 weeks old, 25±5 g BW) which were given purple sweet potato synbiotic drink for 14 days with doses of A1, A2, and A3 (50, 100, 150 mg/kg BW) given once a day. On the 14th day, the mice were induced with *S. aureus* bacteria given intraperitoneally (1 mL, 108 cfu/mL). The immunomodulation-related parameters measured were phagocytic activity, the number of lymphocyte cells, and the relative spleen weight of mice. The results showed that the synbiotic drink of purple sweet potato (A1, A2, and A3) can increase phagocytic activity and lymphocyte cell count and have a significant effect on relative spleen weight (p<0.05). The higher the dose of synbiotic drink, the higher the phagocytic activity and the number of lymphocyte cells, and the smaller the relative spleen weight of the mice.

Keywords: exopolysaccharides, phagocytosis, immunomodulators, synbiotic drinks, purple sweet potatoes

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INTRODUCTION

A global health and socioeconomic crisis has been brought on by the COVID-19 pandemic. COVID-19 (Corona Virus Disease 2019) is a disease outbreak that can infect the respiratory tract in humans. According to the COVID-19 Task Force (2021), as of July 27, 2021, in Indonesia, there are a total of 2.95 million cases. COVID-19 is troubling because its spread can occur very quickly through contact between humans and humans. The immune system is very important to protect the body from viral infections. A good immune system is needed by the body to be able to fight various diseases that develop in today's society. If the immune system works properly, the body will be resistant to bacterial and viral infections and foreign substances. One of the efforts to prevent bacterial and viral infections is by increasing the body's resistance and the effectiveness of the body's immune system. Recovery from a viral infection can be carried out without special treatment if a person's immune system is strong. This is because the virus can be self-medicated (Syahrir et al., 2020).

One of the efforts that can be made to build an immune system in the pandemic and post-pandemic era is to maintain nutritional intake in foods and drinks containing immunomodulatory compounds. The function of immunomodulators is to improve the immune system, namely by stimulation (immunostimulant) or suppressing/normalizing abnormal immune reactions (immunosuppressants). Immunomodulators are needed because they can affect the immune system and the patient's condition against the spread of the disease, as in the case of adjuvant therapy involving bacterial, fungi, or viral infections (Tjandrawinata et al., 2017). In research conducted by (Wasser, 2011) it is known that exopolysaccharides (EPS) are one of the immunomodulatory substances that can increase the secretion of anti-inflammatory cytokines.

An EPS is a polysaccharide resulting from the secretion of lactic acid bacteria (LAB) released on the extracellular around the cell. It has been well documented that lactic acid bacteria are capable of producing long-chain extracellular polysaccharides (EPS) (Imran et al., 2016). EPS produced by lactic acid bacteria in fermented food products acts as an enhancer of taste and texture. Currently, the exploration of EPS-producing LAB is increasing because the ability of LAB to synthesize EPS is considered important for health. Some health facts relate to the ability of probiotic strains to attach to the intestinal mucosa. EPS produced from LAB can attach to the mucosa of the small intestine, thereby increasing the ability to suppress the growth of pathogenic bacteria (Ruas-Madiedo & de los Reyes-Gavilán, 2005). EPS contributes to human health because it has anti-tumoral, antiulcer, anti-inflammatory, and anti-infective activity and boosts the body's immune system (immunostimulator). In addition, EPS is useful as a natural stabilizer and thickener in products such as yogurt.

One of the products that can produce EPS is synbiotic drinks. Synbiotics are a combination of prebiotics and probiotics that have a very important role in health. Probiotics are living microbes that have therapeutic or preventive health benefits when consumed. The most widely used prebiotic component is the oligosaccharide group, such as those found in bananas, apples, corn, potatoes, and tubers, including purple sweet potatoes. According to research by (Sancho et al., 2017) purple sweet potatoes have a high content of oligosaccharides compared to taro and cassava. Oligosaccharides found in purple sweet potatoes can be one of the good prebiotics for LAB nutrition in purple sweet potato synbiotic drinks. The fermentation process in synbiotic products can increase the amount of EPS produced by LAB (Surono & Hosono, 2011). The purpose of this study is to be able to determine the effectiveness of purple sweet potato synbiotic drinks in increasing the immune system in vivo.

MATERIALS AND METHOD

Materials

The materials were used for this research are fresh milk obtained from farmers in Pujon district, East Java, Indonesia. Purple sweet potato and skimmed milk were bought at the traditional market. *Bifidobacterium longum* FNCC 0210, *Lactobacillus bulgaricus* FNCC 0041, and *Lactobacillus plantarum* FNCC 0020 obtained from the microbiology laboratory of Gadjah Mada University

Indonesia. The test bacteria used is *Staphylococcus aureus* (1 x 10^8 CFU/mL) obtained from the Bioscience Laboratory, Faculty of Mathematics and Natural Sciences, University of Brawijaya Malang.

Methods

Preparation of purple sweet potato synbiotic drink

Synbiotic drinks were prepared by a modified method of Khairani et al. (2020). The ingredients were fresh milk of 150 mL, skimmed milk (4% w/v), glucose (7% w/v), and purple sweet potato paste (0%, 5%, 10% and 15% v/v) was pasteurized at 80 °C for 15 minutes, then cooled to a temperature of 40-45 °C. Furthermore, it was inoculated using a LAB starter (*B. longum*, *L. bulgaricus*, and *L. plantarum*) with concentrations (5% and 7% (v/v)), then homogenized and incubated at a temperature of 37 °C for 12 hours so that the synbiotic drink of purple sweet potato was produced.

Determination of EPS level within purple sweet potato synbiotic drink

The determination procedure of EPS level was conducted based on (Rimada & Abraham, 2003). The LAB isolates of purple sweet potato synbiotic drink were put into a centrifuge tube of 10 mL and heated in boiling aquades for 15 minutes. After cooling, it was added with 1 mL of trichloroacetic acid, and then the sample was incubated at 4°C for 24 hours. It was followed by cell separation through a cold centrifugation process at 4°C, 6000 rpm for 15 minutes. The supernatant obtained was added with ethanol and acetone in a ratio of 1:1. Then, it was continued again with cell separation through a cold centrifugation process at 4°C 6000 rpm for 15 minutes. The pellets obtained were weighed as EPS. The EPS level was expressed in the g/L sample.

Preparation of animal subjects

The immunomodulatory effect of synbiotic drinks was carried out on twenty five 12 weeks old Balb/c mice weighing 25±5 g. The mice were obtained from the Animal Physiology Laboratory, State University of Islamic, Malang, East Java, Indonesia, and were first subject to ethical approval by the competent ethics committee (No.E.5.a/057/KEPK-UMM/III/2022). They were divided into 5 treatment groups. A1 was given a purple sweet potato synbiotic drink at a dose of 50 mg/kg BW. A2 was given a purple sweet potato synbiotic drink at a dose of 100 mg/kgBW. A3 was given a purple sweet potato synbiotic drink at a dose of 150 mg/kg BW. K(+) injected *Staphylococcus aureus* bacteria. K(-) who was not given special treatment. The mice are placed in 5 cages consisting of 5 mice each. They were placed in a clean place at room temperature with sufficient ventilation and lighting. Then, the mice were acclimatized for 7 days with the administration of water, feed, air, and laboratory conditions. The feed and drink given during acclimatization was BP-2 feed and mineral water (*ad libitum*). On the 14^{th} day, the mice in each group were infected with 0.5 mL of suspension of *Staphylococcus aureus* injected intra-peritoneally (IP) and then left for 1 hour before surgery.

Surgery procedure of animal subjects

Mice were anesthetized using ether until fainting and then placed on a surgical board. Their abdomen was cleaned with 70% ethanol and then dissected using a sterile surgical tool. If a small amount of peritoneal fluid was found in the stomach, a sterile phosphate-buffered saline (PBS) was added with pH of 7.8 as much as 1-2 mL, and the abdomen was shaken with two fingers carefully, then the peritoneal fluid was taken using a 1 mL syringe.

Examination of the number of active macrophage cells

The peritoneal fluid that had been taken was dripped on the glass object, and as much as 1 drop was made, a thin smear preparation, after which it was fixed in the air until it dried. After drying, methanol was given and left for five minutes, and then 10% of Giemsa dye was given and left for 20 minutes. After that, it was rinsed under running water and dried. The preparation was added with immersion oil and then observed using an electric microscope with objective magnification of 10x and 100x. The

phagocytic activity of macrophage cells was calculated. Phagocytic activity was established based on the percentage of phagocytes performing phagocytosis from 100 phagocytes (Akrom et al., 2015).

$$Phagocytic\ Activity\ (\%) = \frac{Number\ of\ active\ macrophage\ cells}{Total\ number\ of\ macrophage\ cells} \times 100\% \quad \text{..... (1)}$$

Calculation of relative spleen weight

The spleen was taken from the abdominal cavity of the mice once the peritoneum was opened. After the spleen was removed, the accompanying connective tissue was cleared. After it was completely clean, then the spleen was weighed using a digital scale. Relative spleen weight was calculated by dividing the spleen weight by the total body weight and multiplying by 100 to get a percentage (Akrom et al., 2015).

Calculation of the number of lymphocytes cell

The calculation of the number of lymphocytes was carried out by placing the spleen on a petri dish containing 1.5 mL of RPMI (*Rosewell Park Memorial Institute*) medium and then crushed until smooth. It was washed with 10 mL PBS and then centrifuged at a speed of 10,000 rpm for 5 minutes, 40 °C. The supernatant was removed, and 2 mL of PBS was added. The number of lymphocytes was calculated by the counting chamber (*Neubouer Improve*) by dripping the spleen fluid **(**Ulfah et al., 2017**)**.

Data Analysis

Data analysis was performed using SPSS 17 for normality, homogeneity, and one-way variance analysis (ANOVA) test. ANOVA is used to compare the mean values of test parameters between treatment groups. If the calculated F-value is greater than the F-tabel value at a 95% confidence level, it will be continued with Duncan's Multiple Range Test (DMRT) to compare the results and see the difference in each treatment (doses level).

RESULT AND DISCUSSION

The immunomodulatory effect of exopolysaccharides (EPS) from synbiotic drinks was reflected in several changes in the value of biological parameters such as relative spleen weight, phagocytic activity, and lymphocytes number. The level of EPS increased ($p \leq 0.05$) as more purple sweet potato paste was added to the drinks formulation (Table 1).

Table 1. Exopolysaccharide (EPS) levels of synbiotic drinks

Formulation	EPS level (g/L)
A0F1 (0% purple sweet potato paste and 5% starter)	18.24±1.48[a]
A0F1 (0% purple sweet potato paste and 7% starter)	30.72±1.33[b]
A1F1 (5% purple sweet potato paste and 5% starter)	33.34±1.31[b]
A1F2 (5% purple sweet potato paste and 7% starter)	39.08±1.36[c]
A2F1 (10% purple sweet potato paste and 5% starter)	46.01±1.53[d]
A2F2 (10% purple sweet potato paste and 7% starter)	45.58±1.24[d]
A3F1 (15% purple sweet potato paste and 5% starter)	48.02±1.36[d]
A3F2 (15% purple sweet potato paste and 7% starter)	51.97±1.92[e]

[a,b,c,d,e] The means denoted by the same letter showed no significant difference at α = 0.05

The analysis showed that the purple sweet potato synbiotic drink, on average, produced EPS levels between 18.24 and 51,97 g/L. Sample with the lowest EPS level was sample without the addition of purple sweet potato paste and a 5% starter with the ability to produce EPS of 18.24 g/L. EPS are polysaccharides synthesized from lactic acid bacteria released in extracellular cells (Ates, 2015). EPS is divided into two types based on the composition of monosaccharides, namely (1) homopolysaccharides, which are polymers consisting of one kind of monosaccharides, and (2) heteropolysaccharides which

are polymers consisting of several kinds of monosaccharides (Prajapat & Patel, 2013). The sample with the highest EPS content was found in the sample with the addition of 15% purple sweet potato paste with a 7% starter, which had an EPS content of 51.97 g/L. The addition of a starter showed a very noticeable influence on the EPS levels produced. This was suspected due to the interaction between probiotic bacteria and prebiotics in materials that can produce exopolysaccharides. Lactic acid bacteria (LAB) will release EPS resulting from the synthesis of prebiotics into the surrounding environment to protect itself from unfavorable conditions such as pH and extreme temperatures. LAB exopolysaccharides can attach to the small intestine mucosa, thereby increasing its ability to suppress the growth of pathogenic bacteria (Patten & Laws, 2015). This is in accordance with a research study conducted by (Mundiri et al., 2020) that the bacteria used in this study, namely *Lactobacillus plantarum, Lactobacillus bulgaricus,* and *Bifidobacterium longum,* can act as exopolysaccharide producers.

The difference in the addition of purple sweet potato paste showed a very noticeable influence on EPS levels. This was thought due to the presence of a high content of prebiotics in purple sweet potatoes. EPS produced by LAB was the result of prebiotic secretion in purple sweet potato synbiotic drinks (Beka et al., 2021). According to research by (Sancho et al., 2017), purple sweet potatoes have a higher oligosaccharide content than taro and cassava, which is 29 mg/100g. Oligosaccharides are the main type of prebiotic in purple sweet potato synbiotic drinks that are useful as emulsifiers, stabilizers, gelling or water binders in food, and in the health sector as anti-tumors, anti-ulcers, immunomodulators, and lower cholesterol levels. This is reinforced by research conducted by (Sanalibaba & Cakmak, 2016), which found that oligosaccharides can function as prebiotics that can be utilized by LAB to form EPS.

EPS has functional properties including boosting the immune system and preventing colon cancer (Prajapat & Patel, 2013). The EPS obtained in this study is crude EPS because they were obtained only based on the calculation of gravimetric weight after drying, where the EPS obtained was precipitated using 96% cold ethanol (Rimada & Abraham, 2003). EPS produced by LAB is influenced by several factors, such as fermentation conditions and growth media effects.

Table 2. Relative spleen weight between non-treated mice and mice treated with purple sweet potato synbiotic drink

Treatments	Relative spleen weight (%)*_
K (-) (not given any special treatment)	0.74±0.027[b]
K (+) (injected *Sthapylococcus aureus* only)	1.38±0.036[a]
A1 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 50 mg/kg BW)	0.70±0.014[b]
A2 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 100 mg/kg BW)	0.67±0.012[bc]
A3 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 150 mg/kg BW)	0.58±0.017[c]

[a,b,c,] The means denoted by the same letter showed no significant difference at α = 0.05
*spleen weight (g) / animal weight (g) ratio

The spleen is divided into two large sections called the white pulp and the red pulp. *White pulp* is lymphatic tissue containing lymphocytes and macrophages (Tortora & Derrickson, 2009). If an invasion occurs by bacteria in the body, the innate (non-specific) immune response will carry out a primary response by increasing the number of effectors such as macrophages, and if the invasion continues, the body will carry out a secondary immune response by activating the adaptive (specific) immune response by increasing the number of effectors such as B lymphocytes and T lymphocytes (Agnesa et al., 2017). The administration of purple sweet potato synbiotic drinks to mice induced with *Staphylococcus aureus* increased the phagocytic ability of macrophages (judging from their phagocytic activity and capacity) with the highest increase in A3 treatment. It is assumed that when the phagocytic ability of macrophages increases, the innate immune response can overcome bacterial invasion, and the body does not carry out a secondary response (adaptive immune response is not activated) so that the

weight of the spleen does not change much much from condition when both immune responses are inactive, hence the weight of the spleen on A3 being the smallest. On the other hand, in the K(+) treatment, it is assumed that there is activation of both immune responses (innate and adaptive) because there is no addition of immunostimulants from outside the body, so the innate response is not sufficient to overcome bacterial invasion so that the body activates the adaptive response to help overcome the invasion, indicated by the spleen weight of the K(+) group being the largest of the five treatments (Table 2).

Table 3. Phagocytic activity between non-treated mice and mice treated with purple sweet potato synbiotic drink

Treatments	Phagocytic activity (%)*_
K (-) (not given any special treatment)	18.00±4.58[a]
K (+) (injected *Sthapylococcus aureus* only)	36.00±2.65[b]
A1 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 50 mg/kg BW)	36.50±2.12[bc]
A2 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 100 mg/kg BW)	44.00±2.83[c]
A3 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 150 mg/kg BW)	59.67±4.51[d]

[a,b,c,] The means denoted by the same letter showed no significant difference at α = 0.05

Based on statistical tests, there was a significant difference in the administration of purple sweet potato synbiotic drinks with doses of 50 mg, 250 mg, and 500 mg/kgBW on phagocytosis activity. Table 3 showed that the higher the dose of purple sweet potato synbiotic drink, the more phagocytosis of *Staphylococcus aureus there was*. This indicated that the exopolysaccharide content contained in the synbiotic drink of purple sweet potatoes has the potential to increase macrophage activity (Figure 1). As antigen-presenting cells (APCs) and phagocytosis cells, macrophages are among the cells that are crucial to the immune response. As phagocytic cells, macrophages kill using both oxidative and non-oxidative methods (Tarigan et al., 2017). Additionally, macrophages can release IL-12, which promotes CD4+ T cell development into Th1. Th1 cells and NK cells will increase MHC II expression on the APC surface and release IFN γ as macrophage-activating agents. Through antibodies in the opsonization process, Th2 contributes to humoral immunity (Tursinawati & Dharmana, 2015).

Macrophage cells are isolated from peritoneal cavity, because the peritoneal cavity area has a fairly large number of macrophages and is easy to take (Baratawidjaja, 2002). EPS will increase cytokines (Patten & Laws, 2015). The increase in cytokines, which are macrophage cell activating factors, will stimulate immune reactivity, both specific and non-specific. The cytokines contained will increase the ability of macrophage cells to destroy antigens (Wahyudi & Priyanro, 2010).

In statistical tests, there were significant differences between the control group with various doses of purple sweet potato synbiotic drinks. The highest number of lymphocytes was found in the A3 treatment, which was a dose of 150mg/kg BW, while the K (-) treatment got the lowest number, which was 450,000 cells/mL (Table 4). This explains that in purple sweet potato synbiotic drinks dose 150 mg / kg BW, there is an increase in the number of lymphocytes compared to the doses of 100 mg and 50 mg. The increase in the number of lymphocytes is caused by the content of exopolysaccharides contained in purple sweet potato synbiotic drinks. Lymphocytes are cells that are able to recognize and destroy various determinants of antigens that have two properties in a special immune response, namely specificity and memory. Lymphocytes play a role in specific immune responses because each individual adult lymphocyte has a special side binding as a variant of the antigen receptor prototype. Antigenic stimulation induces an immune response carried out by the cellular system jointly played by macrophages, B lymphocytes, and T lymphocytes (Ervando et al., 2019). The increase in the number of lymphocytes is due to the high content of exopolysaccharides in synbiotic drinks as immunomodulators

in increasing lymphocyte cell proliferation through IL-2 production. IL-2 has a role in activating T lymphocyte cells to proliferate. The proliferation of T lymphocytes is stimulated by antigens regulated by the bond between IL-2 and its receptors (Ulfah et al., 2017).

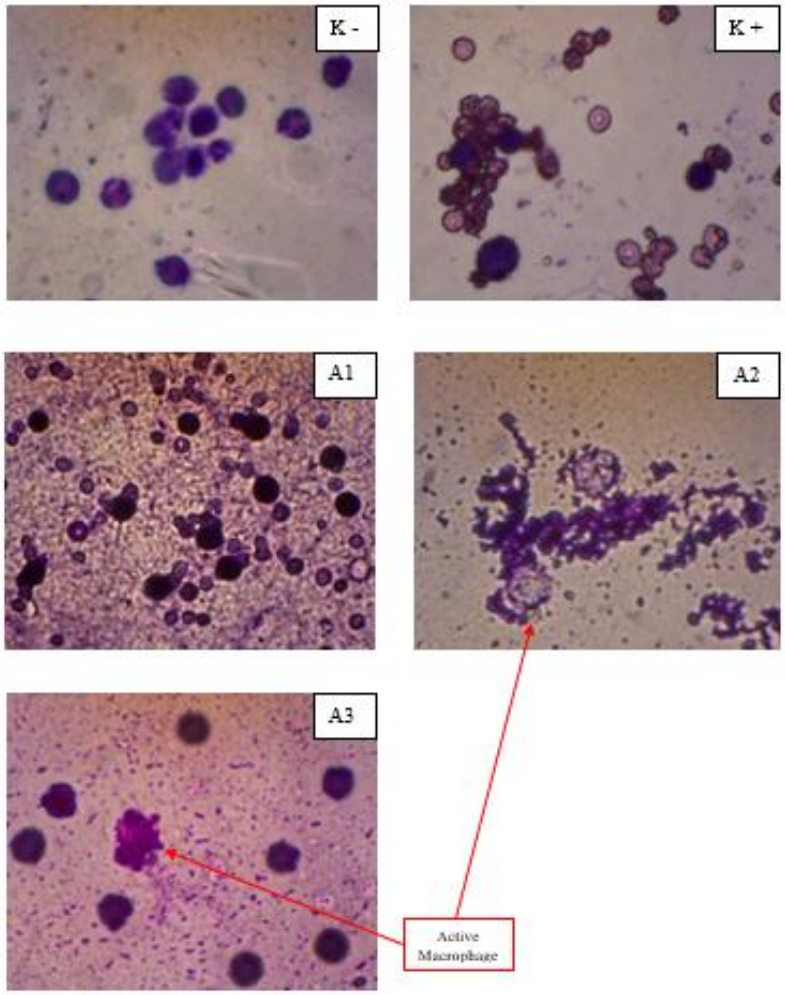

Figure 1.1 Phagocytic activity between non-treated mice and mice treated with purple sweet potato synbiotic drink. K- (not given any special treatment); K+ (injected *S. aureus* only); A1 (injected *S. aureus* & given a purple sweet potato synbiotic drink at a dose of 50 mg/kg BW); A2 (injected *S. aureus* & given a purple sweet potato synbiotic drink at a dose of 100 mg/kg BW; A3 (injected *S. aureus* & given a purple sweet potato synbiotic drink at a dose of 150 mg/kg BW)

It is possible for the number of lymphocytes to increase while the spleen weight decreases in experiments using infected mice. In response to an infection, lymphocytes can leave the spleen and migrate to peripheral tissues or lymph nodes, where they are needed for immune responses. This redistribution of cells could lead to a decrease in spleen weight, even if the overall lymphocyte count increases in the blood or other tissues. During severe immune responses, the spleen can contract to release stored lymphocytes and other immune cells into circulation. This would temporarily increase lymphocyte numbers in the blood but reduce spleen weight. Infections can stimulate clonal expansion

of specific T or B lymphocytes in response to antigens. This expansion might occur in circulation or in lymph nodes, contributing to increased lymphocyte numbers, even as the spleen contracts.

EPS can alter both innate and adaptive immune responses by acting as immunomodulators (Jin et al., 2010). Exopolysaccharides can prevent cancer of the gastrointestinal tract, infections, immunodeficiency-induced diseases, and inflammation. By controlling specific chemicals, such as cytokines, immunomodulators (EPS) typically change the immune system's function (Prajapat & Patel, 2013) by exerting their immunomodulatory effects through two phases (Lynch & Lavelle, 2022). A phosphate group that has a negative charge on EPS triggers the immune system in the first phase. Additionally, EPS's phosphate group stimulates the activation of immune cells, including macrophages and lymphocytes. Because of its larger molecular weight, EPS suppresses the immune system during the second phase (Hidalgo-Cantabrana et al., 2014).

Table 4. Number of lymphocytes between non-treated mice and mice treated with purple sweet potato synbiotic drink

Treatments	Lymphocytes (cells/mL)
K (-) (not given any special treatment)	450,000[a]
K (+) (injected *Sthapylococcus aureus* only)	1,016,666[b]
A1 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 50 mg/kg BW)	1,180,000[b]
A2 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 100 mg/kg BW)	1,710,000[c]
A3 (injected *Sthapylococcus aureus* & given a purple sweet potato synbiotic drink at a dose of 150 mg/kg BW)	2,113,333[d]

[a,b,c,] The means denoted by the same letter showed no significant difference at $\alpha = 0.05$

CONCLUSION

The exopolysaccharides (EPS) contained in purple sweet potato synbiotic drinks exert immunomodulatory effects on mice exposed to *S aureus*. Variations in the dose of synbiotic drinks administration provided significant differences ($p \leq 0.05$) in phagocytic activity, relative spleen weight, and number of lymphocytes in mice. As the dose increased, there was an increase in the value of phagocytosis activity and the number of lymphocytes, as well as a decrease in relative spleen weight. The administration of synbiotic drinks of 150 mg/kg BW is recommended to produce the best improvement in the immune system.

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