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Research Article

Investigating the antibacterial effect of *Bacillus subtilis natto* on human intestinal pathogens

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ABSTRACT

Treatment of diarrhea caused by bacteria is a progressing phenomenon in which probiotics play an essential role. The aim of this study is to investigate the antibacterial effect of the probiotic *Bacillus subtilis natto* on a number of common pathogens of the digestive system. In this study, *Bacillus subtilis natto* ATCC G_8S_3 strain was selected as a probiotic and its antimicrobial effect on four gastrointestinal pathogens including *Bacillus cereus* ATCC 11778, *Salmonella typhimurium* ATCC 1429, *Shigella flexneri* ATCC 11222 and *Escherichia coli* ATCC 25922 was investigated. In order to detect the antimicrobial activity of *Bacillus subtilis natto*, various methods such as agar well diffusion, disc diffusion, MIC and MBC were used based on microbiology standards and with four replications. In the agar well diffusion method, among the four pathogens, *Bacillus subtilis natto* showed an antimicrobial effect only on the pathogen *Escherichia coli*, a non-growing halo with a diameter of 10 mm but this probiotic did not show antibacterial effect on *Bacillus cereus*, *Salmonella* and *Shigella* pathogens. In the disk diffusion method, *Bacillus subtilis natto* did not show antimicrobial effect on any of the pathogens of this research. MIC for *Escherichia coli* pathogen was 50, while no antibacterial effect was observed on *Bacillus cereus*, *Salmonella* and *Shigella* pathogens. MBC results showed that this probiotic had no lethal effect on the studied bacteria. *Bacillus subtilis natto* ATCC G_8S_3 is safe and resistant as a probiotic and it was able to show inhibitory results on *Escherichia coli* pathogen.

1. Introduction

Diarrhea is one of the most important diseases of the digestive system. This disease has a high prevalence in developing countries. On the other hand, the American Academy considers acute diarrhea or acute gastroenteritis to be the second cause of death worldwide (Gomes et al., 2016). Various bacteria such as *Bacillus cereus, Salmonella*, *Shigella*, *Escherichia coli*, *Campylobacter* and *Vibrio cholera*, the cause of various diseases in humans, especially ductal diseases and many intestinal diseases and diarrhea disease. Some of them, such as *Salmonella* and *Shigella*, have always been associated with disease and some others like *Escherichia coli* and *Klebsiella pneumoniae* are normal natural flora which can cause opportunistic infections and antibiotics are usually used to treat them but the excessive use of antibiotics has increased all over the world which has caused an increase in strains resistant to antibiotics (Ivan et al., 2020; Sandra et al., 2021; Hussen et al. 2019). Today, probiotics can

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be used as a suitable way to treat digestive diseases (Wan et al., 2018; James et al., 2019). Probiotics are defined as living microorganisms which are beneficial to the host if consumed in sufficient quantities (Thilagavathi, T. 2020). Probiotics are used as an alternative to chemical feed additives, especially antibiotics, for animal health and human food production, and for the treatment and prevention of infectious diseases caused by diseases of the mouth, intestines, and reproductive system (Wan et al., 2018; Chang et al., 2021; Plaza-Diaz et al., 2019; Tang et al 2019; Zommiti et al., 2020). The important characteristic of probiotic bacteria includes the ability of these bacteria to suppress the proliferation of pathogenic organisms and their pathogenic power. Probiotics can destroy intestinal pathogenic bacteria through different functions such as the production of bacteriocin, short-chain fatty acids, proteins, carbohydrates, vitamins, lactic acid, and hydrogen peroxide (Winschau et al., 2020; Tang et al 2019; Ghasemian et al., 2018; Ghorani 2022; Zommiti et al., 2020). Today, spore-bearing probiotics that are resistant to pH and bile salts are used because *Lactobacillus* and *Bifidobacteria*, which are important and main probiotics. It does not have the ability to resist in the digestive system therefore, bacilli can be a substitute and resistant probiotics in the digestive system (Wan et al., 2018; Elisashvili et al., 2019; Winschau et al., 2020; Tang et al 2019; Zommiti et al., 2020; Richelsen et al., 2020; Zhang et al., 2020). Meanwhile, one of these resistant probiotics is *Bacillus subtilis natto*, which has been used as a probiotic for many years. *Bacillus subtilis natto* can survive for a long time in the digestive tract due to its spores, antimicrobial activity such as bacteriocin production, and resistance to bile acids and salt (Sandra et al., 2021; Zhao et al., 2019). *Bacillus subtilis natto* is used as natto food and probiotic for humans and animal feed and it also causes the growth and survival of *Lactobacilli* and stabilizes the intestinal flora of humans and animals (Wan et al., 2018; Richelsen et al 2020; Zhang et al., 2020). According to what was stated, dealing with functional relationships between probiotics and common bacteria of the digestive system seems to be interesting and important. *Bacillus subtilis natto* probiotic has been done to a limited extent on human digestive diseases So that Jing Zhang used *Bacillus subtilis natto* B4 strain on the pathogen *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* (Mohd Hafez et al., 2020). In Mohd-Hafez's research, *Bacillus subtilis* strain was used on some pathogens such as *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium* (Sun et al., 2010). In this study, *Bacillus subtilis natto* was used as a probiotic model and the analysis of its antimicrobial effect on four bacteria, which are the most common pathogenic bacteria of the digestive system, has been done.

2. Materials and Methods

2.1. Preparation of standard strains

In this research, a probiotic strain of *Bacillus subtilis natto* ATCC G_8S_3 and four strains of gastrointestinal pathogens included *Bacillus cereus* ATCC 11778, *Salmonella typhimurium* ATCC 1429, *Shigella flexneri* ATCC 11222 and *Escherichia coli* ATCC 25922 from it was prepared in lyophilized form from the National Center and Genetic Reserves.

2.2. Revival of bacteria

Probiotic strain and prepared bacteria were cultured using nutrient agar culture medium for survival and it was incubated for 24 hours in aerobic conditions and temperature of 37°C.

2.3. Preparation of probiotic supernatant

Bacillus subtilis natto in tryptic soy broth (TSB) containing nutrients such as ammonium nitrate 3%, sucrose 2%, monopotassium phosphate 1%, potassium sulfate 2%, sodium chloride 0.5% were cultivated and placed in a shaker incubator at 37°C for 48 hours. The use of nutrients in this study has been important which is used to produce and secrete probiotic supernatant upon which. After 48 hours of cultivation, *Bacillus subtilis natto* grew in TSB medium for antimicrobial tests, agar well diffusion, disk diffusion, minimum inhibitory concentration or MIC and minimum lethal concentration (MBC) are used.

2.4. Antimicrobial activity investigation methods

2.4.1. Agar well diffusion method

In this method, the concentration of pathogens *Bacillus cereus*, *Salmonella* *typhimurium*, *Shigella flexneri* and *Escherichia coli* was prepared at a concentration of $1x10^8$ (mg/ml) in physiological serum equivalent to half of McFarland and were cultured in Mueller Hinton Agar medium, by the method of grass cultivation. After 15 minutes, in this culture, the wells were created by using a Pasteur pipette then 200 microliters of probiotic supernatant were added to the wells. After that, the plates were transferred to the incubator at 37°C for 24 hours. The results were checked after 24 hours and the diameter of the halos of no growth around the well was measured and recorded.

2.4.2. Disc diffusion method.

In the disk diffusion method, the concentration of the four pathogens of this study was prepared with a concentration of $1x10^8$ (mg⁄ml), equivalent to half of McFarland in physiological serum. From this, it was cultured on Mueller Hinton Agar culture medium by grass cultivation method. Sterile blank discs were smeared with 10 microliters of probiotic supernatant, and it was placed on the plate with specific intervals. The disc diffusion method was performed according to microbiology standards and with four repetitions. Then, the cultures were placed in an incubator with a temperature of 37°C for 24 hours. After 24 hours, the antibacterial effect of *Bacillus subtilis natto* probiotic on pathogens is evaluated.

2.4.3. The method of minimum inhibitory concentration (MIC) and minimum Bactericidal concentration (MBC)

Muller Hinton Broth culture was used in the MIC method and for the concentration of each pathogen to reach 1×10^5 (mg⁄ml), First, the equivalent of half-McFarland, which is (mg⁄ml) $1x10^8$, was prepared and diluted. These concentrations were added to all the wells except the negative control well. The first well was prepared with a concentration of 100 (μg/ml) of probiotic supernatant and then dilution was done until the concentrations were 50, 25, 12.5 and 6/25 (μg ⁄ ml) and the positive control, with the presence of the pathogen, and the negative control, considered as the absence of the pathogen. Then the cultures were transferred to incubation for 24 hours at 37°C. After 24 hours of incubation, growth or lack of growth is checked. Finally, the results are read. Wells in which no growth was observed were used for minimum bactericidal conventration. For MBC 10 μl was taken from the well without growth and a spot culture was given on Mueller Hinton Agar. MIC and MBC methods were performed according to microbiology standards with four repetitions and after 24 hours of incubation at 37°C, MBC results were observed.

3. Results

3.1. Agar well diffusion

The results of agar well diffusion showed that a non-growth halo was observed around the well based on the antimicrobial effect of probiotics against the pathogen *Escherichia coli* with a diameter of 10 mm. While no halo of non-growth due to the antibacterial effect of the probiotic *Bacillus subtilis natto* was observed around the well containing the medium of the pathogens *Bacillus cereus, Salmonella typhimurium* and *Shigella flexneri*.

3.2. Disc Diffusion

In the disc diffusion test absence of growth halo due to the antibacterial effect of the probiotic *Bacillus subtilis natto* it was not observed against the pathogens of *Bacillus cereus*, *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli* but around the discs of antibiotics, a halo without growth was observed.

3.3. MBC and MIC

MIC results showed that the 50 and 100 (μg⁄ml) concentrations of *Bacillus subtilis natto* has an antimicrobial effect on *Escherichia coli*, which is one of the pathogens under investigation, but this probiotic did not show antibacterial effect on other pathogens. In 6.25, 12.5 and 25 (μg⁄ml) concentrations did not show the growth inhibition effect on *Escherichia coli*. So, the minimum considered as MIC for *Escherichia coli* is 50 (μg⁄ml). MBC results showed that from these two cultured wells, bacteria grew on Mueller Hinton Agar medium. As a result, with these concentrations of the probiotic did not show the killing effect on bacteria.

Table 1. The effects of *Bacillus subtilis natto* probiotic supernatant at different concentrations on human intestinal pathogens in the MIC method.

Bacterial strain	Different concentrations of probiotic supernatant *Bacillus subtilis natto* ATCC G_8S_3 (µg⁄ml) unit				
	100	50	25	12.5	6.25
Escherichia coli ATCC 25922	-	-	+	+	+
Salmonella typhimurium ATCC 1429	+	+	+	+	+
Shigella flexneri ATCC 1222	+	+	+	+	+
Bacillus cereus ATCC 11778	+	+	+	+	+

The sign (+) indicates bacterial growth and the sign (-) indicates the absence of bacterial growth.

4. Discussion

Bacillus subtilis natto is used as natto food and probiotic for humans and animal feed which produces natto kinase and bacteriocin and it acts as an antimicrobial agent for the digestive system (Liu et al., 2021; Guangbo et al., 2021; Zhelyazkov et al., 2022). Preventing the activity of pathogens by probiotics can have an important effect on a person's health against infection with common pathogens of the digestive system. In fact, probiotics are known as management agents for intestinal infections with several mechanisms that basically include the secretion of antibacterial substances, competition in the binding site and colonization in the mucosal surfaces of the intestine and it leads to competition in obtaining food and reproduction between probiotics and pathogens, they operate. Meanwhile, what is clear is the modulation of the immune system and the benefits of probiotics on the health of the host (Tang et al 2019; Nasri et al., 2021).

This study was conducted with the aim of determining the antimicrobial effect of the supernatant obtained from the consumption of *Bacillus subtilis natto* as a spore-forming and resistant probiotic model on gastrointestinal pathogens. The results of the present study show that in the agar well diffusion method, supernatant is effective on *Escherichia coli*, therefore, this supernatant can be effective for bacteria such as *Escherichia coli*, but it was not effective for other bacteria. Looking at the history of the studies done in this field, the results obtained by other colleagues confirm the present study. So that in the study of Nasri et al., which was conducted using the agar well diffusion method to investigate the antimicrobial effect of *Lactobacillus* probiotic on some pathogens such as *Salmonella typhimurium*, *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* (Vuran et al., 2021). In Vuran's study, non-growth halo was observed on *Salmonella timurium*, *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* bacteria which was consistent with our reported result for *Escherichia coli*, but it did not match with the results of other bacteria, including *Bacillus cereus*, *Salmonella typhimurium* and *Shigella flexneri*. These results indicate that *Lactobacillus* probiotic is a stronger probiotic than *Bacillus subtilis natto* and it probably produces more antimicrobial compounds. In another study by Vuran et al., aimed at the antimicrobial effects of lactic acid-producing probiotics on some food pathogens such as *Salmonella typhimurium*, *Listeria monocytogenes*, and *Escherichia coli* by agar well diffusion method (Pazhoohan et al., 2020). In the results of the well agar diffusion method conducted by Vuran et al., an aura of non-growth was observed based on the antibacterial effects of *Lactobacillus* probiotics on

Salmonella typhimurium, *Listeria monocytogenes* and *Escherichia coli* bacteria which is according to the result of our research for the pathogen *Escherichia coli* but it is not compatible with the results of other bacteria such as *Bacillus cereus*, *Salmonella typhimurium* and *Shigella flexneri*. These results show *Lactobacillus* is effective probiotic than *Bacillus subtilis natto*. In addition to stronger probiotics, it may cause the growth of pathogens due to the decrease in pH. In the study of Pazhoohan et al., with the aim of antimicrobial and anti-adhesion effects of healthy *lactobacillus* isolates of human intestinal origin against *Enterotoxigenic E. coli (ETEC)* and *Enteroaggregative E. coli (EAEC)*. it was done by well agar diffusion method (Mohd Hafez et al., 2020). It is consistent with the present study because it showed the inhibition of *Lactobacillus* isolates against the growth of *Escherichia coli* pathogens.

In the research of Vuran et al., the antimicrobial effects of *Lactobacillus* and *Bifidobacteria* on the pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Escherichia coli* were performed by disk diffusion method, the inhibitory effect on *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* bacteria were observed which is not consistent with the result of our research. It can be antimicrobial for lactic acid bacteria caused by the reduction of lactic acid pH. After comparison and analysis, because of the disk diffusion method of our research, the reason for the lack of antimicrobial activity of *Bacillus subtilis natto* supernatant on pathogens can be the limited volume and low concentration of probiotics which covers the discs. In the results of Nasri study, the minimum inhibitory concentration method was used to investigate the antimicrobial effect of *Lactobacillus* probiotics on *Salmonella typhimurium*, *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*. The well without turbidity indicating the lack of growth of pathogens were observed (Nasri et al., 2021) which according to this investigation in 100 and 50 (µg⁄ml) of the supernatant of probiotic *Bacillus subtilis natto*, only the pathogen *Escherichia coli* has been affected with 1×10^5 (mg⁄ml) and no antimicrobial effect was observed on the pathogens Bacillus cereus, *Salmonella typhimurium* and *Shigella flexneri* with a concentration of 1×10^5 (mg⁄ml). Therefore, this shows that Supernatant cannot affect the pathogenic bacteria *Bacillus cereus*, *Salmonella typhimurium* and *Shigella flexneri* with $1x10^5$ (mg⁄ml) and as a result, it has a weak antibacterial effect compared to *Lactobacilli* and *Bifidobacteria*. In Nasri study and the present study, MBC was taken from a well without turbidity to determine the lethality rate. The results of Nasri research showed a 98% decrease in the viability of the bacterial population (Nasri et al., 2021). While in the present study, the results of culture of clear wells in MIC for *Escherichia coli* pathogen, which was carried out on Mueller Hinton Agar culture, showed that the supernatant of this bacterium did not have a lethal effect and only had an inhibitory effect. These results are not consistent with the current research and the reason for this could be the higher antibacterial compounds of lactic acid probiotic compared to *Bacillus subtilis natto* probiotic in the present study. Probably, this probiotic is due to the different antimicrobial methods that were investigated in this research and only on Escherichia coli in the MIC method, an antimicrobial result was obtained. It can be concluded that this probiotic does not produce many antibacterial compounds and since the purity analysis of these compounds has not been done, this probiotic can be worked on in the future. In the research of Zhang et al., which was conducted with the MIC method, the antimicrobial effect of the probiotic supernatant of *Bacillus subtilis natto* B4 strain against the pathogen *Escherichia coli* was carried out. The obtained results showed inhibition of pathogen growth against *Bacillus subtilis natto* B4 strain (Mohd Hafez et al., 2019) which is similar to the results of the present study.

Conclusion

Diarrhea is one of the most important diseases of the digestive system, which is caused by parasites, viruses, and bacteria. Antibiotics are widely used to treat this disease, and excessive use of antibiotics has caused resistance of pathogens in the digestive system. Today, probiotics can be used as a suitable way to treat digestive diseases. Probiotics are live microorganisms that are beneficial to the host if consumed in sufficient amounts. The most important probiotics are *lactobacilli* and

Bifidobacteria but due to the little resistance that these probiotics have against salt and bile acids in the digestive system. Spore-forming probiotics that have high resistance in the digestive system have been used. In this study, resistant spore-forming probiotic *Bacillus subtilis natto* was used as a probiotic model and to analyze the antimicrobial potency of the probiotic supernatant against four common gastrointestinal pathogens including *Bacillus cereus*, *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. The results of this study showed that, the antibacterial effect of *Bacillus subtilis natto* was effective only on the *Escherichia coli* while no antimicrobial activity was observed on other pathogens. The reason for this, could be the sensitivity of the *Escherichia coli* pathogen to the probiotic supernatant. According to the results obtained from this study, it can be claimed that the use of *Bacillus subtilis natto* bacteria, which is part of the family of probiotics. It has a therapeutic and preventive effect on diarrhea. Although expanding the antibacterial range of probiotics requires extensive research and investigations.

Conflict of interest

There is no conflict of interest between the authors.

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