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Decreasing effect of *Bacillus coagulans* on allergy symptoms and IgE level in wistar rat model with ovalbumin-induced allergy

Marjan Salehi[1], Parvaneh Jafari[2]*

[1] *MSc of Microbiology, Department of microbiology, Islamic Azad University, Qom branch, Qom, Iran*
[2] *Assistant professor, Department of microbiology, School of Science, Islamic Azad University of Arak, Iran*

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Abstract: Probiotics are living microorganisms with beneficial effects on the host health by modulating the intestinal microbial flora. In this study, the effect of *Bacillus coagulans* as a probiotic was investigated on reducing serum immunoglobulin (IgE) levels in Allergic Rats model. Also, microflora changes were evaluated in Wistar rats.
First, the ability of *Bacillus coagulans* for binding to Caco2 cell line was investigated. Then, in order to evaluate its effect on allergy, 30 adult Wistar rats were randomly divided into 3 groups, then the allergy induced by peritoneal injection of OVA and probiotic treatment was done during 21 days, then serum immunoglobulin levels were measured, also by determining the microflora pattern, the effect of probiotic function was investigated on intestinal microflora. The results showed that B. coagulans was able to bind to the Caco2 cell line properly. Consumption of this probiotic resulted in a significant reduction in serum immunoglobulin levels (32.41%) $P<0.0001$ in rats with induces allergies by OVA. In addition, after 3 weeks, Probiotic could increase the amount of lactic acid bacteria, yeasts and spore-bearing bacteria in the experimental group compared to the positive allergic control group by 48.8%, 25.45% and 80.59%, respectively. Weight loss was 5.9% in the negative control group and weight gain was 47% in the positive control group.

Keywords: Allergy, Bacillus coagulans, Caco2 cell line, Probiotics

*e-mail: P-jafari@iau-arak.ac.ir

1 Introduction

Allergy is Inflammation of tissues and dysfunction of various organs of the body.

It is also called hypersensitivity. Hypersensitivity is divided into four types based on the reaction time in the body and the mechanisms involved in it. The asthma disease that we are considering in this study is classified in type one and in fact it includes involvement of bronchial or lung tissue, actually it is an inflammatory disease of the nasal mucosa and eventually the lungs (Dispenza, 2019). This disease is caused by the activation of inflammatory cells, including mast cells, by immunoglobulin E (IgE) (Piao et al., 2020). IgE and hypersensitivity of the respiratory tract are increased by environmental allergens (Liang et al., 2019). Studies have shown that IgE levels indicate the rate of allergic reactions to environmental allergens (Hyun et al., 2018). In this case, more secretion of cytokines such as interleukin 4 and interleukin 5 leads to stimulate B cells to produce more IgE (Yao et al., 2020).

Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body, when administered in adequate amounts confer a health benefit on the host. Allergies are said to be caused by defects in the gut microbial flora, and allergies can be largely cured by taking probiotics and boosting the gut microbial flora (Eslami et al., 2020).

Probiotics are thought to interact with three intestinal lines of defense including: microbiota, mucosa, intestinal immune system (GALT), In first level probiotics interfere with growth of survival of bacteria in gut lumen. in lamina propria increase mucosal barrier function and mucosal immune system that modulate signal transduction and innate/adaptive immunomodulation which enhance the cytokines (IL-10 and TGFβ) and leads to produce immunoglobulins, and beyond the gut they affect systemic immune system, nervous system, liver, etc… (Azad et al., 2018).

Also Studies have shown that probiotics can improve the quality of life in people with the asthma (Barcik et al., 2020). Therefore, probiotics are thought to secrete metabolites that play an important role in improving the function of immune cells and the balance of the microflora of the gastrointestinal tract (Ivashkin et al., 2018; Li et al., 2020) The use of antihistamines is effective in relieving inflammation and other symptoms of this disease, but it causes various complications, especially in children. In contrast, the use of available and low-cost probiotics with no side effects is a good choice (Nogueira and Gonçalves, 2011; Parker et al., 2016). Probiotics are found in food products, including fermented dairy products. These microorganisms suppress IgE while regulating the function of some cytokines (Azad et al., 2018). Lactobacillus paracasei reduces dust allergies (Parker et al., 2016). Bacillus coagulans was also shown to reduce thrombotic allergic symptoms (Fu et al., 2017). The findings suggest that lactic acid bacteria may reduce the effects of Allergic rhinitis, while among probiotic bacteria and their effects on allergic reactions, there has been less focus on Bacillus coagulans. Therefore, the present investigation confirmed the effect of this probiotic based on the native strain of Bacillus coagulans in binding to the CaCO2 cell line and reducing the negative effects of ovalbumin allergen in male Wistar rats.

2 Materials and Methods

2. 1. Preparation of lyophilized bacteria powder

The probiotic strain of Bacillus coagulans was prepared from Takgene Zist Company. To prepare the probiotic powder, fermenter 10 L containing 5 L nutrient broth medium (Oxoid, Victoria, Australia) was inoculated with 5% bacterial preculture,the Incubation was adjusted to 30°C, pH was 6.5 and aeration volume was 1 vvm with agitation speed of 250 rpm. After 72 hours' incubation, the sporulation phase was completed, the cells were isolated from the culture medium by centrifuge 10 minutes in 5000 rpm. Next, they were washed with phosphate buffer 3 times and suspended in freeze-dryer solution (Hitachi, Ltd., Japan). The counts of Bacillus coagulans were expressed as spores per gram of powder.

2. 2. Methodology

First, the dilution serial was prepared from bacterial lyophilized powder in phosphate buffer with a pH of 7.2 and the dilution serial tubes were heat treated for 15 minutes at 85°C. Active spores in Tryptic Soy Medium Agar (TSA) (Sigma-Aldrich, USA) were cultured through Pour Plate method. For this purpose, 1 ml of each suspension was transferred to the plate and TSA medium was added. After incubation in 37°C for 24 hrs., plates containing 30-300 colonies were selected and the number of spores of per gram of lyophilized powder was calculated.

2. 3. Investigation of binding ability to the Caco-2 cell line

Caco-2 cell line was purchased from Pasteur Research Institute and cultured in Dulbeco`s modified eagle`s medium, essential medium (DMEM; Sigma, USA). Culture medium was supplemented with 10% V/V OF inactive calf serum (65°C for 30 minutes), 25 mM of HEPES 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid) (BioBasic, Canada), 100 um of penicillin and 100 mg/ml of streptomycin (Sigma-Aldrich, USA). The cell culture was heated to 37°C in an atmosphere containing 5% carbon dioxide. By adding trypsin-EDTA (25%) (Sigma-Aldrich, USA), the cells were isolated and centrifuged at 60,000 rpm for 10 minutes at room temperature.

The cells were then suspended in DMEM medium and counted by a hemocytometer. By adding 5×105 cells to the 12-well cell culture plate, single-layer cells were prepared. The plate was heated for 2 weeks and the culture medium was changed every other day.

After washing the cell monolayer with phosphate buffer, 300 µl of Bacillus coagulans suspension in phosphate buffer with a concentration of 109 CFU/ml was added to each well. The plate was heated for 90 minutes, during which time it was gently shaken. The wells were washed 3 times with phosphate buffer to remove unbound bacteria. To separate the cell line, 300 µl of trypsin (USA Sigma-Aldrich) was added to each well and heated for 30 minutes at 37°C. The enzyme activity was then stopped by adding 30 µl of 10% fetal calf serum and the cell line was completely isolated. Cell proliferation was prepared on the slide from the resulting suspension and the slides were fixed in acetone for 15 minutes. After staining the cell proliferation through Gram method, the number of bacteria attached to each Caco2 cell was counted in 25 areas.

2. 4. Housing and grouping rats

In this study, 30 male Wistar rats were prepared from Arak University of Medical Sciences, with an approximate age of 5 weeks and an average weight of 180-200 g. They were kept at 23±2°C and 50%±5 humidity with a light/dark cycle of 12/12 h. After a 10-day adjustment period, they were randomly grouped into 3 groups of 10.

Table 1. Grouping rats

groups	number	Treatment
Negative control	10	1 ml phosphate buffer (pH: 7.2)
Positive control	10	1 ml phosphate buffer (pH: 7.2)
experimental	10	1 ml probiotic suspension ($2^*=10^9$ CFU/ml)

2. 5. Induction of allergy and gavaging the rats

Ovalbumin powder (USA Sigma) was used to induce allergy in rats by intraperitoneal injection (to induce asthma-like respiratory allergy) and oral gavage (to induce allergy in the mucosa and gastrointestinal tract).

For intraperitoneal injection, 2 mg of ovalbumin for every 100 gr of rats weight was suspended with 100 mg of aluminum hydroxide powder in 1 ml of phosphate buffer at neutral pH. This suspension was intraperitoneal injected to rats on the first and eighth days. It should be noted that in order to equalize the conditions, intraperitoneal injection of negative control rats was performed with phosphate buffer.

To prepare an oral allergen suspension, 50 mg/ml of ovalbumin was prepared in phosphate buffer and orally gavaged to the rats in the experimental and positive control groups on days 12, 15, 18, and 20.11 The negative control group was also gavaged with phosphate buffer on the mentioned days. For 21 days, rats in the experimental group were gavaged with 1 ml of probiotic suspension in PBS at 109 cfu/ml. The control group was daily gavaged with the same amount of buffer.

2. 6. Measurement of Ig E

Finally, blood samples were taken from 3 experimental groups. Blood samples were centrifuged for 15 minutes at 6000 rpm and their serum was isolated. The amount of Ig E in the samples was determined using ELISA method and IgE Anti OVA kit prepared by CRYSTAL DAY, China.

2. 7. Determination of microflora pattern and weight of rats

To determine the pattern of intestinal microflora, 1 gr of feces was collected from each rat in the first and third weeks and suspended in 9 ml of physiological serum containing tween 80 (Merck, Germany) and then serial dilution was prepared. The number of lactic acid and yeast bacteria were determined by Pour plate method in MRS (Merck, Germany) and YGC Agar (Merck, Germany) media (ISIRI 4721), respectively. To count spore-forming bacteria, the samples were first heat treated at 85°C for 15 minutes and then cultured in TSA medium.

2. 8. Weight changes of rats

Weight changes of rats were determined at the beginning and the end of the period using a digital scale accurate to 0.01 grams.

2. 9. Statistical analysis

Data were analyzed using Graph pad prism 5 software with one-way ANOVA and t-test.

3 Results and Discussions

3. 1. Ability to connect to the Caco2 cell line

After culturing the cell line, the bacterial binding to the Caco2 cell line was examined under a microscope. The results of microscopic observation showed that the binding rate of Bacillus coagulans to each 100 Caco2 cells was 201±13/125. This indicates the ability of this bacterium to bind to intestinal cells (Figure 1).

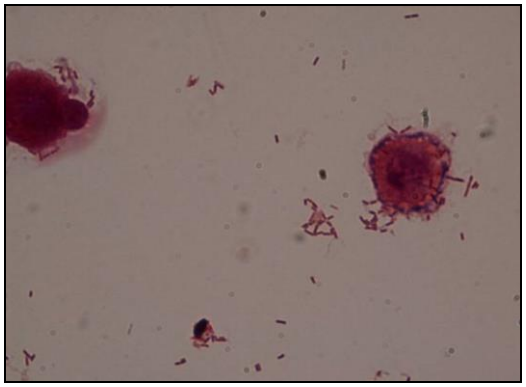

Figure 1. Binding of Bacillus coagulans to Caco2 cells

3. 2. Changes in serum IgE levels

The results of this experiment (Table 1) showed that the oral and intraperitoneal use of ovalbumin caused the serum IgE concentrations to increase from 85.78 to 221.5 mg/dl. This indicates the effectiveness of the method used to induce type I allergy. Daily gavage of Bacillus coagulans (1 ml) at a concentration of 10^9 cfu/ml for 21 days significantly reduced the serum IgE concentrations to 149.7 mg/dl (Table 2). These results indicate the efficacy of probiotics used to help improve albumin-induced allergy. Figure 2 shows the serum IgE concentrations in different groups.

Table 2. Serum IgE concentrations and weight of rats in different groups at the end of the third week of the experiment

End of week 3			groups variables
probiotics	Positive control	Negative control	
149.7 ± 7.509 [b]	221.5±5.421 [a]	85.78 ± 5.492 [c]	Ig E (mg/dl)
54.40 ± 3.530 [a]	-13.17 ± 1.740 [c]	20.17 ± 1.778 [b]	weight (gr)

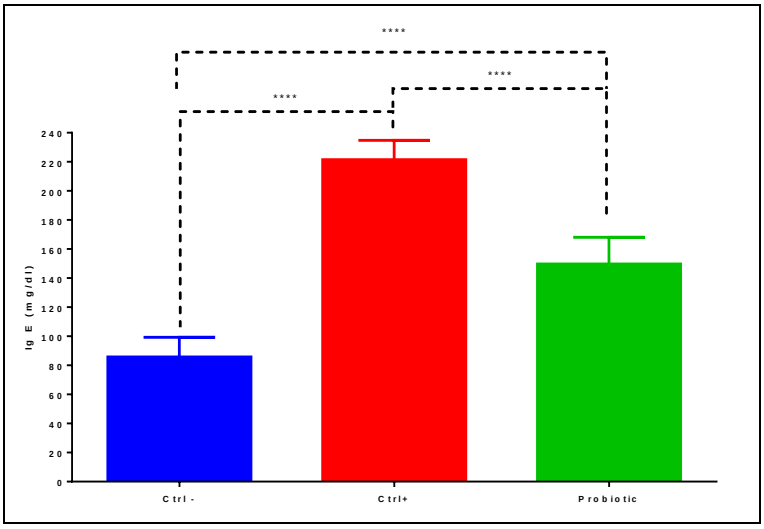

Figure 2. Serum IgE levels in the third week

3. 3. Pattern of intestinal microflora

Due to the allergy, the number of lactic acid bacteria in the positive control group was significantly less than the negative control group in the first and third weeks. In the experimental group receiving probiotics, the number of beneficial lactic acid bacteria in the gastrointestinal tract increased so that it was higher than both the negative control group and the positive control group.

This indicates the positive effects of probiotics used in improving the pattern of intestinal microflora in rats which caused the number of beneficial lactic acid bacteria to be even higher than the control group.

Table 3. Microflora pattern in different groups

Week 2			Week 1			Groups and time variables
probiotics	Positive control	Negative control	probiotics	Positive control	Negative control	
7.491 ± 0.001	5.971 ±0.002	7.040 ±0.040	7.386 ± 0.005	5.983 ±0.001	6.988 ± 0.001	Mold and yeast
9.448 ± 0.057	5.934 ± 0.055	5.979 ± 0.006	8.429 ± 0.046	5.843 ± 0.143	5.975 ± 0.007	Bacilli
9.825 ± 0.027 [a]	6.613 ± 0.232 [d]	7.896 ± 0.0.089 [c]	9.926 ± 0.056 [a]	7.980 ± 0.008 [c]	8.678 ± 0.010 [b]	LAB

Counting the number of yeasts showed that allergy caused the number of yeast microorganisms in the positive control group to be significantly less than the negative control group in the first and third weeks. In the experimental group receiving probiotics, the number of yeasts in the gastrointestinal tract increased so that it was higher than both the negative and positive control groups (Figure 3).

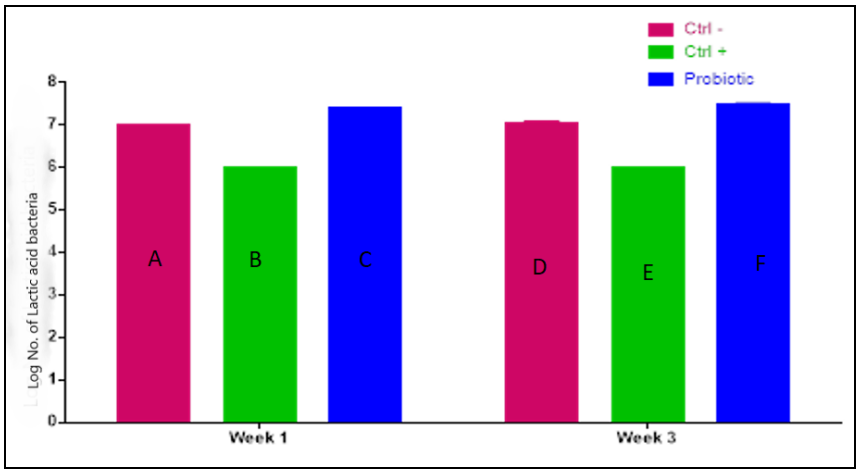

Figure 3. Number of lactic acid bacteria in male Wistar rats in negative control group (A and D), positive control group (B and E) and experimental group (C and F) which were compared in the first and third weeks.

This indicates the positive effects of probiotics used to improve the pattern of intestinal microflora in animals, which has led to an increase in the amount of mold and beneficial yeasts in the experimental group.

This examination showed that allergy had no effect on the number of fecal bacilli so that the number of bacilli in the positive control group and the negative control group did not differ significantly in the first and third weeks. In the experimental group receiving probiotics, the number of bacilli in the gastrointestinal tract increased so that it was higher than both the negative and positive control groups (Figure 4).

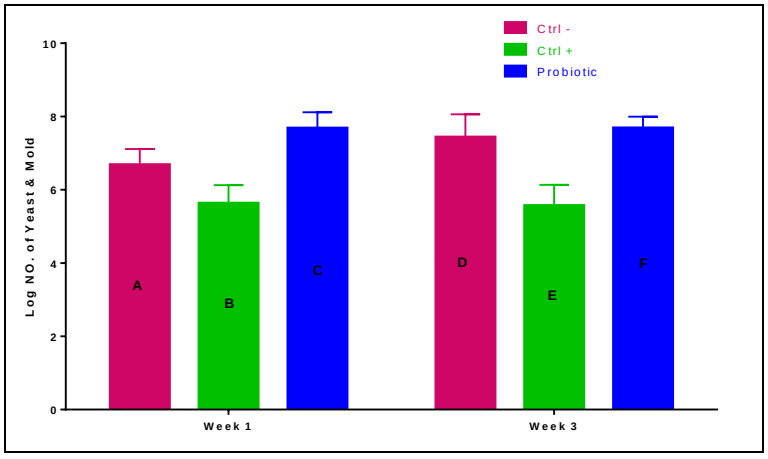

Figure 4. Number of molds and yeasts in male Wistar rats in negative control group (A and D), positive control group (B and E) and experimental group (C and F) which were compared in the first and third weeks.

This indicates the effective placement of probiotics used in the microflora pattern and its improvement in the animal, which has caused the number of bacilli to be even higher than the control group (Figure 5).

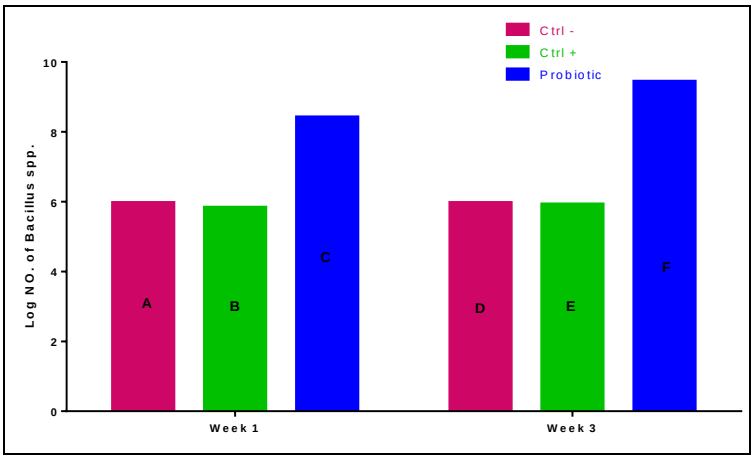

Figure 5. Number of Bacillus in male Wistar rats in negative control group (A and D), positive control group (B and E) and experimental group (C and F) which were compared in the first and third weeks.

3. 4. Weight changes in rats

Because of the allergy, the difference in rat weight in the positive control group was significantly less than the difference in rat weight in the control group in weeks 3 and 1. In the experimental group receiving probiotics, the difference in rat weight increased in weeks 3 and 1 so that it was greater than both the negative and positive control groups. This weight gain in the experimental group seems to be due to the consumption of probiotics (Figure 6).

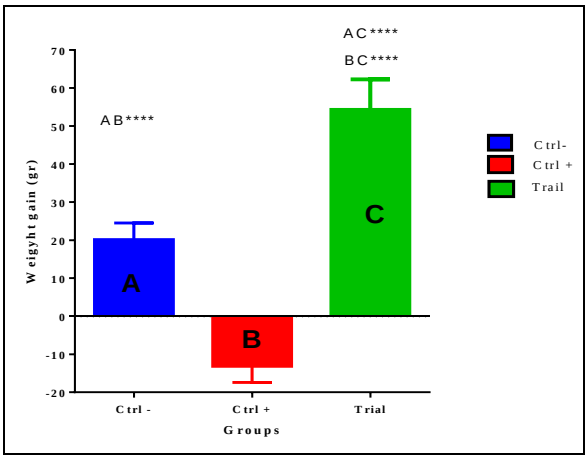

Figure 6. Graph of weight changes in rats in negative control group (A) and positive control group with allergy (B) and experimental group receiving probiotics during three weeks (C).

3. 5. Discussion

In this study, the protective effect of probiotics was investigated on OVA-induced allergic airway inflammation. In recent years, the relationship between the microflora of the gastrointestinal tract and the incidence of allergies has been considered. Hence, many researchers are looking to study the effects of probiotics in the prevention and treatment of allergies and asthma (Marko and Pawliczak, 2017).

The effect of intestinal bacteria is not limited to the gastrointestinal tract. The most important roles of intestinal bacteria include protective, immune and digestive function (Hardy et al., 2013). It means that intestinal bacteria play a significant role in shaping the natural immune response. The balance of intestinal microflora balances the Th1/Th2 ratio, and any disturbance in this ratio causes more allergens to be absorbed, which in turn increases IgE production (Nie et al., 2021). Changes in the intestinal flora are considered as important indicators of allergic diseases (Simms, 2015). Increased prevalence of asthma is closely related to microbiota (Barcik et al., 2020). Various experiments have shown that targeted manipulation of the intestinal ecosystem through probiotics has preventive and therapeutic effects on asthma (Carr et al., 2019). For example, the results of a study on the intestinal microflora of children showed that allergies were more likely to occur in children with high levels of aerobic bacteria and fewer Lactobacilli (Bjorksten et al., 1999). Also, the results of a study in 2018 showed that controlling the intestinal microbiota was very effective in improving patients with atopic asthma, and patients treated with probiotic microorganisms had lower IgE levels (Ivashkin et al., 2018). We also obtained the same results in the microflora study section and it was observed that how Bacillus coagulans was able to replace in the intestine and cause changes and improvements the intestinal flora.

Probiotics are live strains of certain bacteria which have a positive effect on the immune system and health (Özdemir, 2010). Probiotics inhibit allergic diseases by suppressing the Th2 cell response. In a study on mouse models of asthma, Th17 cells developed inflammation, but probiotics suppressed it and increased beta TGF levels (Jan et al., 2012).

Allergens stimulate the immune system to release cytokines to increase the expression of antigens that activate T cells to produce Th2 responses. Cytokines such as IL-4, IL-5, IL-9 cause asthma-like changes in the lungs (Dunn and Wechsler, 2015; Otani et al., 2013). A study has shown that administration of Lactobacillus reuteri improves infants with asthma in the early stages (Abrahamsson et al., 2013).Consumption of a mixture of probiotics can reduce OVA-induced allergic inflammation by reducing the number of irritating molecules on the surface of dendritic cells (Zhang et al., 2021). Lactobacilli and Bifidobacteria are the most common probiotic bacteria that help cure respiratory diseases (Wang et al., 2020). Mice raised under aseptic or sterile conditions have been shown to show stronger allergic reactions to allergen stimulation due to the lack of intestinal microbiota (Herbst et al., 2011). One study showed that Lactobacillus rhamnosus has anti-inflammatory effects in mice and suppressed IgE production which confirms the results of our study (Jang et al., 2012). In addition, a product from combination of two probiotic strains showed moderating activity in adults with asthma (Drago et al., 2015). The results of an experiment showed that the prophylactic supplement of Bifidobacterium longum increased the acetate levels in the feces of mice and reduced allergic inflammation (Wang et al., 2020).

Kim et al. also studied the effects of Lactobacillus acidophilus and Bifidobacterium lactis on OVA-induced allergy and found that OVA-sensitive rats that did not receive probiotics had higher levels of anti-OVA IgE serums, IgG1 as well as higher IgA in the feces. And in the group receiving probiotics, IL4 levels was lower in comparison with the levels of IL10 and INFγ (Kim et al., 2008). In this study, the results are completely similar to our results which is due to interleukins and cytokines. On the other hand, we know that a probiotic bacterium is required

to be resistant to the acidic pH of the stomach and bile salts of the intestine in order to reach and settle in the intestine. Therefore, resistance to acids and bile salts is the main characteristics for the selection of probiotic bacteria. The necessary condition for the probiotic bacteria to settle in the intestine is the ability to attach to the gastrointestinal epithelial cells. Therefore, investigation of bacterial survival in the gastrointestinal tract is one of the most vital factors in selecting a probiotic strain.

Caco-2, HT-29 cell culture line and HT-20MTX mucus producing cell line are usually used in these studies as there are some limitations in studying the settlement of probiotic bacteria in the intestines of humans and animals (Herbst et al., 2011). Sansawat et al. (2008) showed that two strains of Bacillus subtilis has the ability to bind to the Caco2 cell line and, given their properties, can be used as a probiotic in freshwater shrimp and prevent the Aeromonas hydrophila-induced disease to a large extent (Sansawat and Thirabunyanon, 2009).

The results of this study showed that all the examined strains had the ability to bind to the Caco-2 cell line, but their binding power was significantly different. Among the strains, 17, 204 and 15 had extensive binding ability, while 3, 28 and 75C were weakly bound to the cell line. The rest of the studied strains showed less ability to bind to the cell line.

In the present study, consumption of Bacillus coagulans 6063 probiotic in rats for 21 days caused weight gain in the experimental group. In contrast, both the positive control group and the experimental group with allergy lost weight. The strain in this study significantly reduced blood IgE levels in rats. Studies show that as non-pathogenic living microorganisms, probiotics have beneficial effects on health, including decreasing IgE levels, improving different types of allergies, promoting immunity levels and increasing resistance to pathogens. Probiotics have also the ability to increase and enhance the function of beneficial intestinal microbes by affecting the intestinal microflora; Therefore, this theory was formed that the microflora can be a good target in identifying people prone to allergies, and improving the microflora can be effective in reducing allergic symptoms (Barcik et al., 2020; Zech et al., 2016). So, it can be concluded that the native Bacillus coagulans used in this study can also be effective in the treatment and prevention of type 1 allergies, in addition to its appropriate probiotic properties. This bacterium can prevent the destructive effects of allergies by improving the microflora pattern and preventing weight loss. Of course, its use as a drug requires more complementary examinations.

4 Conclusion

Considering the findings of the present study, it can be concluded that Bacillus coagulans, a probiotic from Iran, possibly improves immune system function, strengthens and improves intestinal microflora, and reduces IgE levels and subsequently, the clinical manifestations of allergies. Some factors are involved in the decrease in allergy symptoms and increased tolerance against the bacteria, including TH2 balance followed by IL-4, IL-10 and other cytokines effective in inflammation and other factors involved in the allergenic process against antigen. Bacillus coagulans also caused weight gain in rats and increased resistance against allergies.

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Conflict of interests

The authors state that there are no conflicts of interest regarding the publication of this article.

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