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Research

An Investigation on the Anti-allergic Impacts of the Native Probiotic Bacteria Isolated from the Honey Crop of Asian Honey Bees on Allergic Mice Model

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Abstract *: Probiotic bacteria exert positive effects on human health. Among a wide range of health beneficial effects, the anti-allergic effect is an important one. One indication of allergic reaction is the increase in IgE level in our body. The mechanism initialized by probiotic bacteria leads to the decrease of IgE level that ultimately inhibits the allergic reactions. The present study is aimed to screen anti-allergic effect of native probiotics isolated from nectar. The probiotic bacteria were isolated from the nectar sample collected from Rangpur district of Bangladesh. The isolated probiotic bacteria were then subjected to morphological and biochemical tests. These morphological and biochemical tests allowed us to ensure that the isolated bacteria were the probiotic type. Then animal trial was conducted using these probiotic isolates. In the study, mice were used as the animal model. At the end of the trial, the blood samples isolated from different mice groups were analyzed. It has been found that, the probiotic isolates had significant effect ($p<0.05$) on the IgE level of the mice used this experiment. IgE levels of treatment group 1, treatment group 2 and treatment group 3 were found (9.76±0.2 IU/ml), (5.69±0.14 IU/ml) and (3.22±0.11 IU/ml), respectively. While the probiotic isolates had no significant effect ($p>0.05$) on the eosinophil count and peripheral lymphocyte count of the mice used the experiment.*

Keywords: *Antiallergic Property, Probiotic Isolates, Ige Level, Eosinophil Count, Peripheral Lymphocyte Count*

Introduction

Probiotic is a Latin- and Greek-derived word, meaning 'for life,'which was first used by Kollath (1953). Lilly and Stillwell (1965) were the first to propose a definition of probiotics as the microbes that assist in the growth of other microbes existing in out digestive system. In 2002, an FAO/WHO joint panel defined probiotics are the viable strains of precisely selected microorganisms that, exert a positive physiological impacts on the host, when they are ingested in sufficient quantity (FAO/WHO, 2002). Dr. Guarner and his fellow workers focused on the necessity of the proper concentration of probiotic bacteria with a view to obtaining their respective positive effects (Guarner and Schaafsma, 1998). Most probiotics are bacteria, among which lactic acid bacteria (LAB) are the most common type, but a few molds and yeasts can also be used as probiotics (Oyetayo and Oyetayo, 2005). Genera commonly used as probiotics include *Lactococcus, Lactobacillus, Carnobacterium, Bifidobacterium, Streptococcus, Enterococcus, Pediococcus, Leuconostoc, Propionibacterium, Saccharomyces* yeasts, *Aspergillus* molds, and *Bacillus* species (Amara and Shibl, 2015). A good probiotic candidate must fulfill the following criteria: it must be an organism that is capable of exerting a beneficial effect on the hosts, increased growth or resistance to disease; it must be nonpathogenic and non-toxic; it must be capable of surviving and metabolizing in the gut environment by resisting the low pH of the stomach, organic acids, bile acid and enzymes present in the intestines; and it should be stable under storage and field conditions (Fuller, 1989). Probiotics are called beneficial organisms because they can provide beneficial effects such as altering the intestinal microflora balance, inhibiting the growth of pathogenic bacteria, synthesizing and enhancing the bioavailability of nutrients, promoting good digestion, reducing the effect of allergens, boosting immune function, lowering cholesterol, stimulating the immune system, alleviating lactose intolerance, and increasing resistance to infection. New evidence supports a role for probiotics in the prevention and treatment of other disease conditions, including urogenital infections, cystic fibrosis, various cancers, dental caries, periodontal diseases, and oral malodor (Kaur *et al*., 2015)

Food allergens are the particular constituents of food items which are normally detected by human immune system and ultimately cause several allergic reactions (Boyce *et al*., 2011). Anaphylaxis is, no doubt, one of the most dangerous as well as potentially lethal allergic reactions. There are some other types of allergic reactions like –gastrointestinal manifestations such as diarrhea, vomiting, abdominal pain, dysphagia, feeding disorders, reflux, bloody stools

and growth failure; cutaneous manifestations such as eczema, flushing, angioedema, pruritus and urticaria and respiratory manifestations such as dyspnea, wheezing, nasal congestion, rhinorrhea and sneezing (Nowak-Wegrzyn *et al*., 2016). Probiotic microorganisms have been assumed to activate of endogenous macrophages, inflection of regional and systemic release of Immunoglobulin A (IgA) and change of the levels of pro-inflammatory and anti-inflammatory cytokine, leading to the modulation of the response against allergens present in our day- to- day food items (Vandenplas *et al*., 2015). The term allergens refer to the antigens responsible for allergy (Johansson *et al*., 2004). These types of antigens are able to come in the contact of immune system by a number of ways. For example, respiration, ingestion, contact to skin and so on. Sometimes these allergens have direct access into our body due to an insect bite (Weiner *et al*., 2011). Atopy is a proclivity (either personal or familial), basically during childhood or adolescence, to produce immunoglobulin E (IgE) in response to the general exposure to allergens (usually proteins) and to exhibit typical symptoms such as rhinoconjunctivitis, asthma and dermatitis or eczema. However, all the cases cannot be attributable to mechanisms related to IgE (Johansson *et al*., 2004; Brozek *et al*., 2010; Fiocchi *et al*., 2012).

Probiotic bacteria are postulated to regulate and fight with reproductive tract infections as well as urinary tract infections (Reid and Bocking, 2003; Barrons and Tassone, 2008). In case of dermal applications, probiotic microorganisms may be ingested orally to exert an immune response which has particular systemic impacts. For example, to control dermatological diseases and inflammation of skin in general probiotic preparations can be applied. Probiotic bacteria have also been utilized for the protection against certain infections of respiratory tract (Caramia *et al*., 2008; Hojsak *et al*., 2010). A number of experimental works have presented the specific influences exerted by probiotic bacteria on the epithelial cells of intestine and immune cells with anti-allergic capability (Caramia *et al*., 2008).

Honeybees possess a diverse LAB (Lactic Acid Bacteria) microbiota in their honey crop, acquired by consuming pollen and nectar and through contact with older bees of the colony. All the extant honey bee species possess *Lactobacillus* and *Bifidobacterium* that possess antimicrobial and antiallergic activities (Forsgren *et al*., 2009).

The present study was designed to measure the effects of presumptive probiotic strains on the IgE level, the eosinophil count and the peripheral lymphocyte count of mice.

Materials and methods

Presumptive probiotic bacteria (*Lactobacillus* spp.) were used in various concentrations (mL) after subdividing the mice into six groups namely negative control group (NCG), positive control group (PCG), standard group (SG), treatment group 1 (TG 1), treatment group 2 (TG 2) and treatment group 3 (TG 3) for a 4-week trial **(Table 1)**.

Feeding procedure used in the mice trial

In the negative control group, only basal feed was provided from week 1 to week 4. In case of positive control group, basal feed was given from week 1 to week 2 and a histamine (Minarin) along with basal feed was given from week 3 to week 4. For the standard group, basal feed was given from week 1 to week 2 and a histamine (Minarin, at the rate of 1% of body weight) and an anti-histamine (Fenadin, at the rate of 1% of body weight) were provided parallel with basal feed from week 3 to week 4. For the treatment group 1, basal feed was given from week 1 to week 2 and a histamine (Minarin, at the rate of 1% of body weight) and nectar (at the rate of 0.5mL/mouse) were provided parallel with basal feed from week 3 to week 4. For the treatment group 2, basal feed was given from week 1 to week 2 and a histamine (Minarin) and nectar (at the rate of 1mL/mouse) were provided parallel with basal feed from week 3 to week 4. For the treatment group 3, basal feed was given from week 1 to week 2 and a histamine (Minarin) and nectar (at the rate of 1.5mL/mouse) were provided parallel with basal feed from week 3 to week 4 **(Table 2)**.

Table 1: Number of mice used for the trial

Group	No of mice
Negative Control Group	6
Positive Control Group	6
Standard Group	6
Treatment Groups	18 (three subgroups were made, each having 6 mice)
Total	36 (for a single experiment)
Total 108 mice were used for three experiments	

Table 2: Feeding system during the trial

Group	Week 1	Week 2	Week 3	Week 4
Negative Control Group	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water
Positive Control Group	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water + Minarin	Basal feed + Pure drinking water + Minarin
Standard Group	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water + Minarin + Fenadin	Basal feed + Pure drinking water+ Minarin + Fenadin
Treatment Group 1	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water + Minarin + Nectar (0.5 mL/mouse)	Basal feed + Pure drinking water+ Minarin + Nectar (0.5 mL/mouse)
Treatment Group 2	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water+ Minarin + Nectar (1.0 mL/mouse)	Basal feed + Pure drinking water+ Minarin + Nectar (1.0 mL/mouse)
Treatment Group 3	Basal feed + Pure drinking water	Basal feed + Pure drinking water	Basal feed + Pure drinking water+ Minarin + Nectar (1.5 mL/mouse)	Basal feed + Pure drinking water+ Minarin + Nectar (1.5 mL/mouse)

After then, several physical symptoms like fatigue, loss of appetite, decreased motility, increased prone to any disease and death rate were observed and the following steps were carried out.

Eosinophil level count

The blood was gently mixed in the EDTA vial, so that the cells mixed well with plasma. The blood was drawn in the WBC pipette up to mark 1. Then the excess blood was wiped off from sides of the tip of the pipette. Then the tip was dipped of the pipette in the Dunger's fluid and the fluid was drawn up to mark 11. The dilution was 1 in 10. Holding the pipette horizontally in its long axis, it was rotated slowly to ensure thorough mixing of blood and diluent. This was facilitated by the white bead in the bulb. The cover slip was placed on the cleaned ruled area of the counting chamber. The first 2 to 3 drops (since the fluid has not mixed with blood) of WBC fluid was discarded from the pipette. The chamber was changed by placing the tip of the pipette just beside the cover slip and fluid flows under it by capillary action. Allowed till the counting chamber is just filled. Eosinophils were allowed for 5 minutes to settle in the chamber. The number of eosinophils in the 4 corner squares was counted using a low power objective. Eosinophils were identified because of their bright red granules and count was done within 30 minutes. Absolute eosinophil count (AEC) = (Total number of eosinophil in 4 squares) x 25 (Klion and Weller, 2014)

IgE level count

IgE level was determined using ELISA kit (POINTE SCIENTIFIC INC.,5449 Drive, Canton MI 48188, USA). All the reagents and the samples were brought at room temperature. 20µL sample/standard was added in each well. 100 µL zero buffer was added in each well and mixed well for 10 seconds. The mixture was kept at room temperature for 30 minutes. The well content was discarded and wells were washed 5 times with distilled water. The droplets of water were from the well using absorbent paper. 50 µL enzyme conjugate was added to each well and mixed well for 10 seconds. The mixture was kept at room temperature for 30 minutes. The well content was discarded and wells were washed 5 times with distilled water. The droplets of water were from the well using absorbent paper. 100µL TMB reagent was added to each well and mixed well for 5 minutes. The mixture was kept at room temperature for 20 minutes in dark. Then 100µL stop solution was added to each well and mixed well for 30 seconds. The reading was taken by ELISA reader at 450 nm wavelength within 15 minutes.

Peripheral lymphocyte count

The blood was gently mixed in the EDTA vial, so that the cells mix well with plasma. The blood was drawn in the WBC pipette up to mark 1. The excess blood was wiped off from sides of the tip of the pipette. The tip was dipped of the pipette in the Dunger's fluid and the fluid was drawn up to mark 11. The dilution was 1 in 10. Holding the pipette horizontally in its long axis, it was rotated slowly to ensure thorough mixing of blood and diluent. This was facilitated by the white bead in the bulb. The cover slip was placed on the cleaned ruled area of the counting chamber. The first 2 to 3 drops (since the fluid has not mixed with blood) of WBC fluid was discarded from the pipette. The chamber was changed by placing the tip of the pipette just beside the cover slip and fluid flows under it by capillary action. Allowed till the counting chamber is just filled. Lymphocytes were allowed for 5 minutes to settle in the chamber. The number of lymphocytes in the 4 corner squares was counted using a low power objective. Lymphocytes were identified because of their bright red granules and count was done within 30 minutes. Absolute lymphocyte count (APC) = (Total number of eosinophil in 4 squares) x 25 (Godkar, 2005).

Results and discussion

Isolation of probiotic bacteria

Morphological and biochemical test of seven isolated probiotic bacteria were presented in the **Table 3** where seven isolated bacteria were rod shaped, gram positive, catalase negative, non-motile, and coagulase positive. All the isolated bacteria were tolerant to harsh condition at pH (6.3-6.9), temperature (35^{o} C), NaCl (4%), phenol (0.2%), and bile salt (0.3%). All the results showed that the bacteria were typical *Lactobacillus* spp.

Table 3: Morphological and biochemical test of seven isolated probiotic bacteria

Biochemical tests	Results
Gram Staining	+
Shape	Rod
Catalase test	–
Motility test	Non motile
Coagulase test	+

Sugar fermentation test	Glucose (+), Lactose (+), Dextrose (+), Sucrose (+), Fructose (+), Maltose (-)
pH tolerance test	6.3 to 6.9
Bile salt tolerance test	0.3%
NaCl tolerance test	4%
Phenol tolerance test	0.2%
Temperature test	35^{o}C

+ indicate positive results and – indicate negative resuts

IgE levels, eosinophil count and peripheral lymphocyte count

Immunoglobulin E (IgE) levels, eosinophil count and peripheral lymphocyte count of the treatment groups with the negative, positive and standard control group have been presented in **Figure 1, 2 and 3.**

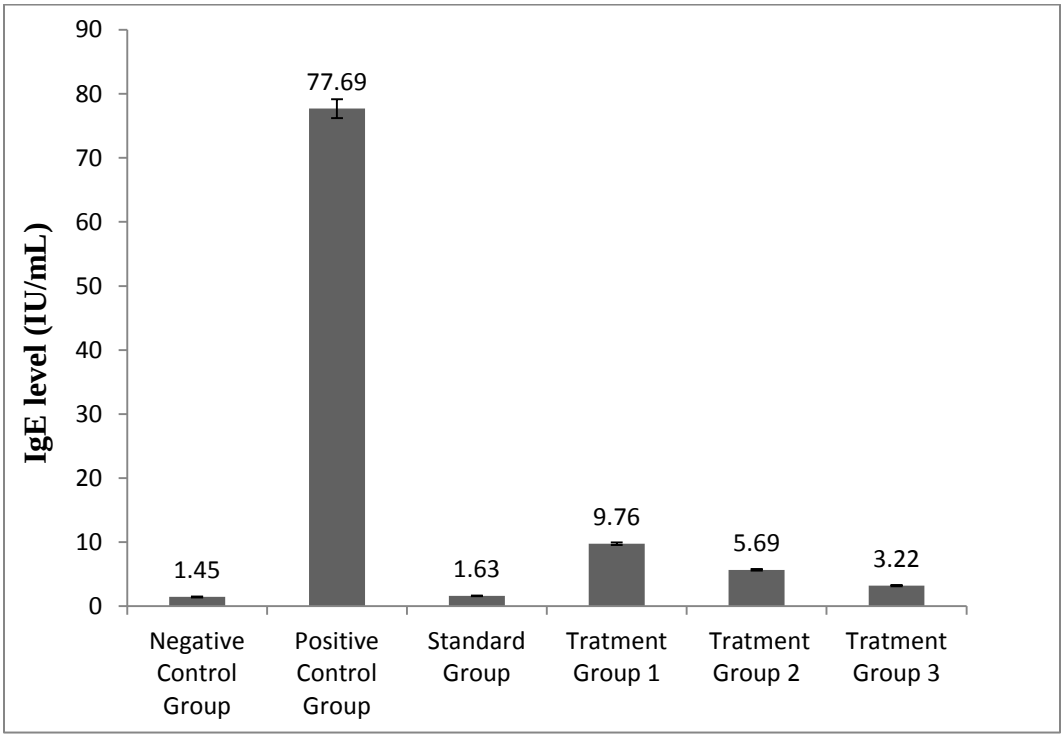

Figure 1: Comparison of Immunoglobulin E (IgE) levels of the treatment groups with the negative, positive and standard control group. (IgE) levels were expressed as International Units per Millilitre (IU/mL). Six mice were used for each group in a single experiment. Values are presented as mean ± SD (bars), n=3.

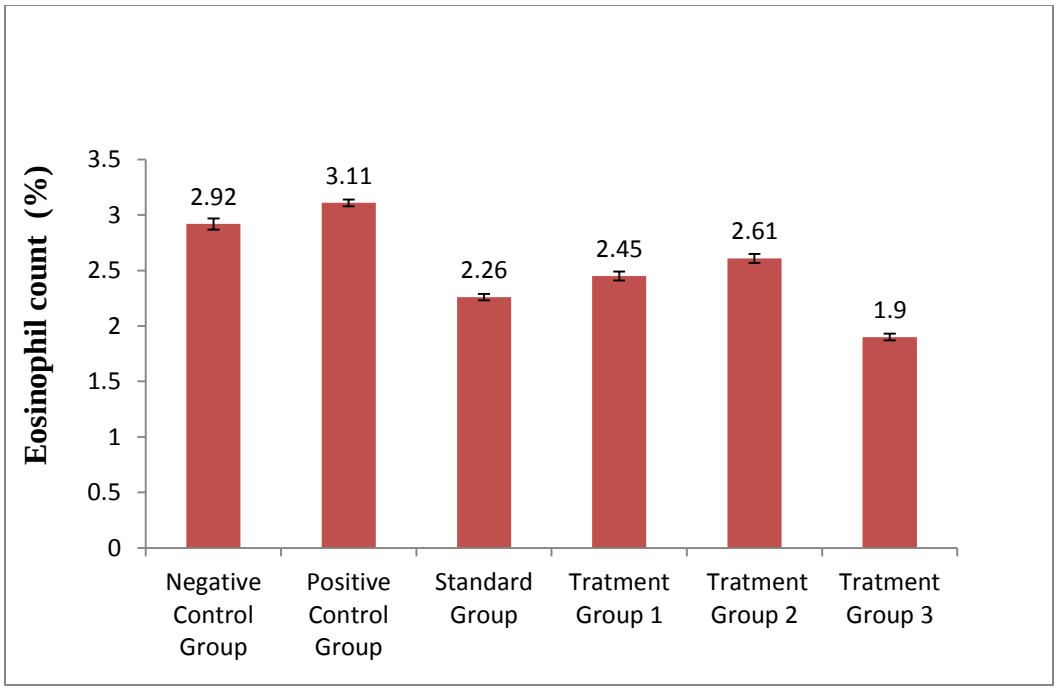

Figure 2: Comparison of eosinophil count of the treatment groups with the negative, positive and standard control group. Six mice were used for each group in a single experiment. Values are presented as mean ± SD (bars), n=3.

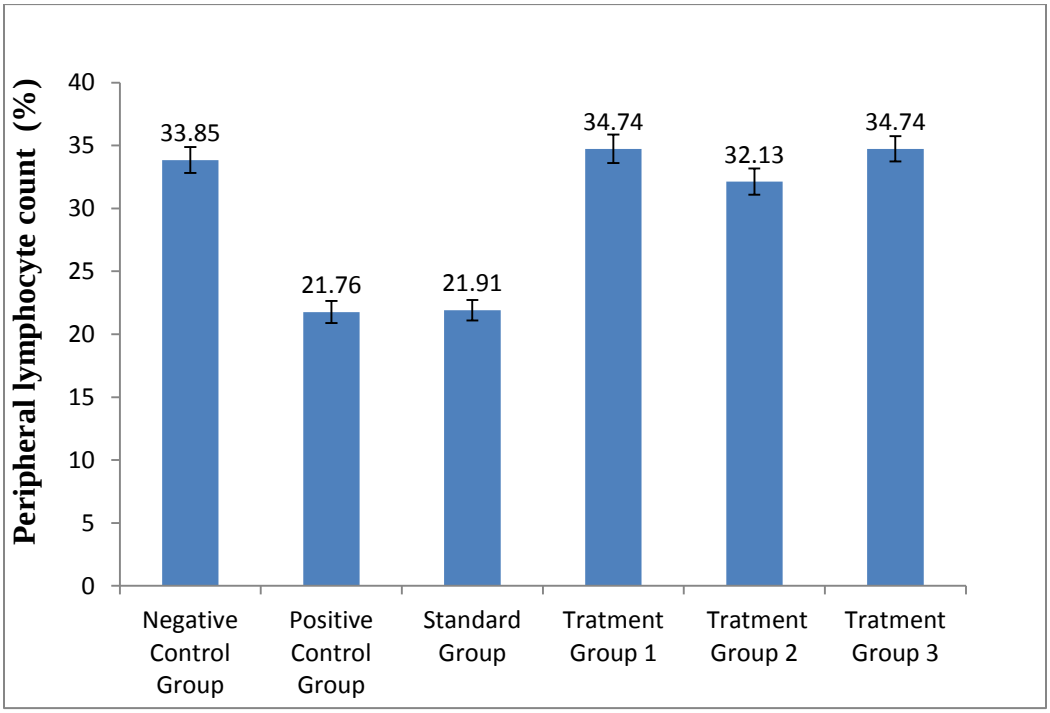

Figure 3: Comparison of peripheral lymphocyte count of the treatment groups with the negative, positive and standard control group. Six mice were used for each group in a single experiment. Values are

presented as mean ± SD (bars), n=3.

In this study, three parameters were considered. They are – IgE level, eosinophil count and peripheral lymphocyte count. From the **Figure 1**, it has been found that, the nectar had significant effect on the IgE level of the mice used this experiment. The negative control group had the lowest IgE level (1.45±0.06 IU/mL, where the positive control group had the highest value of IgE level (77.69±1.48 IU/mL). The standard group accounted for 1.63±0.04 IU/mL. In case of treatment groups, treatment group 1, treatment group 2 and treatment group 3 showed (9.76±0.2 IU/mL), (5.69±0.14 IU/mL) and (3.22±0.11 IU/mL), respectively. These results indicated that the intervention with living probiotics which can prevent allergic response in the mice model. There are some variations in the results of this current study with the result of similar studies including the study of Kim et al. (2005) in which the total serum IgE levels in the three treated groups were not significantly different at week 7 from the levels in mice (naive, 295 ± 25 ng/mL; BGN4, 389 ± 31 ng/mL; *L. casei*, 333 ± 69 ng/mL; *E. coli*, 314 ± 78 ng/mL). These variations may be due to different sample size, probiotic content of the nectar and the process of probiotic ingestion. In the study of Kim, the sample size was 30 and in the current study, the sample size was 36. In the study of Kim, bacteria were provided in powder form but in the current study, the bacteria were provided through nectar.

In this current study**,** the eosinophil count of the negative control group was (2.92±0.05)% and the positive control group presented (3.11±0.03)%. The eosinophil count was found (2.26±0.03)% in the standard group. Among the treatment groups, treatment group 1, treatment group 2 and treatment group 3 displayed (2.45±0.04)%, (2.61±0.04)% and (1.9±0.03)%, respectively **(Figure 2)**. There are some variations in the results of this current study with the result of similar studies. For example, the study of Zhong et al. (2012) where the negative control group showed the eosinophil count per HPF (30.73 ± 10.3), the positive control group showed (75.2 ± 13.7) and the probiotic treated group showed (33.8 ± 9.1).

The value of peripheral lymphocyte count was observed (33.85±1.02)% in case of negative control group whereas, the positive control group accounted for (21.76±0.88)%. The standard group stood for (21.91±0.81)%. The peripheral lymphocyte counts of treatment group 1, treatment group 2 and treatment group 3 were found (34.74±1.12)%, (32.13±1.05)% and (34.74±1)%, respectively **(Figure 3)**. It has been observed that, the nectar increased the level of peripheral lymphocyte at the significant level ($p<0.05$). Here, it has been found that, the nectar

had no significant effect on the peripheral lymphocyte count of the mice used this experiment. There are some variations in the results of this current study with the result of similar studies like the study conducted by Victoria et al. (1999). In the study conducted by Victoria, the value of peripheral lymphocyte count was (48.46±1.07)% in case of negative control group but the positive control group showed (12.55±0.59)%. The standard group accounted for (90±0.07)% and the peripheral lymphocyte counts of treatment group was (55.23±1.2)%.

All such variations may be due to different sample size, different probiotic content of the nectar, difference in the process of probiotic ingestion and the difference in the process of blood sample handling.

Conclusion

It can be concluded that, the presumptive probiotic bacteria have profound effect on the IgE level of mice. But *Lactobacillus* has not any significant effect on the eosinophil level and peripheral lymphocyte level. So, *Lactobacillus* may be used as an agent to prevent allergy. This research work will serve to present *Lactobacillus* as a potential agent to fight against allergy. It can be expected that in near future we will be able to used *Lactobacillus* not only in nectar but also in other food items for human consumption. The ultimate effect will be the reduction of IgE level, which will contribute a lot for anti-allergic mechanism.

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