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Effect of Multi-Strain Probiotics on Levels of IL-6 in Saliva, and the Gingival Health Condition in 10 Years Old Children

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Abstract

Aim of the study: to evaluate the effect of probiotics on gingival health and levels of IL-6 in saliva. Measuring levels of IL-6 in different intervals at zero time before taking probiotic lozenges, 14 days after daily intake of probiotics, and after 28 days of daily intake of probiotics with measuring the gingival index (GI) for children. **Material and method**: A prospective study was conducted from 18, November 2023 to 18, December 2023, involving 100 children (49 females and 51 males) aged 10 years in Anbar, Iraq. Saliva samples were collected at baseline (day 0), day 14, and day 28 after taking probiotic lozenges to measure cytokine levels (IL-6) using ELISA assays. Gingival health was assessed using the Gingival Index. **Results**: The period at 28 days for Gingival Index (GI) recorded the lowest mean±SD, at 0.517 ±0.23, closely followed by the highest record at the baseline period (day 0) with a mean±SD of 1.51 ±0.18. In contrast, the period at 14 days showed mean±SD, at 0.94 ±0.26. Statistical analysis indicated a significant differences among these periods (p-value < 0.05).

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The results for IL-6 levels indicated that the 14-day group exhibited the highest mean±SD, at 0.55±0.15. This was followed by the 28-day group, which showed a mean±SD of 0.52±0.11. In contrast, the baseline group (day 0) had the lowest mean±SD, at 0.48±0.12. **Conclusion**: The study demonstrated significant improvements in gingival health with probiotic lozenge use over 28 days. There was a marked reduction in the Gingival Index, indicating decreased gingivitis severity, from moderate gingivitis (at baseline) to mild gingivitis (after 28 days). The IL-6 levels peaked at 14 days, suggesting an initial immune response, and then slightly decreased by 28 days, reflecting an adaptation to probiotic treatment.

Keywords: probiotics, cytokine, IL-6, gingivitis.

Introduction

Probiotics are live microbes or food substances that contain living microorganisms. When taken in suitable amounts, they have a beneficial effect on health. Several studies have demonstrated the successful use of probiotics in the prevention and treatment of gut diseases. The gut, which is the largest community of microbes living together in harmony, can experience a shift to a condition of dysbiosis or a pathological condition (1-3)

In recent years, attention has been focused on using probiotics and their effect on oral health because the oral cavity is considered the second largest community of microbes in the human body. The action of probiotics either affects co-aggregation or modulates the immune response (2, 4)

It has been proven that most oral diseases are plaque-related, such as dental caries, periodontal diseases, gingival inflammation, and halitosis. While the use of antibiotics affects the microbes in the oral cavity and disrupts homeostasis, probiotics reduce biofilm formation without affecting the oral equilibrium (5) .

The most common probiotics used are *Lactobacillus* and Bifidobacterium strains, which have been proven safe and effective. These microbes are found early in an infant's mouth, as they are present in breast milk (6). They act by neutralizing the pathological effects of microorganisms, preventing their accumulation through natural adherence to the epithelium, mucosa, and tooth structure, and modulating the immune response to reduce cytokine release and inflammation. Probiotics have been shown to reduce the

number of pathogens and cytokines, decreasing microbial pathogenicity and gingival inflammation (7). The probiotic bacteria used in this study are *acidophilus lactobacillu*s and *bifidobacterium* species.

The action of lactobacillus bacteria (probiotics) depends on the secretion of lactic acid and others, producing an antimicrobial substance that has inhibitory effects and antimicrobial effects (8). It has the ability to stop and reverse the acute effect of pathogens by meeting the entero pathogen with supernatants of Lactobacillus (9). In addition, probiotics reduce the number of pathogens and the number of cytokines leading to a decrease in the pathogenicity of microbes and then lessening the gingival inflammation as in Figure 1.

Probiotics affect the immune system (humeral and cellular) by representing structures present in cell membranes like pathogen-associated molecule pattern (PAMP) present in most pathogens, which binds with pattern recognition receptors (PRR) present in the cell membrane of host cells to produce the first response to the pathogen. Like (TLR) a toll like receptor (which is a membrane protein) present in dendritic, immune, and macrophage cells (immune, and non-immune cells). The effect of probiotics is modulating the immune response, by attachment of probiotics with human cells (immune–non-immune cells) through TLR which leads to suppression of the TLR signaling to immune cells, decrease the expression of TLR gene, downregulation of pro-inflammatory interleukins release such as IL-6, IL-12, IL-8 and increase the release of anti-inflammatory cytokines release and maturation of cytokines such as IL-10 (10). The connection between pattern recognition receptors (PRR) in the host cell and the microorganism-associated molecule pattern (MAMP) in probiotic bacteria like lipopoly- saccharide and lipoteichoic acid in the cell membrane lead to induce modulation signaling pathway cascade to the cell nucleus to modulate the expression of gene response like cytokines and other molecule gene expression (11) as shown in Figure 2.

Oral disease treatments are often painful, expensive, and a significant health burden that affects community life. Recent studies suggest that using probiotics for the treatment and prevention of oral diseases could be the future of oral disease management (5, 12).

Gingivitis is an oral disease in which Pathological microorganisms invade the gingival margin, triggering an immune response by activating polymorphonuclear leukocytes and macrophages, leading to increased levels of cytokines such as IL-1β and IL-6, which further destroy the affected area (13). The mechanism of probiotic action according to the study done by (14) showed that lactic acid bacteria (LAB) can stimulate the production of IL-6 from human peripheral blood mononuclear cells (PBMCs). In addition, it indicated that different strains of LAB vary in their ability to induce IL-6 release, with live bacteria generally being more potent inducers than fixed bacteria (glutaraldehyde-fixed bacteria). They observed that IL-6 production was primed by monocyte adherence and affected by several factors such as methods of cell separation, duration of monocyte culture, and donor variability (15). This supports the idea that probiotics can enhance immune responses through cytokine induction, which might explain the increased IL-6 levels observed at different time points in the current study, reflecting the body's response to probiotic administration and subsequent immune modulation (14).

Cytokines are biological substances act as biomarkers to detect the presence of disease or pathology, serving as disease indicators or treatment response detectors. They hold significant value in predicting and prognosticating pathology (16). Cytokines present in saliva, an oral fluid secreted by salivary glands, contain water, electrolytes, and salivary proteins like lysozymes, cytokines, and immunoglobulins. Their main function as a first-line of defense mechanism against physiological trauma and pathological microorganisms (17). Cytokines, such as glycoproteins; modulate the inflammatory response (18).

The most prominent cytokine in oral disease is IL-6, a pro-inflammatory cytokine released by macrophages, T-cells, fibroblasts, and gingival lymphocytes, stimulated by IL-1. It releases in response to acute inflammation and stimulates osteoclast activity (19). IL-6 plays an important role in the host defense mechanism, as macrophages are the first line of innate defense in inflammatory and immune responses. IL-6 is not always present in gingival crevicular fluid, appearing only during activation, such as in cases of viral infection (20). The aim of this study is to determine the effect of probiotics on oral salivary cytokine; pro-inflammatory IL-6

levels in children aged 10 years old and gingival health status.

2. Material and methods

This prospective study was carried out from November 18, 2023 to December 18, 2023. The study group was selected from a primary school in Anbar, Iraq, Prior to commencement, ethical clearance was acquired by the Al-Mustansiriyah University, College of Dentistry. Furthermore, the necessary permissions were obtained from the Ministry of Education to carry out clinical examinations and laboratory biochemical tests. In addition, each participant was given a written consent form and a patient information leaflet to request approval from the child's parents or guardian.

2.1 Subjects

A total of 100 children including males and females in the age range of 10 years (51 male, 49 female) participated in this study, the samples were 4 primary schools. The sample collection in the study occurred at three specific time points: initially before the intervention, then subsequently on Day 14, and again on Day 28 after the commencement of daily probiotic lozenge administration. In this clinical study, an investigation of the effects of probiotic lozenges on gingival health for the same three intervals was performed.

Saliva collection:

` Saliva samples were collected in a similar systematic manner as described by Tsukinoki et al. (2021). Samples were taken at three distinct times: initially, before the administration of probiotic lozenges to establish a baseline, then on Day 14 after the start of daily lozenge intake, and finally on Day 28 (21, 22). Each collection occurred between 9 a.m. and 12 p.m., and samples were handled using the same rigorous protocol collected in sterile plain tubes, centrifuged at 3000 rpm for 15 minutes, the supernatant was then transferred to labeled Eppendorf tubes, and stored at (-80°C) until analysis via Sandwich enzyme immunoassay (ELISA). After deep freezing all samples were left at room temperature for throwing and analyzed to measure the salivary anti-inflammatory IL-6 concentration by following the manufacturer's directions for the use of a commercially available ELISA kit

2.1.1 Inclusion criteria

1. School children (male and female) 10 years old.

2. School children with mild moderate gingivitis.

3. Can chew lozenges.

4. Race from Iraq.

Exclusion criteria:

1. Children above 10 years.

2. Children with chronic disease.

3. Children with systemic disease.

4. Children cannot chew lozenges.

5. Children with autoimmune diseases.

6. Children under antibiotic treatment.

7. Children under preventive treatment like (professional fluoride application, and systemic fluoride application).

Ethical approval

The research study was conducted in accordance with the Declaration of Helsinki, and it was approved by AL-Mustansiriyah University, College of Dentistry's research ethical committee (No MUPRV0010), from December 2023 to December 2025.

Oral Examination:

During the study, each subject underwent a clinical examination conducted by a dentist three times: before the intervention, then subsequently on Day 14, and again on Day 28. This examination included assessing the periodontal status of all teeth using a periodontal probe (to assess the gingival health by examination of teeth 16-22-24 upper. 44-42-36 lower for each tooth examine four surfaces (distobuccal, buccal, mesiobuccal, and lingual surfaces of each tooth) according to Loe and Sillness 1963 (23) measuring index to measure the severity of gingival disease by using diagnostic instruments mirror and periodontal probe (Loe and Sillness, 1963). The key periodontal parameter measured was the Gingival Index. The samples under the study had mild-moderate gingivitis.

Gingival Index (GI)

The Gingival Index system was used to evaluate gingival inflammation around four surfaces of each tooth according to criteria by Loe and Sillness in 1963:

The scoring criteria are as follows:

Score 0: Normal, no inflammation, no bleeding.

Score 1: Slight change in color and mild edema, indicating mild inflammation without bleeding.

Score 2: Redness, hypertrophy, edema, and glazing, denoting moderate inflammation with bleeding on probing.

Score 3: Marked redness, hypertrophy, edema, and ulceration, representing severe inflammation with spontaneous bleeding.

Results

The current study included 100 children age range from 9.5 to 10.5 years (49 females and 51 males).

Descriptive characteristic

In the demographic analysis, it was shown that the gender distribution among the 100 children (49 females and 51 males). As demonstrated in Table 1. However, there was a statistically non-significant difference in responses between male and female participants as shown in Table 1.

The clinical parameter in the oral examination:

Descriptive statistic: Gingival index

The period at 28 days for Gingival Index (GI) recorded the lowest mean±SD, achieving 0.517 ±0.23 (this value for mean (0.51) and SD (0.23)), while the highest record at baseline period (day 0) with a mean±SD of 1.51 ±0.18. In contrast, the period at 14 days showed mean±SD, at 0.94±0.26. Statistical analysis indicated a significant difference among these periods (p-value < 0.05), as shown in Table 2.

Interleukin -6 (IL-6) pg/ml

Descriptive statistics for the IL-6 levels in the follow-up study group are displayed in Table 3. The results indicate that the 14-day group exhibited the highest mean±SD, at 0.556±0.151. This was followed by the 28-day group, which showed a mean±SD of 0.523±0.113. In contrast, the baseline group (day 0) had the lowest mean±SD, at 0.482±0.12 as shown in Table 3.

Comparisons of Gingival Index (GI) levels between periods

Table 4 details the Gingival Index (GI) analysis at three periods baseline, 14 days, and 28 days following the commencement of probiotic lozenge treatment. Comparisons show a significant reduction in GI between baseline and 14 days, similarly, between baseline and 28 days, and between 14 and 28 days, all with an equal level of statistical significance (p=0.0001). As demonstrated in Table 4 and Figure 3.

Comparisons between levels of IL-6 (pg/ml) between periods

To evaluate the mean differences in the level of IL-6 among all periods, the Least Significant Difference Test (LSD) was utilized, as illustrated in Table 5.

The results showed a significant difference between the baseline period (day zero) and both 14 and 28-day periods ($p<0.0001$). Conversely, the comparison between the 14-day and 28-day periods revealed a statistically non-significant difference ($p=0.076$), as detailed in Table 5 and Figure 4.

Discussion

This study involved 100 children aged 10 years, divided by gender (51 males and 49 females. There is no dropout of any participant through the study.

In this study, the selection of 10-year-old children to investigate the effects of probiotics on gingival health and cytokine levels is strategic for multiple reasons. Firstly, by the age of 10, children have typically transitioned from primary to permanent dentition, marking a critical period for influencing long-term oral health outcomes where interventions could have enduring effects. Additionally, children at this age are usually capable of managing basic oral hygiene practices independently, though they may still be refining their techniques, providing an ideal opportunity to assess the benefits of probiotics as a supplementary treatment. Moreover, ten-year-olds are generally able to understand and comply with study requirements, such as taking probiotics regularly and participating in dental examinations (24).

The non-significant difference between genders in response to probiotic treatment in this study can be explained through several biological and social factors. At the age of 10, the biological differences between males and females, particularly in terms of oral environment and immune response, are minimal. Hormonal changes that might affect the oral microbiome immune responses and oral health status more generally occur during pre-puberty which occurs 1-2 years later than from this age (25) .

According to a study by (24) probiotics had a generally uniform effect on oral health across genders in young children, supporting the findings of the current study.

Gingival Index (GI)

The Gingival Index (GI) values at the baseline period (day 0) were significantly higher. It indicates that the patients had a higher level of gingivitis at the start of the study (samples with moderate gingivitis). In contrast, the GI values at 14 days were lower. On the other hand, the GI values at 28 days after starting the probiotic regimen were the lowest (mild gingivitis). This

suggests that the probiotic treatment had a positive effect on gingival health, as the GI values decreased over time.

The improvements in GI might be attributed to probiotics' ability to interfere with the pathogenesis of periodontal disease by competitive inhibition of pathogenic bacteria, production of antimicrobial substances, and modulation of the host immune response. Specifically, probiotics may enhance the production of anti-inflammatory cytokines or directly modulate inflammatory pathways in the gingival tissue. This finding is consistent following (26), which showed similar improvements in periodontal health with extended probiotic use. In addition, Zhang et al. (2022) emphasize the possible therapeutic advantages of adding probiotics to the treatment of periodontal disease, such as enhanced clinical parameters and decreased counts of bacteria below the gingival margin.

A comprehensive review and meta-analysis by (27) discovered that probiotic supplementation led to improvements in clinical parameters, decreased subgingival bacterial counts, and lower levels of pro-inflammatory mediators in patients with periodontal disease.

(28) explore the immune-modulatory effects of probiotics on both illness and wellness. These effects include reducing inflammation and the ability to regulate immune/inflammatory pathways linked with active diseases.

(29) examined the use of probiotics in the treatment of periodontal diseases and peri-implant health. This included their role in controlling biofilm, reversing dysbiosis, and modulating the host response.

In contrast, (30) observed no substantial disparities in Plaque index, Gingival index, and Bleeding on probing, in Gingival crevicular fluid samples after administering yoghurt with beneficial bacteria to patients. This finding contradicts previous studies suggesting that the temporary consumption of probiotics has a positive impact on plaque buildup and gingivitis indicators.

(31) proposed that the advantages of probiotics may reach a point of no further improvement after an initial time, which contradicts the ongoing enhancement shown in this research.

Interleukin - 6 (IL-6) pg/ml

Regarding IL-6, the result found that IL-6 levels were highest at 14 days after starting the probiotic regimen, with a mean value of 0.556± 0.151. By 28 days, the IL-

6 levels had slightly decreased to a mean of 0.523 ± 0.113. Initially, at baseline (0 days), the IL-6 levels were at their lowest, with a mean value of 0.482 ± 0.121.

Different species of Lactobacillus can variably induce cytokine production, including IL-6, IL-12, and TNF-α, which indicates that Lactobacilli may differentially affect dendritic cell (DC) maturation and subsequent immune responses (Christensen, et al., 2002). The capacity of Lactobacilli to induce IL-6 is consistent with their role in enhancing intestinal IgA responses and promoting the terminal differentiation of B cells into plasma cells. The initial rise in IL-6 could be a result of the immune system's attempt to balance the pro-inflammatory and regulatory cytokine responses induced by the probiotics. The eventual decrease in IL-6 suggests an adaptation to the probiotic presence, reducing the inflammatory response as homeostasis is re-established within the oral microbiota (Heufler et al., 1996). According to a study by (14) which showed that lactic acid bacteria (LAB) can stimulate the production of IL-6 from human peripheral blood mononuclear cells (PBMCs). In addition, it indicated that different strains of LAB vary in their ability to induce IL-6 release, with live bacteria generally being more potent inducers than fixed bacteria. Specifically, they observed that IL-6 production was primed by monocyte adherence and affected by factors such as methods of cell separation, duration of monocyte culture, and donor variability. This supports the idea that probiotics can enhance immune responses through cytokine induction, which might explain the increased IL-6 levels observed at different time points in the current study, reflecting the body's response to probiotic administration and subsequent immune modulation.

This study is consistent with (32), they indicate that the probiotic strains could stimulate the production of significant amounts of cytokines, such as IL-6, IL-12, and IL-1β, which are involved in immune responses. In addition, (6) shown that the introduction of probiotics (*Lactobacillus reuteri*) can initially enhance the immune response, which may include an increase in cytokines such as IL-6, followed by a stabilization phase as the body adapts to the probiotics.

Macrophage induced by monocyte activation through a (probiotic supernatant) application like certain types of bacteria such as (*Lactobacillus rhamnosus* , *helveticus* , and *L. casei*) leads to the highest levels of IL-6. Monocyte cells

present in the bloodstream when there is a trigger to immune response such as infection, trauma, or others, they leave the bloodstream and enter the tissue and become activated and transformed into macrophages which leads to an increase in the level of pro-inflammatory IL-6 release (32)

This discrepancy in levels of IL-6 could be attributed to differences in the strains of probiotics used, the dosages administered, or the baseline immune status of the subjects. Probiotics may compete with pathogenic bacteria, reducing their numbers and thereby decreasing the need for an inflammatory response (33).

Conclusion:

The results showed significant improvements in gingival health over 28 days, as evidenced by reductions in the Gingival Index (GI) and changes in cytokine levels (IL-6). In addition, the GI decreased from baseline to 14 days and further to 28 days, indicating a reduction in gingivitis severity (from moderate to mild gingivitis). This demonstrates the positive impact of probiotics on gingival health.

IL-6 levels peaked at 14 days, suggesting an initial immune response, and then slightly decreased by 28 days, reflecting an adaptation to probiotic treatment.

Conflict of interest

The authors reported that they have no conflicts of interest.

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Tables

Table 1: Demographic information of gender in this study.

		Base line record
Gender	Female No. (%)	49 (49.0%)
	Male No. (%)	51 (51.0%)
Total		100 (100.0%)

NS= non-significant at p>0.05, significant at p<0.05.

Table 2: Descriptive statistics for the follow-up study concerning Gingival Index.

Gingival Index			
ANOVA table	Group		
	Base line record (day 0)	14 Days	28 Days
Mean	1.51	0.94	0.51
±SD	0.18	0.26	0.24
±SE	0.01	0.027	0.024
Minimum	1.160	0.400	0.200
Maximum	2.000	1.600	1.100
F	455.923		
p-value	0.0001 sig		

Significant, p<0.05, non-significant, (p>0.05), SD: Standard Deviation, SE: Standard Error, df=2

Table 3: *Descriptive* statistics for the follow-up study concerning IL-6.

IL-6 pg/ml			
ANOVA Table	Group		
	Day 0	14 Days	28 Days
Mean	0.48	0.55	0.52
±SD	0.12	0.15	0.11
±SE	0.01	0.01	0.011
Minimum	0.27	0.05	0.32
Maximum	0.75	0.94	0.81
F	8.17		
p-value	0.0001		

Significant, p<0.05, non-significant, (p>0.05), SD: Standard Deviation, SE: Standard Error, df=2

Table 4: Comparison of Gingival index /probiotic between day 0, 14 days and 28 days.

	Least Significant Difference Test (LSD) to Comparisons	Mean Difference	Adjusted P Value
Gingival Index (GI)	Day 0 X 14 Days	0.56920*	0.0001
	Day 0 X 28 Days	0.99660*	0.0001
	14 Days X 28 Days	0.42740*	0.0001

Significant, p<0.05, non-significant, (p>0.05).

Table 5: Multiple comparisons between follow-up study groups of IL-6 levels.

	Least Significant Difference Test (LSD) to Comparisons	Mean Difference	Adjusted P Value
IL-6	Day 0 X 14 Days	-0.073750*	0.000
	Day 0 X 28 Days	-0.041240*	0.01
	14 Days X 28 Days	0.032510	0.076

Significant, p<0.05, non-significant, (p>0.05)

Figures

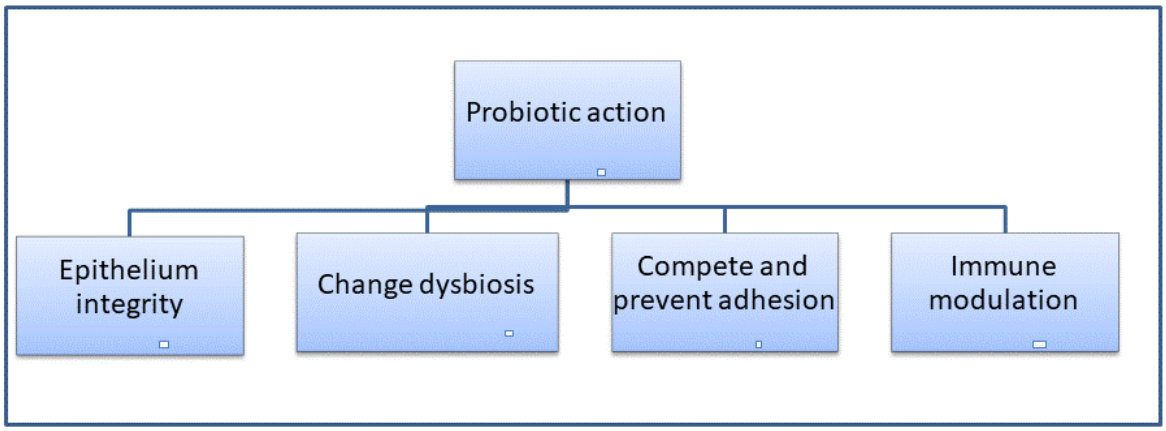

Figure 1: The general mechanisms of probiotics action

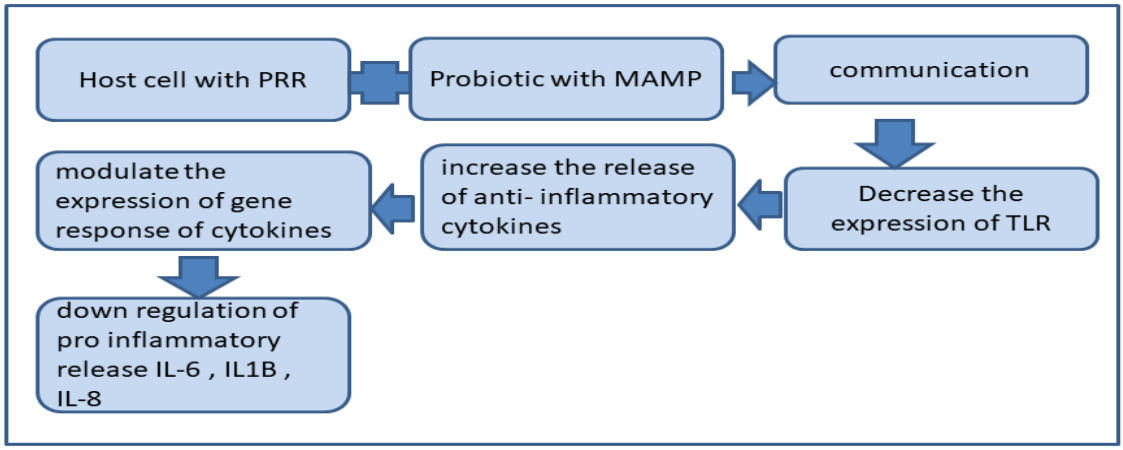

Figure 2: Probiotics and host cell (cellular aspect).

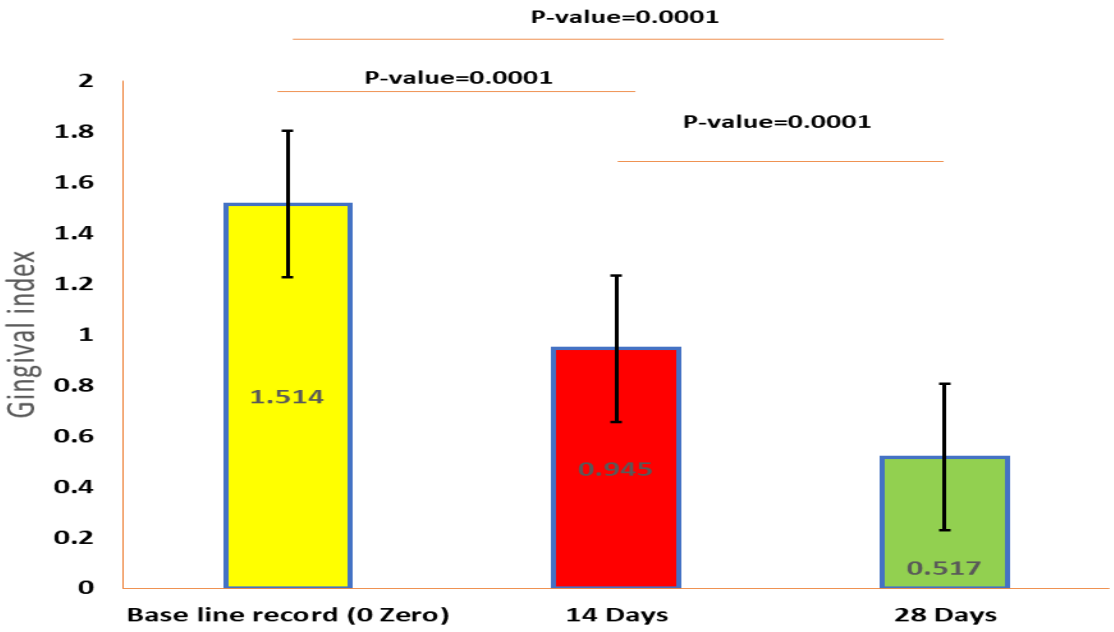

Figure 3: comparison of GI level between follow-up study groups

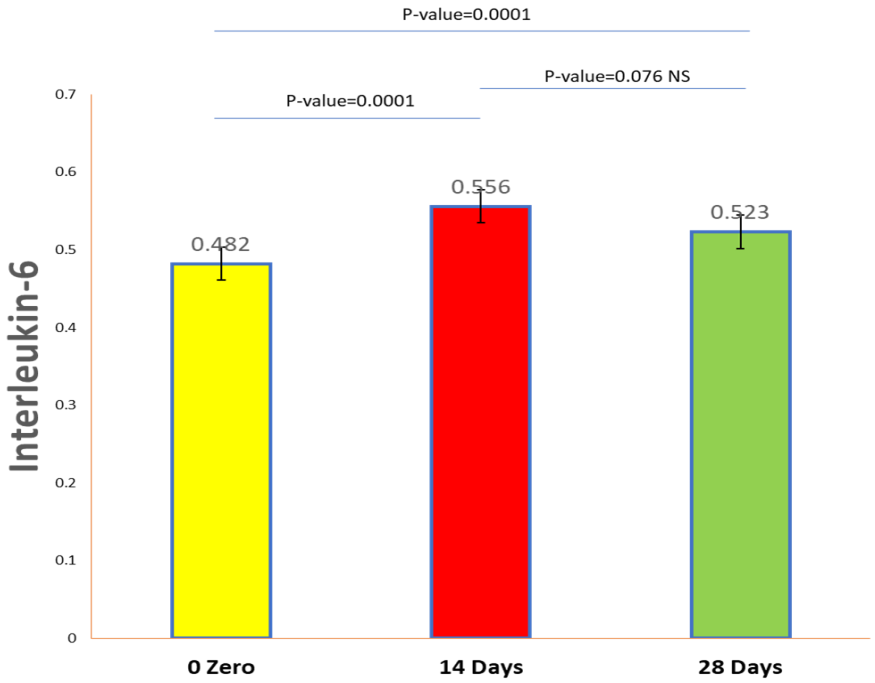

Figure 4: comparison of IL-6 levels between follow-up study groups