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Prospective study of probiotic supplementation results in immune stimulation and improvement of upper respiratory infection rate

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ABSTRACT

The human gut microbiota is an important environmental factor for human health with evolutionarily conserved roles in immunity, metabolism, development, and behavior of the host. Probiotic organisms are claimed to offer several functional properties including stimulation of immune system. The purpose of this study is to investigate the effects of a probiotic supplementation on adult volunteers who have contracted the common cold four or more times in the past year. This study is a single center, double-blind, randomized, controlled, prospective trial. Subjects received a probiotic drink containing *Lactobacillus paracasei* (at least 3×10^7 colony forming units (CFU) ml^{-1}), *Lactobacillus casei* 431® (at least 3×10^7 CFU ml^{-1}) and *Lactobacillus fermentium* PCC® (at least 3×10^6 CFU ml^{-1}) or an identical placebo without probiotics for a 12-week study period. The consumption of probiotics significantly reduced the incidence of upper respiratory infection ($p < 0.023$) and flu-like symptoms with an oral temperature higher than 38 °C ($p < 0.034$) as compared to the placebo group. Subjects that consumed probiotics demonstrated a significantly higher level of IFN-γ in the serum ($p < 0.001$) and sIgA in the gut ($p < 0.010$) as compared to the placebo group and a significant higher level of serum IFN-γ ($p < 0.001$) and gut sIgA ($p < 0.001$) as compared to their baseline test results. In contrast, there were no significant differences in the serum IL-4, IL-10, IgA, IgG or IgM between the probiotics and the placebo groups. Results of this study demonstrated that probiotics were safe and effective for fighting the common cold and influenza-like respiratory infections by boosting the immune system.

1. Introduction

The intestinal microbiota is an ecosystem containing tens of trillions of microorganisms including many species of known bacteria. Bacterial cells outnumber human cells in the body by approximately ten times with 10–100 trillion microbes living in the gastrointestinal (GI) tract alone [1,2]. Probiotics are "live microorganisms which could possess a health benefit on the host when administered in appropriately adequate amounts" [3]. A number of genera of bacteria and yeasts are used as probiotics, including *Lactobacillus, Bifidobacterium, Leuconostoc, Pediococcus, and Enterococcus*. Species belonging to the genera *Lactobacillus* and *Bifidobacterium* are found as a part of the gastrointestinal normal microflora; they are safe and widely used in yogurts and other dairy products [4,5]. There are no universal probiotic strains that could meet all clinical needs [3]. Health benefits derived from the consumption of foods containing probiotic bacteria, such as *Lactobacillus casei, Lactobacillus acidophilus, and Bifidobacterium* spp., have been well studied and reviewed. The probiotics health benefits include controlling gastrointestinal infections, improvement in lactose metabolism, anticarcinogenic and antimutagenic properties, cholesterol reduction, immune system stimulation, and improvement in inflammatory bowel disease [6].

The immune system is complex and needs to be maintained and constantly stimulated by antigens in order to recognize and neutralize pathogens efficiently. The immune system can be

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classified into subsystems, such as, innate immunity versus adaptive immunity or humoral immunity versus cell-mediated immunity. Probiotics play a role in balancing the host defensive mechanism including innate and adaptive immune responses. Probiotics should be safe for use and benefit the host colonic mucosa and systemic immunity by definition [7]. The mechanism of how probiotics work on the host organism and immune system is complicated and still not fully elucidated. However, it is believed that probiotics could promote the production of bacteriocins and short chain fatty acids, lower gut pH, complete available nutrients in the colon, colonization site interference, colonize and compete for binding sites on gut epithelial cells, stimulate mucosal barrier function and modulate the immune system [8]. There are numerous studies which demonstrated that probiotics stimulate the innate and acquired immune response by inducing secretory and systemic IgA secretion, promoting phagocytosis, modifying T-cell responses, maintaining the homeostasis of Th1 and Th2 activities by enhancing Th1 responses and attenuating Th2 responses [9e11]. Animal and human clinical studies showed the various *Lactobacillus* strains modified the Th1 responses by the induction of IFN-γ, IL-2 and tumor necrosis factor (TNF)-β [12–17]. Several animal studies demonstrated the reduction of IL-4, IL-5, IL-10, and IL-13 by either oral feeding or intraperitoneal injection with *Lactobacillus casei, Bifidobacterium animalis, or Bifidobacterium breve* [15,18,19]. Several animal and human clinical studies showed the induction of serum IgA and IgA secreting cells by either *Lactobacillus* or *Bifidobacterium* strains [20–23].

Numerous human and animal studies have been conducted and suggest that probiotics are safe and effective for clinical application on human diseases, such as antibody-associated diarrhea [24,25], inflammatory bowel disease [26], ulcerative colitis [27], GI tumors [28], allergy and eczema [17,19,29,30], and virus infection [31,32]. A randomized, double-blind, placebo-controlled study showed the reduction of the incidence of the common cold, the duration of the common cold symptoms and the pharyngeal symptoms that accompany the common cold by consumption of *Lactobacillus plantarum* HEAL 9 and *Lactobacillus paracasei* 8700 [32]. *Lactobacillus acidophilus* has been used as a live vehicle for oral immunization against chicken anemia virus [33]. A murine study showed that milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus* could be used as a prophylactic against gastrointestinal infections caused by *Shigella* [34]. Also yogurt supplemented with *Lactobacillus acidophilus* and *Bifidobacterium spp*. stimulated the mucosal and systemic IgA responses to the cholera toxin immunogen [35].

2. Materials and methods

2.1. Participants

Subjects were recruited from Beijing Chaoyang Hospital in the city of Beijing, China. The inclusion criteria were: male or female, 25–45 years old, succumbed to the common cold or influenza at least four times in the past calendar year, fully understood the risks and potential benefits of participation in this study, and signed informed consent forms before entering the study. Subjects were excluded if: they were diagnosed with decreased immunity caused by any diagnosed chronic illness, they had any GI illness with medical treatment when being enrolled, they had any diagnosed respiratory illness with symptoms similar to the common cold or influenza, they were currently taking any pain medication, they received any vaccine for upper respiratory infection within 6 month before enrollment, they received any purgative drug or digestion-related drug two weeks prior to enrollment, they took any dairy product containing prebiotics and probiotics ten days prior to enrollment, they took any preventive drug for upper respiratory infection, they received any drug which may impact the immune system (such as antibiotics) three months before enrollment, they were alcoholic or drug addicted, they were pregnant or breast-feeding mothers, or they participated in another clinical trial three months prior to enrollment.

2.2. Study design

This study was a single center, randomized, double-blind, placebo-controlled, prospective trial with a 12-week probiotics intervention. Any changes in medication, health status or adverse events were recorded. All probiotic products except the test drink were forbidden during the entire study. All subjects were given a list of probiotic foods and supplements available in the market to ensure that no forbidden products were consumed. The Human Ethics Committee of Beijing Chaoyang Hospital approved the study protocol. All subjects provided written informed consent.

2.3. Sample size and randomization

Subjects (136) were screened, randomized and enrolled; 67 subjects completed each group with one dropout per group. Subjects were assigned to the groups randomly by the physician and the intervention began immediately following randomization. The subjects, the investigators, the physicians, the study nurses and other study personnel were blinded using randomization codes and were kept confidential until the end of the data analysis. With a power of 80% and at a significance level of 0.05, the difference between the groups would be statistically significant with 60 subjects per group.

2.4. Cultures and probiotic drink

All bacterial strains were supplied as a lyophilized powder from Chr. Hansen (Hørsholm, Denmark). Cultures and were stored at (−18 °C). *Lactobacillus casei* 431® *and L. paracasei* cultures were grown on LC medium (Land Bridge, Beijing. China) and *Lactobacillus fermentum* PCC® was grown on MRS (de Man, Rogosa and Sharpe) agar with tetracycline (tetracycline hydrochloride, Sigma Chemical Company, T-8032) at 37 °C for 3 days. The yogurt drink was fermented from milk (homogeneous, pasteurized at 90–95 °C for 5–10 min, then cooled to 42–43 °C) (800 g L^{-1}), glucose (10 g L^{-1}), and sucrose (Hangzhou Weichuan Foods Company, LTD, Shanghai, China) (70 g L^{-1}) plus the starter culture. Starter culture consisted of *Lactobacillus bulgaricus* and *Streptococcus thermophiles.*

Placebo yogurt drink: The placebo yogurt was fermented by starter culture only. The starter culture contained 1×10^5 CFU ml^{-1} *Lactobacillus bulgaricus* and 1×10^{10} CFU ml^{-1} *Streptococcus thermophiles*. After fermentation for seven hours, stirring and cold storage, the placebo yogurt contained starter culture at 2×10^7 CFU ml^{-1}. The shelf life of the placebo yogurt drink was 28 days when stored at 4 °C. The starter culture remaining in the placebo yogurt after 28 days in storage was 5×10^6 CFU ml^{-1}.

Probiotic yogurt drink: The probiotic yogurt contained starter culture at 2×10^7 CFU ml^{-1} plus *Lactobacillus paracasei* at 1×10^8 CFU ml^{-1}, *L. casei* 431® at 1×10^8 CFU ml^{-1} and *Lactobacillus fermentum* PCC® at 6×10^7 CFU ml^{-1}, otherwise the procedures were the same for preparation. The shelf life of the probiotic yogurt drink was 28 days in cold storage (4 °C) after which it contained starter culture (5×10^6 CFU ml^{-1}), *Lactobacillus paracasei* at 3×10^7 CFU ml^{-1}, *L. casei* 431® at 3×10^7 CFU ml^{-1} and *Lactobacillus fermentum* PCC® at 3×10^6 CFU ml^{-1}.

2.5. Interventions

During the intervention, all subjects received once daily doses; probiotic drink (150 mL) or placebo drink (150 mL) was administered after lunch for a total of 12 weeks. Subject compliance was followed by daily questionnaires.

2.6. Collection of blood and fecal samples

Blood and fecal samples were collected at two time points: baseline, before the intervention began, and at 12 weeks, at the conclusion of the study. Fecal samples were collected into two plastic tubes and immediately frozen at −20 °C for the fecal sIgA analysis. At each time point, 5.0 mL of blood was drawn for IFN-γ, IL-4, IL-10, IgA, IgG and IgM analysis, and 2.0 mL for the complete blood count analysis.

2.7. Statistical analysis

Descriptive statistics were provided for baseline subject characters and outcome variables by study groups. Mean and standard deviation (SD) were reported for continuous variables; frequency and percentage were reported for categorical variables.

Incidence of flu-like illness and upper respiratory infection (URI), as well as, medical treatments and absence from work due to flu-like illness and URI during the study were calculated for each study group. The difference between study groups in these variables was evaluated using logistic regression models.

For continuous outcomes (IL-4, IL-10, IFN-γ, IgA, IgG, IgM and sIgA concentrations) differences between study groups were evaluated using F-test; pre- and post-intervention difference within each study group was evaluated using paired *t*-test.

Statistical analysis in this study was performed using SAS 9.3 statistical software (SAS Institute Inc., USA). All tests employed a 0.05 significance level.

3. Results

A total of 136 participants (25—45 years old) who sustained common cold or influenza—like respiratory illness (collectively upper respiratory infections (URI)) at least four times in the

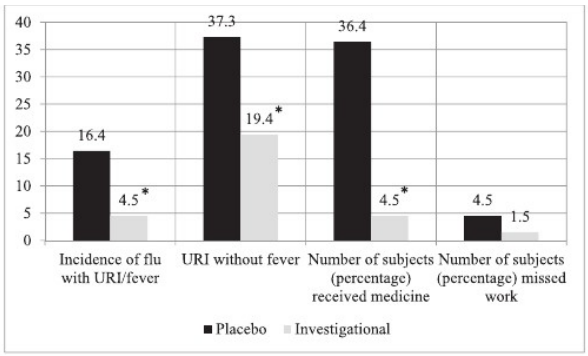

Fig. 2. Incidence of subjects with URI with fever, subjects with URI without fever, subjects who took medication, subjects who missed work due to URI (percentages) (* indicates statistical significance).

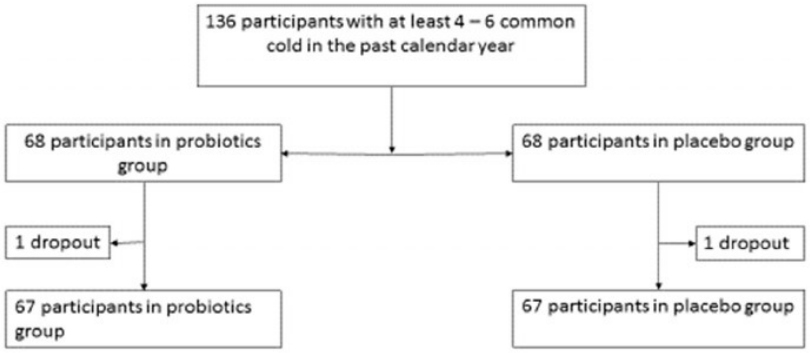

Fig. 1. Flowchart of the study participants throughout the study.

Table 1
Differences of demographic, body condition and the incidence of the common cold at baseline between the placebo and investigational groups (p > 0.05) (n = 134).

Baseline	Placebo (n = 67)	Probiotic (n = 67)	All subjects(n = 134)	Group difference (p value)
Sex				
male	33 (49.3%)	33 (49.3%)	66 (49.3%)	N/A
Female	34 (50.7%)	34 (50.7%)	68 (50.7%)	
Age(year)	32.6 ± 6.5	34.3 ± 6.0	33.4 ± 6.3	0.118
Body weight(KG)	68.3 ± 11.2	68.7 ± 11.6	68.5 ± 11.3	0.838
Height(cm)	166.9 ± 7.1	167.3 ± 8.4	167.1 ± 7.8	0.791
BMI	24.4 ± 2.8	24.4 ± 2.9	24.4 ± 2.9	0.913
Body temperature(°C)	36.3 ± 0.2	36.3 ± 0.2	36.3 ± 0.2	0.684
Systolic (mm Hg)	127.4 ± 5.3	126.7 ± 7.2	127.1 ± 6.3	0.504
Diastolic (mm Hg)	79.0 ± 4.0	78.6 ± 5.1	78.8 ± 4.6	0.665
Incidence of the common cold and flu in the past calendar year	4.9 ± 0.9	4.7 ± 0.8	4.8 ± 0.9	0.313
History of smoking	8 (11.9%)	9 (13.4%)	17 (12.7%)	0.795
History of alcohol use	14 (20.9%)	11 (16.4%)	25 (18.7%)	0.507

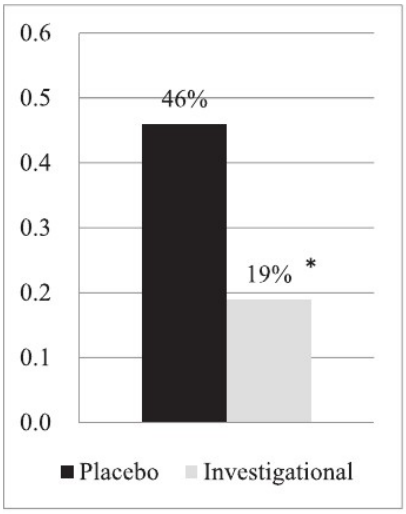

Fig. 3. Incidence of study participants with URI with and without probiotics (percentages) (* indicates statistical significance).

previous year were enrolled; 67 participants completed the trial from each group (Fig. 1). The gender ratio of completed participants was 66 males to 68 females (49.3%:50.7%) (Table 1). There were a total of two dropouts during the study, one from each group.

Statistically significant differences between the two groups regarding the incidence of upper respiratory infection during the study are presented in Supplemental Table 1. There were a total of 14 participants (11 from the placebo group (16.4%) and 3 from the investigational group (4.5%), $p < 0.034$) who had an influenza-like illness with body temperature higher than 38 °C and at least one of the URI symptoms, such as cough, nasal congestion, headache, or muscle pain, etc. There were a total of 38 participants (25 from the placebo group (37.3%) and 13 from the investigational group (19.4%), $p < 0.023$) who had no fever but showed at least one of the URI symptoms during the study. There were a total of 27 participants (24 from the placebo group (35.8%) and 3 from the investigational group (4.5%), $p < 0.001$) who received drug treatment for their URI symptoms during the study. Even though there were 3 participants from the placebo group that missed work and one participant from investigational group that missed work due to URI, there was no statistically significant difference between the two groups. The incidence of URI with and without flu and fever is presented in Fig. 2.

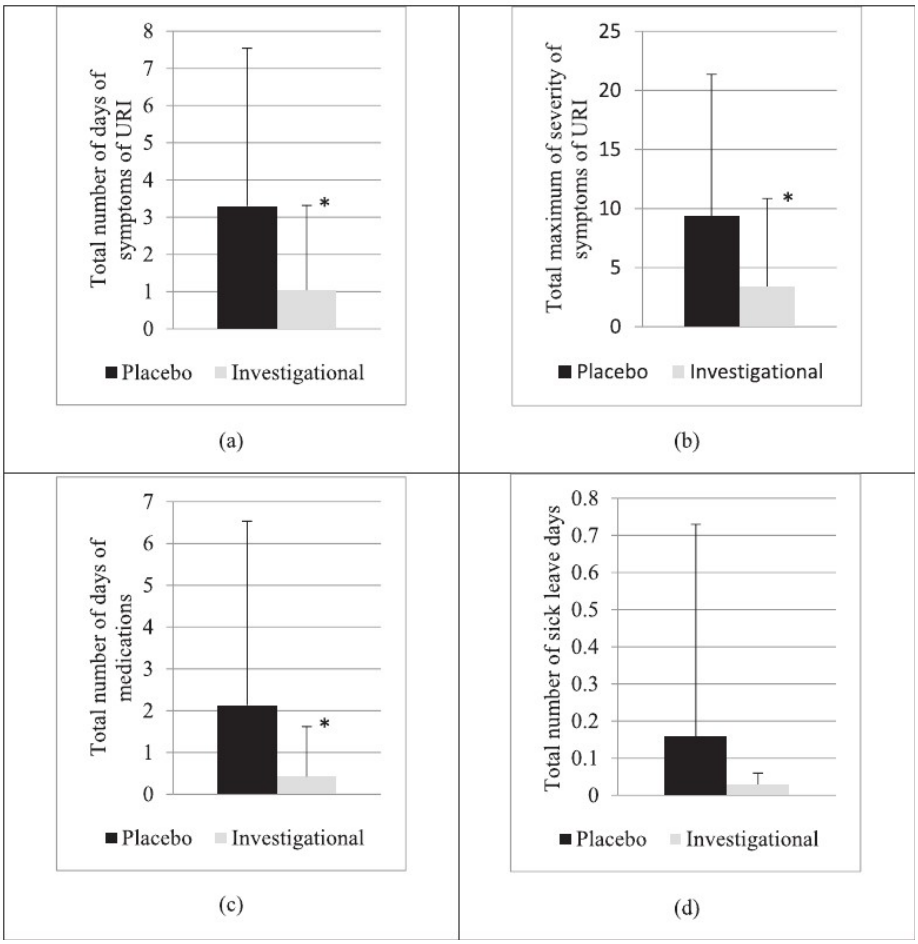

Fig. 4. Duration, severity, days of medication, and sick leave days (Panels a–d, respectively) in each group with URI symptoms (Total participants) (* indicates statistical significance).

The results (Supplemental Table 2) showed that the differences between the groups during the trial with no incidence of URI, with one incidence of URI, and with two incidences of URIl are statistically significant ($p < 0.004$). Supplemental Table 2 shows the average cases of URI without flu among the placebo and probiotics groups; the total cases of URI among the individuals who received probiotics were less than half that of the placebo group (Fig. 3). The ANOVA test showed significantly fewer incidences of common cold/flu in the probiotics investigational group than the placebo group ($p < 0.002$). FISHER statistical method was used for the analysis.

The results (Supplemental Table 3) showed that the average days of URI symptoms, total scores of severity of URI symptoms, and the average days receiving medicine (Fig. 4, panel a–c) during the trial, counting all participants. The probiotics group is statistically less than the placebo group ($p < 0.001$, $p < 0.001$ and $p < 0.002$, accordingly). However, the average days of sick leave between the two groups (Fig. 4, panel d) did not show any significant differences ($p < 0.074$). ANOVA statistical method was used for the analysis.

The results (Supplemental Table 4) showed that the average days of having URI symptoms and total scores of severity of URI symptoms (Fig. 5, panel a, b) during the trial when counting only participants with URI in the probiotics group are statistically less than in the placebo groups ($p < 0.002$ and $p < 0.028$, respectively). However, the average days receiving medicine and the average days of sick leave (Fig. 5, panels c, d) between the two groups did not show any significant differences ($p < 0.064$ and $p < 0.290$, respectively). ANOVA statistical method was used for the analysis.

Participants in the probiotics group showed significantly higher levels of serum IFN-γ than the placebo group at the end of the probiotics intervention (Table 2, Fig. 6) ($p < 0.001$) but without significant differences at baseline ($p < 0.654$). Also, participants in the probiotics group showed significantly increased serum IFN-γ levels after probiotics intervention compared to baseline levels (Table 2) ($p < 0.001$). ANOVA and paired *t*-test statistical method

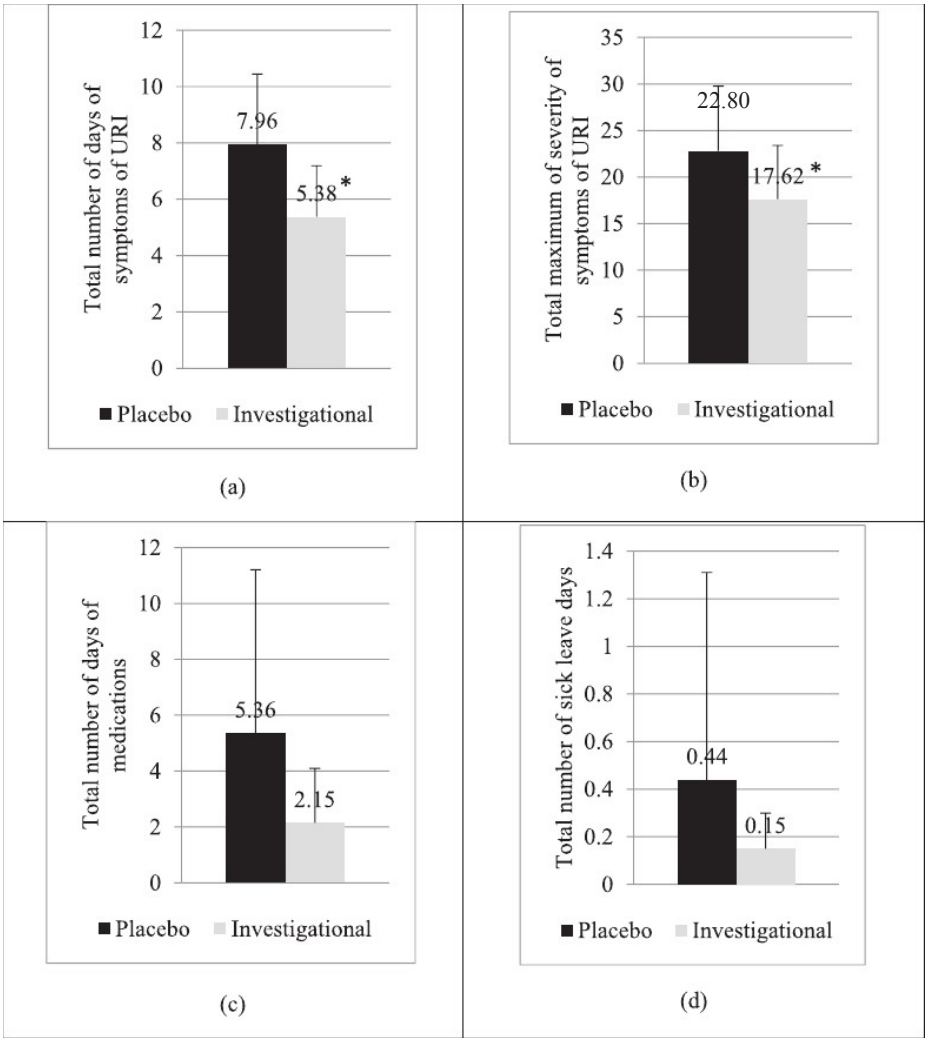

Fig. 5. Duration, severity, days of medication, and sick leave days (Panels a–d, respectively) in each group with URI symptoms (Only participants with common cold/flu counted) (* indicates statistical significance).

Table 2
Fecal sIgA level and serum immune markers at baseline and after intervention; mean value ± standard deviation, differences between groups (ANOVA).

	Blood index	Placebo	Investigational	Group difference (p value)
Baseline	human interleukin 4(IL-4) (ng/ml)	0.77 ± 0.09	0.79 ± 0.10	0.261
	human interleukin10(IL-10) (pg/ml)	25.03 ± 1.31	25.11 ± 1.22	0.716
	interferon IFN-γ (pg/ml)	121.97 ± 16.35	123.25 ± 16.59	0.654
	immunoglobulin A IgA (g/L)	2.12 ± 0.51	2.15 ± 0.53	0.706
	immunoglobulin G IgG (g/L)	12.08 ± 2.05	11.84 ± 1.97	0.501
	immunoglobulin M IgM (g/L)	1.10 ± 0.45	1.07 ± 0.37	0.688
	sIgA(ng/ml)	39.71 ± 23.93	39.35 ± 23.73	0.930
After intervention	human interleukin 4 IL-4(ng/ml)	0.78 ± 0.09	0.76 ± 0.09	0.292
	human interleukin 10 IL-10(pg/ml)	24.77 ± 1.11	24.82 ± 1.06	0.794
	interferon IFN-γ (pg/ml)	123.09 ± 17.15	147.10 ± 17.49	<0.001***
	immunoglobulin A IgA (g/L)	2.10 ± 0.52	2.23 ± 0.61	0.204
	immunoglobulin G IgG (g/L)	11.97 ± 1.73	12.10 ± 2.00	0.695
	immunoglobulin M IgM (g/L)	1.12 ± 0.43	1.14 ± 0.44	0.834
	sIgA(ng/ml)	40.09 ± 26.60	52.93 ± 29.90	0.010*
Difference before and after intervention(after intervention-baseline)	human interleukin 4 IL-4(ng/ml)	0.01 ± 0.13	−0.03 ± 0.12	0.061
	human interleukin 10 IL-10(pg/ml)	0.25 ± 1.71	0.29 ± 1.52	0.886
	interferon IFN-γ (pg/ml)	1.11 ± 22.67	23.84 ± 23.51	<0.001***
	immunoglobulin A IgA (g/L)	−0.01 ± 0.31	0.08 ± 0.49	0.212
	immunoglobulin G IgG (g/L)	−0.10 ± 1.40	0.26 ± 1.57	0.164
	immunoglobulin M IgM (g/L)	0.02 ± 0.29	0.07 ± 0.30	0.372
	sIgA(ng/ml)	0.38 ± 15.32	13.59 ± 20.99	<0.001**

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; no mark = $p \geq 0.05$.

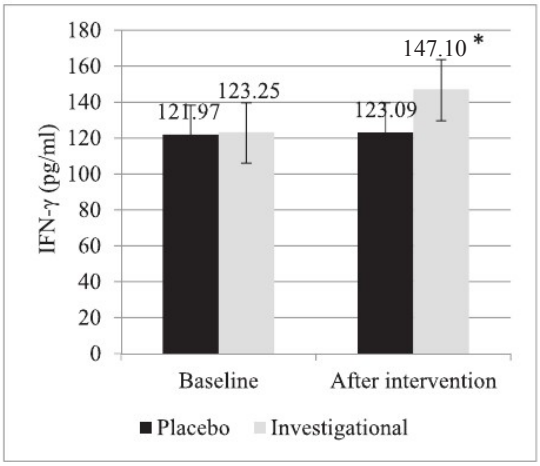

Fig. 6. Serum IFN-γ level at baseline and after probiotic intervention (* indicates statistical significance).

were used for the analysis.

Participants in the probiotics group showed significantly higher levels of fecal sIgA than the placebo group at the end of probiotics intervention (Table 3, Fig. 7) ($p < 0.001$) but without significant differences at baseline ($p < 0.930$). Also, participants in the probiotics group showed significantly increased fecal sIgA levels after probiotics intervention compared to baseline (Table 2) ($p < 0.001$).

The test results of serum IL-4, IL-10, IgA, IgG and IgM did not show any statistically significant difference between the two groups, baseline and after the probiotics intervention (Table 2).

Only one participant in the probiotics group experienced the adverse event of diarrhea that was attributed to the probiotics used in the study and the participant was withdrawn from the trial by the investigators (Supplemental Table 5). One participant in the placebo group experienced increased defecation and bowel sounds leading to withdrawal by the investigators. The symptoms of URI due to the common cold or flu are listed as adverse events but are also the primary outcome measures by design. Those incidences of the common cold and flu are not related to the study products.

The complete blood count showed no statistical differences between the two study groups (data not shown).

4. Discussion

The findings of our study indicate that the combination of probiotics (*Lactobacillus paracasei*, *Lactobacillus casei* 431® and *Lactobacillus fermentum* PCC®) could reduce the incidence of the upper respiratory infection, which is possible by increasing the level of IFN-γ in the blood and sIgA in the gut. The safety of probiotics (*Lactobacilli and Bifidobacterium*) that have been used in food supplements have been demonstrated by numerous clinical studies [36,37]. The Th1 response is characterized by the production of IFN-γ, which activates the bactericidal activities of macrophages, induces B cells to make opsonizing and complement-fixing antibodies, and leads to cell-mediated immunity. In contrast, the probiotics combination did not show any statistically significant effect on changing the level of IL-4 and IL-10 indicating that Th2 helper cells were not activated during the probiotics intervention. (Th2 cells produce IL-4, which facilitates B cell isotype switching.) Nor did the probiotics combination have any impact on the level of IgA, IgG and IgM, which must be explained as the combination of probiotics in this study have little or no activation of Th2 cells.

Numerous studies have illustrated the effects of the intestinal microflora on the functioning immune response, therefore, it seems reasonable that changing the microflora with probiotics could potentially modulate the immune response and, in fact, improve the immune status of individuals. Live probiotic cultures can induce mucin expression, phagocytosis and modulate cytokine profiles. The induction effects can also be seen when using specific parts of the probiotic cells, such as, peptidoglycan, LPS or DNA, without the whole live bacteria. Yet, the immune stimulation and cytokine expression is strain specific, may vary with Gram positive and Gram negative bacteria, and also may vary with mixtures of the probiotic bacteria.

Further randomized, controlled studies, including a larger number of subjects and a healthy group of participants, should be performed to understand the immunomodulatory effects of selected probiotics and its consequences in terms of disease prevention. Though there is good evidence that probiotics stimulate

Table 3
Group differences in serum immune biomarkers and fecal sIgA at baseline and group differences after intervention (paired *t*-test).

Blood index	Placebo		Investigational	
	Mean difference before and after intervention(after intervention-baseline)	Difference before and after(p value)	Mean difference before and after intervention(after intervention-baseline)	Difference before and after(p value)
human interleukin 4(IL-4) (ng/ml)	0.01	0.640	−0.03	.068
human interleukin 10(IL-10) (pg/ml)	−0.26	0.223	−0.29	.128
interferon IFN-γ (pg/ml)	1.11	0.689	23.84	<.001***
immunoglobulin A IgA (g/L)	−0.02	0.678	0.08	.213
immunoglobulin G IgG (g/L)	−0.11	0.533	0.25	.189
immunoglobulin M IgM (g/L)	0.02	0.520	0.07	.070
sIgA(ng/ml)	0.38	0.841	13.59	<0.001***

***: $p < 0.001$; no mark = $p \geq 0.05$.

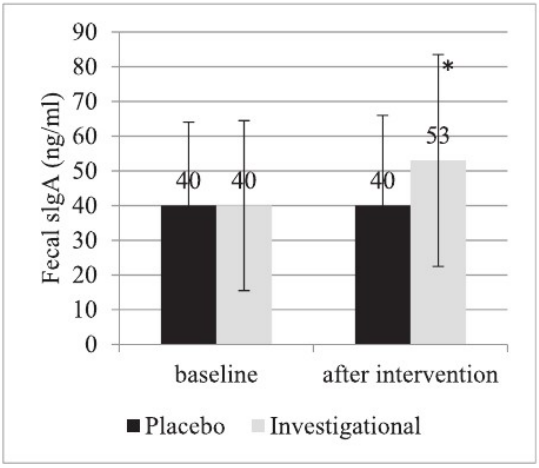

Fig. 7. Fecal sIgA level at baseline and after probiotic intervention (* indicates statistical significance).

the immune system, additional in vivo studies are needed to confirm that probiotic-mediated immune stimulation can promote prolonged resistance to various infections and diseases in humans.

5. Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent: "Informed consent was obtained from all individual participants included in the study. No authors had any conflict of interest while participating in this study. No animals were used by any authors while participating in this study.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2018.03.001.

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