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Bacillus coagulans GBI-30, 6068 decreases upper respiratory and gastrointestinal tract symptoms in healthy Mexican scholar-aged children by modulating immune-related proteins

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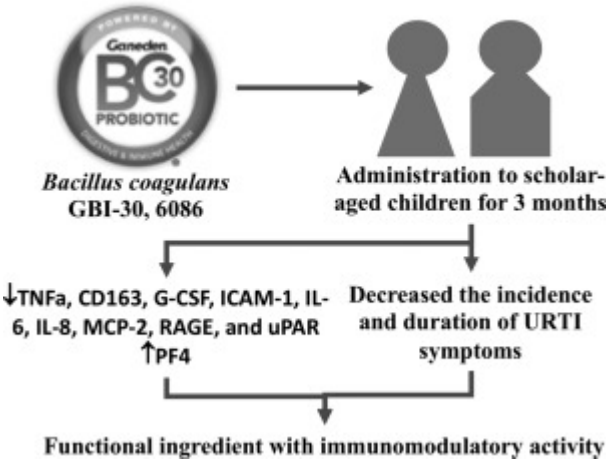
Highlights

- Ganeden**BC**^{30} probiotic ameliorates URTI in healthy scholar-aged children.
- TNFa, CD163, G-CSF, ICAM-1, IL-6, IL-8, MCP-2, RAGE, uPAR, and PF4 were modulated.
- Ganeden**BC**^{30} may be used as functional ingredient with immunomodulatory activity.

Abstract

This randomized, double-blind, parallel and placebo-controlled study aimed to evaluate the effect of *Bacillus coagulans* GBI-30, 6086® probiotic (Ganeden**BC**^{30}®) against upper respiratory tract infections (URTI) and gastrointestinal tract infections (GITI) in eighty healthy school-aged children (6–8 years old). The participants received daily a sachet containing either Ganeden**BC**^{30} (1×10^9 colony-forming units) or placebo (maltodextrin) for three months. Ganeden**BC**^{30} significantly decreased the incidence of URTI symptoms including nasal congestion, bloody nasal mucus, itchy nose, and hoarseness. The duration of the URTI-associated symptoms of hoarseness, headache, red eyes, and fatigue was also decreased. Ganeden**BC**^{30} supplementation also significantly reduced the incidence rate of flatulence. These beneficial effects were associated with the modulation of serum TNFα, CD163, G-CSF, ICAM-1, IL-6, IL-8, MCP-2, RAGE, uPAR, and PF4. Therefore, probiotic *B. coagulans* GBI-30, 6086 modulated immune-related proteins in healthy children, decreasing several URTI and GITI symptoms, thus, this functional ingredient may contribute to a healthier lifestyle.

Graphical abstract



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Keywords

Bacillus coagulans GBI-30; GanedenBC^{30}®; Probiotic; Upper respiratory; Gastrointestinal; Immune system

1. Introduction

Respiratory infections are the most common infections in children, with a mortality of >4 million deaths per year in the world (WHO, 2015). Moreover, acute respiratory infections represent 30–40% of medical consultations and 20–30% of hospitalizations worldwide (WHO, 2015). In Mexico, over 3.2 million cases of acute respiratory infections were reported in 2016 in school-aged children (6–8 years; SSA, 2016). Upper-respiratory tract infections (URTI) affect nose, sinuses, pharynx, and larynx, and encompass diagnoses such as common cold, sore throat, nasal obstruction, pharyngitis, laryngitis, and sinusitis (Simoes et al., 2006). The URTI are usually caused by some virus families such as the rhinovirus, parainfluenza, respiratory syncytial virus (RSV), adenovirus, human metapneumovirus, and bocavirus (Cotton, Innes, Jaspan, Madide, & Rabie, 2008).

Acute gastrointestinal infections are the second most frequent infectious disease (WHO, 2015). Infectious diarrhea is the eighth most common cause of morbidity worldwide, with 1.4 million deaths in 2015, and the second most common cause of morbidity in developing countries, with 57.2 deaths/million. In Mexico, 4,476,041 cases of gastrointestinal infections were reported in 2016, from which about 451,211 correspond to school-aged children (5–9 years old; SSA, 2016). The most common symptoms of GITI include diarrhea, constipation, intestinal inflammation, and vomiting (WHO, 2015).

The prescription of antibiotics for URTI and GITI has been associated with the development of antibiotic-resistant bacteria (Hao, Lu, Dong, Huang, & Wu, 2011). Therefore, alternative sources of immunomodulatory agents are of great interest. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). Probiotics act at three different levels: i) directly with the intestinal microflora, ii) in the intestinal mucus layer and epithelium, modulating intestinal barrier function and the mucosal immune system, and iii) by affecting organ function, such as the peripheral immune system, liver and brain (Jirillo, Jirillo, & Magrone, 2012).

The importance of probiotics on the modulation of the immune system is of increasing interest. It has been reported that the ingestion of Lactobacilli and Bifidobacterium species increases the immunoglobulin A (IgA) response, suggesting that probiotics stabilize the gut immune barrier and modulate local inflammatory responses (Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001).

Several studies have reported the beneficial effect of probiotics on URTI and GITI symptoms. For instance, *Lactobacillus casei* Zhang reduced respiratory and gastrointestinal symptoms in healthy young and elderly adults, decreased IL-1 pro-inflammatory cytokine production, increased production of IL-4 and IL-10 anti-inflammatory cytokines, and up-regulated CD4, CD8, CD44, CD27 and CXCR5 expression in whole blood (Hor et al., 2018). Regarding children, Lau et al. (2018) recently reported that *Bifidobacterium longum* BB536 decreased the incidence of URTI in preschool children, which was associated with the modulation of gut microbiota.

The ability of probiotics to eliminate some pathogens has been evaluated. For example, Johnston and colleagues found a reduction of 66% in *C. difficile*-associated diarrhea cases in patients taking a combination of probiotics and antibiotics (Johnston et al., 2012). Corr et al. (2007) also found that bacteriocin Abp118 produced by *Lactobacillus salivarius* was sufficient to protect mice from infection with *Listeria monocytogenes*.

Bifidobacterium and *Lactobacillus* have been widely recognized as probiotics (Ranadheera, Naumovski, & Ajlouni, 2018), and thus their immunomodulatory activities have been extensively evaluated. Nevertheless, there is an increasing interest in *Bacillus* species as probiotics, including *B. subtilis, B. cereus, B. coagulans,* and *B. licheniformis* (Cutting, 2011).

Bacillus coagulans GBI-30, 6086® (Ganeden**BC**^{30}®) probiotic has been reported to enhance the immunological response *in vitro* of healthy human adult cells to common viral causes of URTI (Kimmel, Keller, Farmer, & Warrino, 2010). Moreover, Kalman et al. (2009) reported that Ganeden**BC**^{30} reduced GITI symptoms in adults with post-prandial intestinal gas-related symptoms. Nevertheless, the immunomodulatory effect of this probiotic strain has not been evaluated in children. Therefore, the aim of this study was to assess the effectiveness of Ganeden**BC**^{30} probiotic, as compared with a placebo, on the incidence rate, duration, and severity of acute URTI and GITI symptoms in school-aged children, and to identify its mechanisms through the analysis of thirty-nine immune response-related proteins.

2. Materials and methods

2.1. Study products

Bacillus coagulans GBI-30, 6086 strain (Ganeden**BC**^{30}) and placebo (maltodextrin) were donated by Ganeden, Inc. (Mayfield Heights, OH, USA). The Ganeden**BC**^{30} probiotic consists of *B. coagulans* obtained by fermentation, recovered by centrifugation, and spray-dried with maltodextrin, whereas the placebo consisted of only maltodextrin (88.1% pentasaccharides and above, Maltrin M100, Grain Processing Co., Muscatine, IA, USA). Both products were packaged into 2 g sachets. The probiotic sachet contained ~1×10^9 CFU/2 g of *B. coagulans*. Both products were stable at room temperature and in the form of dispersible powder throughout the study. Both sachets were identical in taste and appearance.

2.2. Selection of subjects

Eligible participants were screened based on the following inclusion and exclusion criteria. Inclusion criteria included children aged 6–8 years in generally good health. Exclusion criteria included diagnosis with any chronic disease and acute respiratory or gastrointestinal disease. Volunteers were required to refrain from consuming pharmaceuticals such as anti-inflammatory, immunosuppressive, and laxative agents. Moreover, volunteers were asked to avoid the

consumption of probiotic and prebiotic supplements for the duration of the study and 4 weeks before the study.

Ninety-four children were recruited from a local elementary school in Querétaro, México. Fourteen children were excluded, since eight children did not attend the initial evaluation, three were not compliant with the study protocol, and three dropped out during the study. Eighty children completed the study and their data were included for statistical analysis. This study was conducted following the guidelines of the Declaration of Helsinki for experiments involving human. The informed consent of parents or legal tutors as well as the informed assent of the children were obtained. The study protocol was approved by the Bioethics Committee of the Natural Sciences School of the Autonomous University of Querétaro (identifier: 02FCN2017).

2.3. Study design

To evaluate the effect of Ganeden**BC**^{30} probiotic consumption in the immune system of healthy children, a three-month, double-blinded, parallel, randomized and placebo-controlled study design was used. Eligible subjects were randomized 1:1 according to a computer-generated blocked randomization list and assigned to the probiotic and placebo group with treatment codes. The allocation sequence and test product codes were not available to any member of the research team until the end of the study.

The intervention consisted of the daily administration of a 2 g sachet containing either Ganeden**BC**^{30} probiotic (~1×10^9 CFU/2 g sachet) or maltodextrin (placebo) for three months. All treatments were dissolved in ~50 mL of flavored water and were immediately administered to the participants by a member of the research team on work days and by parents or legal guardians on weekends. Blood samples were collected at months 0 and 3 for serum separation, which were stored at –80 °C until analysis Fig. 1.

Fig. 1. Flowchart of participant recruitment and study flow.

2.4. Questionnaires

Health condition questionnaires were completed weekly by the parents or legal guardians of the participants throughout the study. The questionnaires comprised the record of the incidence, duration, and severity of single specific symptoms of URTI (congested nose, runny nose, yellow nasal mucus, bloody nasal mucus, crystalline nasal mucus, itchy nose, itchy throat, sore throat, hoarseness or rough voice, dry cough, cough with phlegm, headache, muscle pain, red eyes,

sneezing, headache, appetite loss, and fever) and GITI (constipation, abdominal discomfort and stomach pain, flatulence, diarrhea, and vomiting). Mean duration values were included only when an incidence >2 was recorded. The severity of URTI symptoms was recorded as none, light, moderate, and severe, which were scored as 0, 1, 2, and 3, respectively, except for sneezing which was graded as no (0) or yes (1).

2.5. Hematologic analysis

Venous blood samples were obtained in fasting conditions. The analysis of Total Blood Count (TBC) was performed at the Nutrition Clinique of Autonomous University of Querétaro. TBC included hemoglobin (HGB), hematocrit (HCT), red and white blood cell count (RBC and WBC), mean corpuscular volume (MCV), mean concentration of hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), number and percentage of lymphocytes (LYM), granulocytes (GRA), neutrophils (NEU), and platelets (PLT). This analysis was performed using an automated hematology analyzer Sysmex, Model KX-21n (Sysmex Corporation, Japan). High, normal, and low blood controls D-check D plus where used to guarantee the quality of the analysis (Diagon, Inc.) Each sample was analyzed in duplicate.

2.6. Immune-related protein quantification

Immunoglobulin A (IgA), E (IgE), G (IgG), and M (IgM) serum levels were quantified with enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, Saint Louis, MO, USA) following the manufacturer's instructions. In addition, thirty-nine immune response-related biomarkers were determined with a Quantibody Array kit (QAH-IMR, Raybiotech, Norcross, GA, USA), which consists of a multiplexed sandwich ELISA-based quantitative platform. The proteins included in the array were the following: CD40, CRP, *E*-selectin, IL-1a, IL-1b, IL-2 Ra, IL-10, IL-13, IL-18, ST2, TNFα, CD14, CD163, FAS, FASL, G-CSF, ICAM-1, IL-2, IL-4, IL-6, IL-8, IL-12 p70, Lipocalin-2, MCP-1, MCP-2, MIF, MIP-1a, MIP-1b, OPN, PAI-I, PF4, Procalcitonin, RAGE, Resistin, Thrombomodulin, TREM-1, uPAR, VCAM-1, and VEGF. The antibody array included both positive and negative controls and each antibody was arrayed in quadruplicate.

The simultaneous quantification of the immune-related proteins was assessed according to the manufacturer's protocol. The protein spots were detected using Cy3 labeled-streptavidin, and the density of each spot was determined with a laser scanner (Genepix 4100 Microarray Scanner). An intra- and inter-glass slide normalization was carried out for all samples, using two positive controls for normalization. Standards were included in the antibody array for the generation of a standard curve for each protein, which was analyzed either by linear regression or log-log algorithms to meet their analytical needs.

2.7. Statistical analysis

Data were grouped by treatment (probiotic or placebo) and experimentation time (month 0 or 3). Then, extreme outliers were identified by constructing boxplots (Box & Whiskers charts), which

were confirmed by using an interquartile range (IQR) with a cutoff of >3 × IQR (interquartile range). To determine whether data should be analyzed with parametric or non-parametric tools, data distribution was assessed with Kolmogorov-Smirnov's test, and homoscedasticity was assessed with Levene's test. Parametric data were analyzed with the student *t*-test, whereas non-parametric data were analyzed by Wilcoxon test. Qualitative variables were analyzed by Chi-square test. Differences were considered as significant if $p<0.05$. These statistical analyses were carried out in JMP software (v13).

3. Results and discussion

3.1. Baseline characteristics

The anthropometric characteristics of the children included in the study at month 0 and 3 are shown in Table 1. A total of 80 children were enrolled in the study, which were randomized into the probiotic group ($n=40$) and the placebo group (n = 40). No significant differences were observed in gender distribution and age between groups. Insignificant differences were observed in baseline demographic characteristics and lifestyle habits between both groups (data not shown). All children showed a similar rate of increasing height, weight, waist circumference, and hip circumference throughout the study (Table 1), which is due to childhood growth, showing similar growth rates among treatment groups.

Table 1. Anthropometric and hematologic parameters of the children included in the study.

Parameters	**Placebo ($n=40$)**		**Probiotic (n = 40)**	
	Month 0	**Month 3**	**Month 0**	**Month 3**
	Mean ± SE	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**
Female, n	23		24	
Male, n	17		16	
Age, years	7.03 ± 1.03		7.38 ± 1.33	
Height, m	123.13 ± 1.30	124.77 ± 1.30	120.95 ± 1.38	122.28 ± 1.40***
Weight, kg	25.38 ± 0.75	26.32 ± 0.96***	24.66 ± 1.21	26.03 ± 1.32***
Waist circumference, cm	58.71 ± 1.18	57.94 ± 1.15*	58.99 ± 1.50	57.94 ± 1.51**
Hip circumference, cm	67.00 ± 1.01	68.06 ± 1.01**	66.15 ± 1.23	67.60 ± 1.31***
W/H ratio	0.48 ± 0.01	0.47 ± 0.01**	0.49 ± 0.01	0.47 ± 0.01**

Parameters	Placebo ($n=40$)				Probiotic (n=40)			
	Month 0		Month 3		Month 0		Month 3	
	Mean ± SE		Mean ± SE		Mean ± SE		Mean ± SE	
z-score BMI	0.43 ± 0.20		0.47 ± 0.20		0.29 ± 0.21		0.50 ± 0.19*	
z-score W/A	0.44 ± 0.17		0.49 ± 0.18		–0.05 ± 0.22		0.04 ± 0.22	
z-score H/A	0.44 ± 0.17		0.22 ± 0.13*		–0.05 ± 0.22		–0.58 ± 0.14	
WBC, $\times 10^3/\mu L$	5.49 ± 0.19		5.79 ± 0.23		5.70 ± 0.26		6.02 ± 0.23	
RBC, $\times 10^6/\mu L$	4.61 ± 0.04		4.74 ± 0.04*		4.64 ± 0.05		4.77 ± 0.04*	
HGB, g/dL	13.84 ± 0.12		14.27 ± 0.12*		14.04 ± 0.13		14.43 ± 0.14*	
HCT, %	36.28 ± 0.32		37.59 ± 0.30**		36.33 ± 0.33		37.65 ± 0.30**	
MCV, fL	78.81 ± 0.37		79.32 ± 0.37		78.41 ± 0.47		78.95 ± 0.48	
MCH, pg	30.08 ± 0.18		30.11 ± 0.20		30.33 ± 0.26		30.24 ± 0.23	
PLT, $\times 10^3/\mu L$	270.08 ± 7.77		278.23 ± 8.45		294.56 ± 7.84		305.64 ± 9.85	
LYM, %	42.89 ± 1.40		42.15 ± 1.74		41.99 ± 1.44		44.76 ± 1.49	
MXD, %	8.84 ± 0.47		9.34 ± 0.43		8.80 ± 0.57		8.76 ± 0.42	
NEU, %	48.27 ± 1.46		48.51 ± 1.70		49.21 ± 1.53		46.49 ± 1.53	
LYM, $\times 10^3/\mu L$	2.33 ± 0.10		2.61 ± 0.11*		2.33 ± 0.09		2.66 ± 0.12*	
MXD, $\times 10^3/\mu L$	0.48 ± 0.03		0.59 ± 0.03*		0.49 ± 0.03		0.53 ± 0.03	
NEU, $\times 10^3/\mu L$	2.68 ± 0.14		3.15 ± 0.20*		2.88 ± 0.22		2.84 ± 0.17	
MPV, fL	10.32 ± 0.16		10.73 ± 0.20		9.81 ± 0.13		10.17 ± 0.12*	

Asterisks indicate significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$) by t-Student or Wilcoxon test between month 0 and month 3 for each treatment group. W/H: weight/height; BMI: body mass index; W/A: weight/age; H/A: height/age; WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; PLT: platelets; LYM: lymphocytes; MXD: mixed population of monocytes, basophils, and eosinophils; NEU: neutrophils; MVP: mean platelet volume.

The hematologic parameters of the children included in the study at month 0 and 3 are shown in Table 1, which are associated with inflammatory diseases in response to chronic infections as well as blood disorders. The administration of placebo and probiotic for 3 months significantly

($p < 0.05$) increased red blood cells (RBC, ~2.8%), hemoglobin (HBG, 2.8–3.1%), hematocrit (HCT, ~3.6%), and lymphocytes (LYM, 12–14%) as compared to initial conditions (month 0).

The administration of the probiotic significantly ($p < 0.05$) increased the mean platelet volume (MVP, 3.7%) which was not modified with the placebo. Conversely, the placebo significantly ($p < 0.05$) increased monocyte, basophil and eosinophil counts (MXD, 22.9%) and neutrophil counts (NEU, 17.5%), which were not modulated by the probiotic.

It is important to mention that all hematologic data are within the reference values. Moreover, the variability observed at the initial and final conditions of both experimental groups are within the normal variability of these hematologic parameters; therefore, no biological significance can be concluded with these results. One possible explanation of the increased levels of these hematologic parameters is the hemoconcentration due to a lower water intake, since the study was carried out from September to November, and cold season is associated with dehydration. Accordingly, mild dehydration has been associated with a slight increase of HGB and HCT (Vivanti, Harvey, Ash, & Battistutta, 2008). Nevertheless, we did not include water intake in the questionnaires to support this hypothesis.

3.2. Upper-respiratory and gastrointestinal tract infection symptoms

The primary outcome measure of the URTI and GITI questionnaires was the incidence rate (number of events) of each symptom during the intervention period, which is shown in Table 2. Ganeden**BC**^{30}-supplemented children showed significantly ($p < 0.05$) lower incidence rates of several URTI symptoms like congested nose, bloody nasal mucus, itchy nose, and hoarseness along the whole intervention study; along with GITI symptoms like flatulencies; and other symptoms such as headache, red eyes, and fatigue. Moreover, in the 1st period (1–4 weeks) of the intervention study, Ganeden**BC**^{30}-supplemented children showed a lower incidence of runny nose, itchy throat, sneezing, and appetite loss, whereas yellow mucus was only decreased in the second month of the intervention study.

Table 2. Effect of probiotic Ganeden**BC**^{30} treatment on the incidence of upper respiratory and gastrointestinal tract infection symptoms.

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)
Upper respiratory tract symptoms								

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)
Congested nose	13(33%)	6(15%)*	5(13%)	3(8%)	9(23%)	5(13%)	4(10%)	2(5%)
Runny nose	13(33%)	8(20%)	3(8%)	0(0%)*	8(20%)	7(18%)	5(13%)	3(8%)
Yellow mucus	3(8%)	4(10%)	2(5%)	0(0%)	0(0%)	3(8%)*	1(3%)	1(3%)
Bloody mucus	3(8%)	0(0%)*	2(5%)	0(0%)	1(3%)	0(0%)	0(0%)	0(0%)
Crystalline mucus	7(18%)	5(13%)	2(5%)	4(10%)	5(13%)	2(5%)	2(5%)	0(0%)
Itchy nose	8(20%)	2(5%)*	5(13%)	1(3%)	4(10%)	2(5%)	0(0%)	1(3%)
Itchy throat	8(20%)	5(13%)	3(8%)	0(0%)*	3(8%)	3(8%)	2(5%)	2(5%)
Sore throat	9(23%)	8(20%)	5(13%)	1(3%)	3(8%)	4(10%)	2(5%)	3(8%)
Hoarseness	8(20%)	2(5%)*	2(5%)	0(0%)	4(10%)	2(5%)	3(8%)	0(0%)*
Dry cough	8(20%)	5(13%)	2(5%)	1(3%)	7(18%)	4(10%)	5(13%)	1(3%)
Cough with phlegm	8(20%)	6(15%)	5(13%)	1(3%)	3(8%)	5(13%)	1(3%)	5(13%)
Sneezing	12(30%)	6(15%)	11(28%)	2(5%)**	4(10%)	4(10%)	3(8%)	1(3%)
Gastrointestinal tract symptoms								
Constipation	7(18%)	2(5%)	4(10%)	1(3%)	5(13%)	1(3%)	1(3%)	0(0%)
Intestinal inflammation	5(13%)	2(5%)	2(5%)	1(3%)	2(5%)	1(3%)	1(3%)	2(5%)
Flatulencies	21(53%)	12(30%)*	16(40%)	8(20%)*	13(33%)	6(15%)	8(20%)	7(18%)
Diarrhea	3(8%)	3(8%)	2(5%)	1(3%)	0(0%)	1(3%)	1(3%)	1(3%)
Vomit	1(3%)	2(5%)	1(3%)	0(0%)	0(0%)	0(0%)	1(3%)	1(3%)
Other symptoms								
Headache	13(33%)	5(13%)*	7(18%)	2(5%)	3(8%)	2(5%)	4(10%)	1(3%)
Muscle pain	5(13%)	3(8%)	5(13%)	3(8%)	2(5%)	2(5%)	1(3%)	0(0%)

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)	Placebo *n*[a](%)	Probiotic *n*[a](%)
Red eyes	9(23%)	2(5%)*	8(20%)	2(5%)*	3(8%)	0(0%)*	2(5%)	0(0%)
Fatigue	11(28%)	3(8%)*	8(20%)	2(5%)*	6(15%)	2(5%)	4(10%)	0(0%)*
Appetite loss	9(23%)	3(8%)	6(15%)	1(3%)*	4(10%)	2(5%)	1(3%)	0(0%)
Fever	5(13%)	2(5%)	4(10%)	1(3%)	1(3%)	1(3%)	0(0%)	0(0%)

Asterisks indicate significant differences (*$p<0.05$, **$p<0.01$) by the Chi-square test between placebo and probiotic groups for each intervention period.

a Number of children that reported each symptom.

The secondary outcome measure of the URTI and GITI questionnaires was the duration of each symptom during the intervention study, which is shown in Table 3. Regarding URTI symptoms, the supplementation with Ganeden**BC**^{30} only decreased significantly ($p<0.05$) the duration of hoarseness during the whole intervention study as compared to the placebo group. Moreover, the duration of other symptoms like headache, red eyes, and fatigue was significantly ($p<0.05$) decreased with Ganeden**BC**^{30} treatment during the entire intervention study. Interestingly, the duration of sneezing and headache was decreased with Ganeden**BC**^{30} treatment only during the first month of the intervention study.

Table 3. Effect of probiotic Ganeden**BC**^{30} treatment on the duration of upper respiratory and gastrointestinal tract infection symptoms.

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE
Upper respiratory tract symptoms								
Congested nose	1.79 ± 4.57	1.88 ± 6.80	0.55 ± 2.13	0.79 ± 4.30	0.95 ± 2.79	0.88 ± 2.79	0.29 ± 1.11	0.21 ± 1.1

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± S
Runny nose	1.69 ± 5.14	1.13 ± 2.82	0.36 ± 1.88	–	1.00 ± 3.02	0.88 ± 2.48	0.33 ± 1.43	0.26 ± 1.1
Yellow mucus	0.26 ± 1.17	0.19 ± 0.80	0.24 ± 1.16	–	–	0.07 ± 0.26	–	–
Bloody mucus	0.24 ± 1.12	–	0.07 ± 0.34	–	–	–	–	–
Crystalline mucus	1.36 ± 3.91	0.65 ± 0.37	0.33 ± 1.87	0.26 ± 1.03	0.76 ± 0.41	0.40 ± 0.40	0.26 ± 1.41	0.00 ± 0.0
Itchy nose	0.73 ± 1.84	1.09 ± 6.86	0.48 ± 1.58	–	0.26 ± 0.83	0.58 ± 3.51	–	–
Itchy throat	0.55 ± 1.25	0.42 ± 1.38	0.24 ± 0.10	–	0.24 ± 0.14	0.21 ± 0.14	0.07 ± 0.34	0.21 ± 1.1
Sore throat	0.79 ± 2.25	0.72 ± 1.78	0.43 ± 1.35	–	0.24 ± 0.19	0.40 ± 0.19	0.12 ± 0.14	0.23 ± 0.1
Hoarseness	0.86 ± 2.21	0.15 ± 0.64*	0.24 ± 1.10	–	0.38 ± 1.32	0.14 ± 0.64	0.24 ± 1.25	–
Dry cough	0.98 ± 2.40	0.56 ± 1.68	0.12 ± 0.63	–	0.64 ± 1.67	0.49 ± 1.67	0.21 ± 0.65	–
Cough with phlegm	1.12 ± 3.21	1.23 ± 3.41	0.57 ± 1.66	–	0.31 ± 1.23	0.39 ± 1.51	–	0.74 ± 2.5
Sneezing	1.67 ± 4.14	0.63 ± 2.51	1.17 ± 2.90	0.12 ± 0.63*	0.36 ± 1.29	0.35 ± 1.70	0.14 ± 0.52	–
Gastrointestinal tract symptoms								
Constipation	1.42 ± 0.53	1.50 ± 0.71	1.00 ± 0.00	–	1.00 ± 0.00	–	–	–
Intestinal inflammation	1.40 ± 0.89	6.00 ± 5.66	1.00 ± 0.23	–	2.00 ± 1.41	–	–	2.00 ± 1.0
Flatulencies	7.05 ± 7.09	6.75 ± 7.39	3.06 ± 2.54	3.25 ± 2.05	4.23 ± 2.55	4.83 ± 3.92	5.13 ± 2.59	3.71 ± 3.8
Diarrhea	1.00 ± 0.00	2.33 ± 1.53	0.00 ± 0.00	–	–	–	–	–
Vomit	–	1.00 ± 0.00	–	–	–	–	–	–
Other symptoms								
Headache	0.67 ± 1.30	0.14 ± 0.41**	0.38 ± 1.06	0.05 ± 0.21*	0.14 ± 0.08	0.07 ± 0.08	0.14 ± 0.05	–
Muscle pain	0.48 ± 0.18	0.19 ± 0.18	0.21 ± 0.65	0.14 ± 0.56	0.24 ± 0.14	0.05 ± 0.14	–	–

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± S
Red eyes	1.36 ± 3.67	0.23 ± 1.09*	0.69 ± 1.84	0.23 ± 1.09	0.58 ± 1.86	–	0.19 ± 0.97	–
Fatigue	0.98 ± 2.08	0.19 ± 0.93*	0.41 ± 0.89	0.14 ± 0.77	0.36 ± 1.01	0.05 ± 0.21*	0.21 ± 0.95	–
Appetite loss	1.33 ± 5.06	0.09 ± 0.37	0.83 ± 3.00	–	0.48 ± 2.21	0.05 ± 0.21	–	–
Fever	0.26 ± 0.77	0.07 ± 0.34	0.21 ± 0.72	–	–	–	–	–
Evacuations								
Total evacuations	1.47 ± 0.62	1.49 ± 0.86	1.60 ± 0.74	1.52 ± 0.89	1.40 ± 0.64	1.31 ± 0.53	1.24 ± 0.57	1.31 ± 0.4
Bristol score of 3–4	4.31 ± 3.05	5.57 ± 3.00	4.48 ± 3.16	5.44 ± 2.82	5.24 ± 3.94	7.15 ± 3.62	5.92 ± 3.80	5.69 ± 3.7
Bristol score of 5–6	4.45 ± 2.94	4.29 ± 2.10	4.61 ± 3.26	4.58 ± 2.68	5.40 ± 3.79	5.29 ± 3.45	6.42 ± 2.64	3.38 ± 2.4
Bristol score of 7	1.83 ± 0.21	1.00 ± 0.19*	1.00 ± 0.00	1.80 ± 0.84	1.00 ± 0.00	1.50 ± 0.71	1.00 ± 0.00	1.00 ± 0.0

Data is presented in days. Dashes (–) indicate absence of the symptom or an incidence of only 1 case in the period. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$) by the t-Student or Wilcoxon test between placebo and probiotic groups for each intervention period.

Regarding GITI, the supplementation with Ganeden**BC**^{30} did not ameliorated the duration of these symptoms as compared to the placebo group (Table 3). Stool consistency was reported according to Bristol stool scale as follows: well-formed stools are defined as those with Bristol score of 3 or 4, semi-formed stools are defined as those with Bristol score of 5 or 6; liquid stools are defined as those with Bristol score of 7. It is worth mentioned that the children that participated in the study did not reported hard stools (Bristol scores 1 and 2). The administration of Ganeden**BC**^{30} significantly ($p < 0.05$) decreased liquid stools quantity during the whole study as compared to the placebo group (45%), whereas as semi-formed stools were only significantly ($p < 0.05$) decreased during the third month (47%) (Table 3). However, the quantity of well-formed stools was not significantly ($p < 0.05$) increased.

The final outcome measured in the URTI questionnaire was the mean severity score of each symptom, which is shown in Table 4. No significant difference in severity scores were observed in any symptom.

Table 4. Effect of probiotic Ganeden**BC**^{30} treatment on the mean severity score of upper-respiratory tract infection symptoms.

Symptoms	Whole period (1–12 weeks)		1st period (1–4 weeks)		2nd period (5–8 weeks)		3rd period (9–12 weeks)	
	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE	Placebo Mean ± SE	Probiotic Mean ± SE
Congested nose	1.24 ± 0.37	1.43 ± 0.46	1.48 ± 0.50	1.25 ± 0.43	1.18 ± 0.35	1.33 ± 0.43	1.13 ± 0.25	2.04 ± 0.66
Runny nose	1.14 ± 0.12	1.46 ± 0.14	1.00 ± 0.00	1.71 ± 0.41	1.18 ± 0.37	1.36 ± 0.50	1.38 ± 0.75	1.36 ± 0.48
Yellow mucus	1.56 ± 0.51	1.35 ± 0.47	1.83 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.33	1.00 ± 0.00	1.40 ± 0.00
Bloody mucus	1.50 ± 0.87	0.00 ± 0.00	1.75 ± 1.06	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Crystalline mucus	1.13 ± 0.21	1.06 ± 0.11	1.71 ± 0.38	1.50 ± 0.27	1.10 ± 0.22	1.13 ± 0.18	1.00 ± 0.00	0.00 ± 0.00
Itchy nose	1.25 ± 0.47	1.05 ± 0.06	1.40 ± 0.55	1.08 ± 0.00	1.00 ± 0.00	1.07 ± 0.09	1.00 ± 0.00	1.00 ± 0.00
Itchy throat	1.29 ± 0.45	1.59 ± 0.38	1.67 ± 0.58	0.00 ± 0.00	1.11 ± 0.19	1.51 ± 0.43	1.00 ± 0.00	1.71 ± 0.41
Sore throat	1.31 ± 0.43	1.71 ± 0.53	1.63 ± 0.42	2.00 ± 0.42	1.00 ± 0.00	1.44 ± 0.51	1.00 ± 0.00	1.98 ± 0.54
Hoarseness	1.06 ± 0.16	1.17 ± 0.24	1.33 ± 0.47	0.00 ± 0.00	1.00 ± 0.00	1.17 ± 0.24	1.00 ± 0.00	0.00 ± 0.00
Dry cough	1.23 ± 0.42	1.03 ± 0.08	1.00 ± 0.00	1.00 ± 0.00	1.29 ± 0.49	1.05 ± 0.10	1.07 ± 0.15	1.00 ± 0.00
Cough with phlegm	1.58 ± 0.66	1.42 ± 0.39	1.90 ± 0.60	1.33 ± 0.47	1.00 ± 0.00	1.56 ± 0.52	1.00 ± 0.00	1.30 ± 0.28
Sneezing	1.20 ± 0.39	1.39 ± 0.46	1.22 ± 0.40	1.00 ± 0.00	1.00 ± 0.00	1.38 ± 0.48	1.00 ± 0.00	1.87 ± 0.48

Data is presented as mean severity score. No significant ($p < 0.05$) differences were observed by the t-Student or Wilcoxon test between placebo and probiotic groups for each intervention period.

Altogether, these results indicate that the probiotic Ganeden**BC**^{30} exerts a beneficial effect on the incidence, duration and severity of URTI symptoms and on the incidence of flatulence in school-aged children. Therefore, immune-related proteins in serum were quantified to gain insight into the mechanism of action of Ganeden**BC**^{30} on reduction in incidence and duration of URTI symptoms.

3.3. Immune-related protein analysis

Serum antibodies and immune-related proteins were quantified to understand the immunomodulatory effect of probiotic BC-30 on URTI and GITI symptoms. The effect of probiotic BC-30 on four serum immunoglobulins (IgA, IgE, IgG, and IgM) is shown in Table 5. The supplementation with Ganeden**BC**^{30} for three months significantly ($p<0.05$) increased serum IgE by 17.3% as compared to initial conditions (month 0), whereas this immunoglobulin was not affected in the placebo group. Nevertheless, serum IgE levels remained within normal reference values (<1600 UI/mL).

Table 5. Effect of probiotic Ganeden**BC**^{30} treatment on immune-related protein serum levels at month 0 and 3.

Immune proteins	**Placebo**		**Probiotic**	
	Month 0 Mean ± SE	**Month 3 Mean ± SE**	**Month 0 Mean ± SE**	**Month 3 Mean ± SE**
IgA[1]	15.30 ± 12.9	158.5 ± 9.7	125.29 ± 10.8	144.71 ± 9.1
IgE[1]	94.4 ± 4.2	97.1 ± 3.3	85.74 ± 2.5	100.58 ± 4.8*
IgG[1]	1189.9 ± 27.9	1155.9 ± 28.6	1251.56 ± 25.9	1212.08 ± 33.8
IgM[1]	151.8 ± 3.4	133.0 ± 7.6*	148.43 ± 3.0	138.62 ± 5.3
CD40[2]	404.7 ± 32.2	377.1 ± 25.6	376.1 ± 32.9	405.2 ± 32.7
CRP[2]	30,879.6 ± 2393.5	31,158.1 ± 2204.5	24,887.3 ± 1598.5	27,147.4 ± 1813.3
E-selectin[2]	1929.7 ± 81.6	2196.9 ± 149.0	2042.2 ± 122.9	2244.8 ± 162.5
IL-1a[2]	38.0 ± 3.2	33.4 ± 2.5	30.6 ± 2.0	31.7 ± 2.2
IL-1b[2]	46.8 ± 4.6	34.1 ± 2.9*	33.0 ± 1.8	41.0 ± 3.2*
IL-2 Ra[2]	76.3 ± 8.1	64.5 ± 5.1	86.8 ± 8.7	70.4 ± 5.5
IL-10[2]	6.7 ± 0.3	6.4 ± 0.3	6.4 ± 0.3	5.8 ± 0.2

Immune proteins	Placebo		Probiotic	
	Month 0 Mean ± SE	Month 3 Mean ± SE	Month 0 Mean ± SE	Month 3 Mean ± SE
IL-13[2]	3.3 ± 0.2	3.4 ± 0.2	3.7 ± 0.2	3.3 ± 0.3
IL-18[2]	340.9 ± 21.6	267.6 ± 19.3*	291.8 ± 25.1	350.8 ± 35.2
ST2[2]	35.0 ± 2.4	28.1 ± 2.4*	35.2 ± 3.9	26.6 ± 1.5*
TNFa[2]	592.6 ± 58.2	530.4 ± 63.7	661.2 ± 55.5	526.1 ± 45.5*
CD14[2]	19,501.3 ± 1107.1	18,885.6 ± 756.1	17,523.5 ± 847.4	17,134.5 ± 958.3
CD163[2]	20,885.7 ± 2388.5	18,992.1 ± 1914.3	16,850.0 ± 1878.8	12,133.0 ± 1138.8*
FAS[2]	37.1 ± 4.3	25.8 ± 2.3*	30.8 ± 3.3	19.7 ± 1.1**
FASL[2]	22.0 ± 3.0	13.9 ± 1.5*	16.8 ± 1.8	17.4 ± 2.2
G-CSF[2]	9.0 ± 0.8	9.2 ± 1.0	8.7 ± 1.2	6.5 ± 0.6*
ICAM-1[2]	15,819.4 ± 870.2	15,045.1 ± 618.16	14,901.0 ± 971.4	12,653.3 ± 856.8*
IL-2[2]	7.3 ± 0.3	7.4 ± 0.2	7.3 ± 0.3	6.9 ± 0.3
IL-4[2]	8.2 ± 0.8	7.7 ± 0.8	6.9 ± 0.6	8.2 ± 0.7
IL-6[2]	14.0 ± 0.6	13.6 ± 0.7	13.5 ± 0.8	11.8 ± 0.6*
IL-8[2]	7.1 ± 0.6	8.3 ± 0.4	8.2 ± 0.6	6.8 ± 0.6*
IL-12 p70[2]	3.3 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	3.2 ± 0.2
Lipocalin-2[2]	1164.2 ± 87.1	946.4 ± 48.5*	1180.6 ± 84.1	982.9 ± 50.7*
MCP-1[2]	100.5 ± 7.3	105.0 ± 6.1	95.6 ± 4.9	110.0 ± 7.8
MCP-2[2]	25.1 ± 2.0	28.3 ± 2.5	35.7 ± 22.1	22.1 ± 6.2**
MIF[2]	125.2 ± 11.9	134.6 ± 17.2	107.08 ± 10.9	136.5 ± 18.7
MIP-1a[2]	30.7 ± 2.3	26.7 ± 1.8	30.1 ± 2.0	32.2 ± 3.1
MIP-1b[2]	54.4 ± 3.4	58.7 ± 4.5	60.2 ± 5.2	64.2 ± 6.2
OPN[2]	137,403.4 ± 3245.3	14,149 ± 1730**	131,562.3 ± 24,737.1	136,120.0 ± 24,587.2
PAI-I[2]	7299.1 ± 538.6	7825.1 ± 614.6	6762.3 ± 450.6	7156.3 ± 657.9
PF4[2]	1749.8 ± 119.6	1858.1 ± 106.8	1320.0 ± 99.6	1696.4 ± 107.8*
Procalcitonin[2]	3558.1 ± 309.6	2258.7 ± 209.4***	3300.9 ± 394.7	2681.9 ± 173.6
RAGE[2]	1947.2 ± 88.9	1756.09 ± 77.2	1920.9 ± 85.3	1713.6 ± 65.3*

Immune proteins	Placebo		Probiotic	
	Month 0 Mean ± SE	Month 3 Mean ± SE	Month 0 Mean ± SE	Month 3 Mean ± SE
Resistin	6335.2 ± 1001.2	19,485.1 ± 3807.4**	15,082.2 ± 1836.1	14,659.7 ± 2092.9
Thrombomodulin[2]	477.6 ± 47.7	338.3 ± 29.5*	356.1 ± 23.3	401.7 ± 28.8
TREM-1[2]	453.7 ± 55.0	415.0 ± 37.8	464.6 ± 56.6	400.6 ± 43.7
uPAR[2]	289.6 ± 39.8	284.5 ± 30.5	504.7 ± 55.6	312.6 ± 34.5**
VCAM-1[2]	205,296.2 ± 11,859.1	241,175.4 ± 15,527	194,984.3 ± 11,375.1	179,975.4 ± 11,416.7
VEGF[2]	22.4 ± 2.0	27.8 ± 3.6	35.6 ± 4.3	27.3 ± 2.7

Data are expressed as [1]UI/mL and [2]pg/mL. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) by t-Student or Wilcoxon test between month 0 and month 3 for each treatment group.

In addition to immunoglobulins, thirty-nine human immune response-related proteins were quantified (Table 5). Three proteins (ST2, FAS, and Lipocalin-2) were significantly ($p < 0.05$) reduced with the administration of both Ganeden**BC**^{30} and placebo (19.7–24.4, 30.5–36.0, and 16.8–18.7%, respectively). Decreased serum lipocalin-2 levels in both experimental groups may be associated with the lower waist circumference observed at the end of the study, since this protein is an adipokine involved with white adipose tissue remodeling and is positively associated to body mass index (Ishii et al., 2017).

Il-1b was significantly ($p < 0.05$) increased with Ganeden**BC**^{30} supplementation (24.2%) but significantly ($p < 0.05$) decreased with placebo treatment (27.1%). Moreover, children treated with placebo for three months showed decreased serum levels of IgM (12.3%), IL-18 (21.5%), FASL (36.8%), Osteopontin (OPN, 89.7%), Procalcitonin (36.5%), and Thrombomodulin (29.2%), and significantly ($p < 0.05$) increased Resistin (107.6%) as compared to initial conditions, which were not affected by the probiotic administration.

Interestingly, the administration of the Ganeden**BC**^{30} for three months significantly ($p < 0.05$) decreased the serum levels of nine proteins: TNFα, CD163, G-CSF, ICAM-1, IL-6, IL-8, MCP-2, RAGE, and uPAR, and increased PF4 serum levels as compared to initial conditions, which were not affected with the placebo treatment.

Cluster of Differentiation 163 (CD163) is a scavenger receptor exclusively of monocytes and macrophages that acts as an acute phase-regulated receptor in the clearance and endocytosis of hemoglobin/haptoglobin complexes, protecting tissues from free hemoglobin-mediated oxidative damage. In addition, this protein acts as an innate immune sensor for bacteria and belongs to the characteristic activation response of macrophages during the inflammation process. After three months of Ganeden**BC**^{30} supplementation, children showed significantly ($p < 0.05$) decreased

serum CD163 (28%) (Table 5). Interestingly, this protein is increased in a wide spectrum of both acute and chronic inflammation disorders, and thus has been proposed as a target for pharmacological treatment of macrophage-induced inflammation (Etzerodt & Moestrup, 2013).

CD163 plays a key role in Th17 differentiation by increasing the expression of IL-6, TNFα, and IL-1b *via* TLR2, TLR4, and TLR5 signal pathway, contributing to the regulation of intestinal adaptive immunity (Kayama, Nishimura, & Takeda, 2013). TNFα was significantly ($p<0.05$) reduced after the administration of Ganeden**BC**^{30} for three months by 20.4% (Table 5). Similarly, Yang, Kelesidis, Cordova, and Khanlou (2014) reported that the administration of Ganeden**BC**^{30} for three months decreased CD163 in HIV-1 infected persons as compared to the placebo group, which was associated with the improvement of chronic gastrointestinal symptoms.

Granulocyte Colony-Stimulating Factor (G-CSF) is a hematopoietic growth factor involved in the proliferation and differentiation of hematopoietic precursors of neutrophil granulocytes; it is associated with an increased number and activity of neutrophils (Xu, Höglund, Håkansson, & Venge, 2000). In this study, the supplementation with Ganeden**BC**^{30} for three months significantly ($p<0.05$) decreased G-CSF serum levels by 25.3% (Table 5).

Accordingly, Kim, Sierra, Williams, Gulino, and Tosato (2006) reported that *Lactobacillus rhamnosus* reduced TNFα levels by paracrine G-CSF-mediated effects in macrophages by activating STAT3 which prevents JNK activation thereby suppressing TNFα production. Therefore, the reduction of CD163, G-CSF, and TNFα suggest that Ganeden**BC**^{30} may have exerted a protective effect against macrophage-induced inflammation, which may be a mechanism associated with the effect of Ganeden**BC**^{30} on the amelioration of URTI and GITI symptoms.

Intracellular Adhesion Molecule-1 (ICAM-1) or CD54 is considered a key molecule in immune-mediated inflammatory process due to its functions as a co-stimulatory signal during antigen presentation to T cells. The main function of ICAM-1 is to facilitate the trans-endothelial migration of leukocytes and the activation of T cells. ICAM-1 is associated with the activation of the mucosal innate immune system of the respiratory tract and is increased in serum during upper respiratory tract acute and chronic infections (Sato & Kiyono, 2012).

The administration of Ganeden**BC**^{30} for three months significantly ($p<0.05$) decreased serum ICAM-1 levels by 15.1% (Table 5). Although it has been reported that ICAM-1 is induced under inflammatory conditions by TNFα and IL-1b activation *via* IKK-β/NF-κB signaling (Sato & Kiyono, 2012).

Interleukin-6 (IL-6) is a cytokine secreted by macrophages, T cells, endothelial cells, and fibroblasts; it is induced by IL-1b activation and is increased in response to TNFα. IL-6 is considered a keystone cytokine in both health and disease since it exerts both pro- and anti-inflammatory activities.

Neutrophil-secreted Il-6R is considered an alarmin, an endogenous molecule that signals tissue and cell damage, which promotes IL-6 trans-signaling within the local milieu as a potential danger response to disease, which affects both innate and adaptive immune outcomes. IL-6 has a clear pro-inflammatory effect in acute innate responses, promoting the expression of adhesion molecules, such as ICAM-1.

The administration of Ganeden**BC**^{30} for three months significantly ($p<0.05$) decreased IL-6 serum levels by 12.6% (Table 5). Accordingly, previous studies have demonstrated an association between reduced IL-6 levels and protection from gastrointestinal disease and inflammation (Yan & Polk, 2011; Zanello et al., 2011).

Receptor for Advanced Glycation End Products (RAGE) is a transmembrane receptor of the immunoglobulin superfamily. The interaction between RAGE and its ligands activates NF-κB and thus the transcription of pro-inflammatory factors. RAGE is expressed in macrophages, contributing to their innate immune response (Wolowczuk et al., 2008). The administration of Ganeden**BC**^{30} for three months significantly ($p<0.05$) reduced RAGE serum levels by 10.8% (Table 5). These results are interesting, since RAGE upregulation is associated with inflammatory lesions, including acute respiratory and gastrointestinal infections. Moreover, it has been reported that RAGE participates at the initiation and perpetuation phases of the innate immune response (Liliensiek et al., 2004). Therefore, Ramasany, Yan, and Schmidth (2009) suggested that therapeutic targets associated with RAGE inhibitors must be identified. Interestingly, the effect of probiotics on RAGE has not been reported elsewhere.

Urokinase-type Plasminogen Activator Receptor (uPAR) is an important regulator of extracellular matrix (ECM) proteolysis, cell-ECM interactions, and cell signaling. This protein regulates the activity of the plasminogen system, an extracellular proteolytic cascade that is negatively regulated by plasminogen activator inhibitor-1 (PAI-1, Smith & Marshall, 2010).

The administration of the Ganeden**BC**^{30} for three months significantly ($p<0.05$) decreased uPAR serum levels by 38.1%, whereas PAI-1 was unaffected (Table 5). uPAR participates in tissue remodeling and the normal functions of the hematopoietic and immune systems. Therefore, it has been proposed that uPAR represents an interesting therapeutic target to inhibit proteolytic cascades and intracellular signaling (Mazar, Ahn, & O'Halloran, 2011). Rasouli, Ghadimi-Darsajini, Nekouian, and Iragian (2017) reported that *Lactobacillus reuteri* downregulates uPA and uPAR expression in human gastric adenocarcinoma epithelial (AGS) cells. This study indicates that other probiotic bacteria such as Ganeden**BC**^{30} may also exert effects on the uPAR system.

Finally, Platelet Factor 4 (PF4) is part of the CXC chemokine family and is mainly released from activated platelets, and to a lesser degree from T cells. This protein has been shown to exert both anti- and pro-inflammatory effects depending on the cellular target. It has been reported that PF4 affects the chemotaxis of neutrophils and monocytes, as well as their adhesion to the endothelium and migration through blood vessels. Furthermore, PF4 promotes monocyte

survival and differentiation into macrophages (Trzeciak-Ryczek, Tokarz-Deptuła, & Deptuła, 2013). In this study, the administration of Ganeden**BC**^{30} for three months significantly ($p<0.05$) increased serum PF4 levels by 22.2% (Table 5), suggesting an effect on cellular chemotaxis.

This study demonstrates for the first time that *B. coagulans* modulate specific serum proteins (TNFα, CD163, G-CSF, ICAM-1, IL-6, IL-8, MCP-2, RAGE, uPAR, and PF4) in healthy scholar-aged children, which are related to both innate and adaptive immune system, which can lead to the immunoprotection against acute respiratory and gastrointestinal tract infections. Therefore, further studies can be explored to evaluate the effect of *B.* Ganeden**BC**^{30} as an ingredient in functional foods. In this regard, it has been reported the importance of guaranteeing the survival of probiotics during the elaboration of food products and their shelf life, as well as their passage through the gastrointestinal tract. It has been reported that *Bacillus* strains, including Ganeden**BC**^{30}, survived the shelf life in cheese, pasteurized orange juice and bread, as well as simulated gastrointestinal tract conditions (Soares et al., 2019).

Similarly, Adibpour, Hosseininezhad, and Pahlevanlo (2019) reported that *B. coagulans* and *subtillis* are highly stable during storage when included in rock candies, whereas Marcial-Coba, Pjaca, Andersen, Knochel, and Nielsen (2019) demonstrated that *B. coagulans* survived during the storage of dried date paste under aerobic and anaerobic conditions, as well as simulated *in vivo* gastric conditions. Moreover, it has been reported that *B. coagulans* show a high survival in unroasted green coffee and tea after brewing conditions without altering the sensory profile (Majeed et al., 2019). Interestingly, *B. coagulans* and *subtilis* also survived during the cooking process of cooked sausages, demonstrating the application of these spore-forming probiotics in functional foods subjected to high thermal conditions (Jafari et al., 2017). Therefore, there is a wide range of applications for the development of functional foods added with *Bacillus* probiotics.

4. Conclusions

The consumption of *Bacillus coagulans* GBI-30, 6086 (Ganeden**BC**^{30}) decreased the incidence rate of nasal congestion, bloody nasal mucus, itchy nose, hoarseness, and flatulencies, as well as the duration of hoarseness, headache, red eyes and fatigue in healthy school-aged children over a three-month period. These beneficial effects on upper-respiratory and gastrointestinal tract infection symptoms were associated with modulation of the immune system, since Ganeden**BC**^{30} altered the serum levels of TNFα, CD163, G-CSF, ICAM-1, IL-6, IL-8, MCP-2, RAGE, uPAR, and PF4. Therefore, these results suggest that Ganeden**BC**^{30} may be used as a functional ingredient with positive immunomodulatory properties in children. This study contributes in the biomedical and clinical research with scientific information that supports the beneficial effects of probiotics in the prevention and treatment of immune-related acute infections. Further studies could aim the evaluation of the effect of Ganeden**BC**^{30} on other immune-related infections or metabolic disease to explore their impact in human health.

Declaration of Competing Interest

David Keller was an employee of Ganeden, Inc. during the conduct of this study.

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