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Randomized Clinical Trial for the Evaluation of Immune Modulation by Yogurt Enriched with β-Glucans from Lingzhi or Reishi Medicinal Mushroom, *Ganoderma lucidum* (Agaricomycetes), in Children from Medellín, Colombia

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ABSTRACT: Pattern recognition receptor (PRR) agonists are promising for use in modulating immune responses in clinical settings characterized by immune immaturity or deficiency. β-Glucans derived from *Ganoderma lucidum* have demonstrated immune-modulatory activity both *in vitro* and *in vivo*. To evaluate the immunomodulatory activity of orally administered β-glucans, a randomized, double-blinded, placebo-controlled clinical study was performed in asymptomatic children, aged 3 to 5 years old, from Medellín, Colombia. Primary outcomes were the circulating CD8+ T lymphocyte and natural killer cell counts; secondary outcomes were circulating lymphocyte counts (total, $CD3^+$, and $CD4^+$ T cells), serum concentrations of total immunoglobulin A and cytokines, and various hematological parameters. The treatments were administered daily for 12 weeks, and physical and laboratory evaluations were performed at days 0 and 84. Children in the group receiving a yogurt with β-glucans presented a significantly higher absolute count of peripheral blood total lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$ T cells) than that in the group receiving placebo. The interventions were safe and well tolerated; no abnormal increases in serum creatinine or hepatic aminotransferases occurred, and adherence was higher than 90% in the intervention groups. This study demonstrates that β-glucans from *G. lucidum* increase the frequency of immune system cells in the peripheral blood; these cells are critical in the defense against infectious threats in asymptomatic children 3 to 5 years old. These findings warrant longer controlled clinical trials that aim to evaluate the efficacy of β-glucans in preventing infections in healthy children and to define their potential to enhance lymphoid cell number and functions in various lymphoid immune deficiencies.

KEY WORDS: β-glucans, *Ganoderma lucidum*, immune modulation, lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, medicinal mushrooms, randomized clinical trial

ABBREVIATIONS: DCC, Data Coordinating Center; **DSMB**, data safety monitoring board; **IgA**, immunoglobulin A; **IL**, interleukin; **NK**, natural killer; **PRR**, pattern recognition receptor

I. INTRODUCTION

Throughout life, internal and external factors often alter the competence of the immune system,[1,2] which results in an increased susceptibility to infectious threats and abnormal responses to artificial antigenic challenges by vaccination.[1–3] These facts have motivated interest in promoting research on strategies to positively modulate the immune response in order to overcome the functional limitations these immune alterations may cause. In particular, there is interest in developing modulatory strategies derived from natural mechanisms for activating innate and adaptive immune responses.[4,5]

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Today, it is well established that immune system activation starts with the recognition of molecules associated with pathogens (pathogen-associated molecular patterns), danger signals (danger-associated molecular patterns), or both by means of receptors that recognize pathogens (pattern recognition receptors [PRRs]), such as the well-known Toll-like receptors.[6,7] Indeed, several investigations have demonstrated the positive role of PRR agonists in the artificial activation of innate and adaptive immune cells,[8–10] and they postulated them as potential therapeutic modulators of immune responses *in vivo* to overcome the consequences of transient and permanent immune deficiencies.[11–14]

Among the PPR agonists under evaluation for potential use in human immune dysregulations, several complex polysaccharides known as β-glucans, which are ubiquitous in both bacterial and fungal cell walls, have been implicated in the initiation of antimicrobial immune responses.[15,16] *In vitro* studies have demonstrated that β-glucans are potent immunomodulators, with effects on both innate and adaptive immunity,[17,18] acting through signals delivered by several immune receptors—dectin-1, complement receptor 3, and Toll-like receptors 2 and 6.[18,19] In particular, β-glucans derived from the lingzhi or reishi medicinal mushroom, *Ganoderma lucidum*, have been approved for human use by the US Food and Drug Administration.[20]

It is interesting to note that β-glucans are one of the molecules involved in a novel but remarkable phenomenon called "trained immunity,"[21] which supports the concept of a memory for the innate immune system[22]; it acts as cross-protection between infections, independently of T and B cells, and is due to memory properties that are dependent on epigenetic and metabolic modifications presented by natural killer (NK) cells, monocytes, and macrophages.[23] A primary infection or vaccination induces innate trained immunity, and signals that originate in PRRs mediate it, enhancing protective inflammatory responses and conferring protection against secondary infection or reinfection.[22,23]

Several studies have confirmed the safety and tolerability of various doses of soluble forms of orally administered *G. lucidum*–derived β-glucans.[24,25] Also, some *in vitro* and *in vivo* investigations have suggested these β-glucans have immunomodulatory activity. A phase 1 study evaluated the safety, tolerability, and immune modulation associated with 4 days of oral administration of β-glucans in soluble form (100, 200, or 400 mg/day); no drug-related adverse events occurred, and the immunoglobulin A (IgA) concentration in saliva significantly increased in those in the 400 mg/day arm.[25]

An exploratory, 1-branch, prospective clinical study was recently performed with a cohort of asymptomatic Colombian children.[26] It demonstrated that a nutritional supplement enriched with β-glucans from *G. lucidum*, administered daily for 6 weeks, was safe, was well tolerated, and stimulated the expansion of lymphoid T-cell and NK-cell subsets in peripheral blood.[26] These findings suggest the importance of evaluating the clinical efficacy of β-glucans purified from *G. lucidum* in both children and adults by means of well-designed clinical trials.

Because not enough clinical data are available from randomized studies aimed at assessing the safety and immunomodulatory effectiveness of purified β-glucans derived from *G. lucidum*, we designed a randomized clinical trial to evaluate clinical and immune parameters in a cohort of asymptomatic children from the city of Medellín, Colombia. β-Glucans obtained biotechnologically from *G. lucidum* were mixed in yogurt and administered daily for 12 weeks. Physical and laboratory evaluations were performed at days 0 and 84.

II. MATERIALS AND METHODS

A. Mushroom Material, Medium, and Culture Conditions

The *G. lucidum* strain was provided to the company PROGAL BT S.A.S. by the Universidad EAFIT as a part of an entrepreneurship process. A third-party laboratory identified the strain as *G. lucidum* HQ235632

in the UNITE database. The mushroom was maintained on potato dextrose agar at 4°C. The fermentation process used a medium containing 35 g/L glucose, 5 g/L yeast extract, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$; this was maintained in Erlenmeyer flasks at 30°C and 100 rpm for 10 days, then later in a 200-L reactor with the same medium and under the same conditions.

B. Obtaining β-Glucans

After 10 days, the fermentation process ended with sterilization, and the fermentation broth, including mycelia, was dried using a spray dryer. A fine powder was obtained containing > 70% β-glucans. A dairy company then added the powder to the yogurt used in this study.

C. Study Population

Asymptomatic children, both female and male, between ages 3 and 5 years were eligible to participate in this study. All participants were recruited at the childcare center Lucia Jaramillo at the foundation Atención a la Niñez located in the urban area of Medellín, Colombia. The main exclusion criteria included a negative response after reading the informed consent document; a lack of a health insurance (any plan); and taking medications, mainly glucocorticoids, when the intervention was starting.

The study was conducted in accordance with the tenets of the Declaration of Helsinki, and the Institutional Ethical Review Board of IPS Universitaria, University of Antioquia, approved the protocol. All the participants' parents signed the informed consent, which was prepared according to Colombian legislation (Resolution 008430 of 1993), before their child's participation in the study began. The study is included in the ISRCTN Registry (ISRCTN64202426; http://www.isrctn.com).

D. Study Design

This randomized, double-blind, placebo-controlled clinical trial was conducted between September and December 2015. The treatment assignment ratio was 1:1 and was fixed throughout the study. A statistician in the Data Coordinating Center (DCC) developed the allocation sequence by using randomly permuted blocks generated by a random number generator (SPSS software version 22). Once the sequence was generated, it was matched with sequential numbers between 001 and 170 and given to the manufacturer of the yogurt, who labeled the yogurt as containing β-glucans or not (placebo). The allocation sequence remained confidential (filed at the DCC) until the end of the study, and the personnel performing the study (auxiliary, laboratory, and nursery staff and physicians), and the parents and children, were blinded to the intervention (yogurt with or without β-glucans).

E. Interventions

The treatment was administered in yogurt that was manufactured by a local company (Colanta, Medellín, Colombia). The placebo was the yogurt with no added supplements, whereas the main intervention was the same yogurt enriched with β-glucans (350 mg) biotechnologically obtained from the mushroom *G. lucidum* (Ganogen; PROGAL BT S.A.S., Medellín, Colombia). The final products, namely placebo or β-glucans, were administered daily (Monday through Friday) to each participant for 12 weeks; as mentioned above, those administering the yogurts were blinded to which product was being given (see the "Study Design" section just above). No other changes were implemented in the daily diet of those children.

F. Clinical and Laboratory Follow-Up

After enrollment and daily (Monday through Friday) for 12 weeks, nursery personnel, who were blinded to the type of intervention, administered the yogurt and accompanied each child while and after they ate it. In addition, a physician—also blinded to the intervention—evaluated the participants every week and recorded the following parameters: general physical examination results, occurrence of infectious or non-infectious diseases, hospitalizations, adherence and tolerance to interventions, and any adverse reactions.

Venous peripheral blood samples were collected at days 0 and 84 (week 12, after the last yogurt intake) to test for hematological parameters (red blood cell, platelet, and leukocyte counts; hemoglobin; hematocrit; and blood smear; all tested by VID Laboratory, Medellín, Colombia), total serum IgA measured through the use of nephelometry (VID Laboratory), and frequency of lymphocyte subsets as determined by flow cytometry (VID Laboratory and the Flow Cytometry Unit of the University of Antioquia, Medellín, Colombia).

The following mouse antihuman monoclonal antibodies were used for cell staining: BD Multitest CD3/CD4/CD8 with fluorescein isothiocyanate–labeled anti-CD3 (clone SK7), allophycocyanin-labeled anti-CD4 (clone SK3), and phycoerythrin-labeled anti-CD8 (clone SK7). We identified NK cells in a separate tube using PerCP-Cy anti-CD3 (clone SK7), phycoerythrin-labeled anti-CD56 (clone B159), and fluorescein isothiocyanate–labeled anti-CD16 (clone 3G8) (all from BD Biosciences, San Diego, CA). Appropriate isotype controls were used for each antibody. Whole blood (120 μL) was incubated with the specific monoclonal antibodies at room temperature in darkness for 20 minutes. Erythrocytes then were lysed by incubating with 2 mL BD FACS Lysing Solution 1X (BD Biosciences) for 10 minutes. The cell suspension was centrifuged at 250*g* for 5 minutes, the supernatant was discarded, and the cells were washed with 2 mL cold phosphate-buffered saline and again centrifuged at 250*g* for 5 minutes. Finally, the cells were fixed with 250 mL 2% paraformaldehyde. All the stained and fixed cells were stored at 4°C until acquisition in a FACSCanto II cytometer (BD Biosciences). At least 10,000 events were acquired from the lymphocyte region for each sample. T-cell subpopulations were identified as $CD3^+/CD4^+$ or $CD3^+/CD8^+$, and NK cells were defined by evaluating the expression of CD56 and CD16 in the subpopulation of $CD3^-$ lymphocytes. Acquisition analysis was performed with CellQuest software (BD Biosciences). The absolute number of peripheral blood lymphocytes was calculated on the basis of manually determined total and differential blood cell counts.

G. Cytokine Measurement

Interleukin (IL)-12 (IL-12p70), IL-1β, IL-6, IL-10, and tumor necrosis factor-α serum concentrations were determined by flow cytometry with a BD Cytometric Bead Array human inflammatory kit (Pharmingen; Becton Dickinson Biosciences) and following the manufacturer's recommendations.

H. Study Outcomes

The primary outcome was the immunomodulatory effect of β-glucans on the numbers of circulating $CD8^+$ T lymphocytes and NK cells. The secondary outcomes were the immunomodulatory effect of β-glucans on the numbers of circulating lymphocytes, the numbers of circulating $CD3^+$ and $CD4^+$ T lymphocytes, and the serum concentrations of total IgA and cytokines. Our goals were to define the effect of β-glucans on these various hematological parameters and to determine the safety of β-glucans in yogurt administered to children for 12 weeks.

I. Data Analysis and Sample Size

In a previous 1-arm, open clinical study, we found a mean ± standard deviation increase of 184 ± 427 cells/μL after an intervention with a nutritional supplement enriched with β-glucans.[26] On the basis of that result, our primary hypothesis here was that the use of β-glucans (350 mg/day for 12 weeks) could result in an increase in CD8 T-cell count of 19% or more compared with placebo. This estimated increase was used to define the sample size:

$$N = \frac{2S^2}{(\hat{\mu}_2 - \hat{\mu}_1)^2} * f(\alpha, \beta)$$

where S^2 represents the estimated standard variance; $\hat{\mu}_i$, the estimated mean $CD4^+$ T lymphocytes of each group; and $f(\alpha, \beta)$, a function that depends on confidence and expected power. Thus, each intervention group should include 85 individuals (170 for both groups) to achieve a confidence of 95% and a power of 80%; we considered possibly losing to follow-up 10% of individuals.

J. Interim Monitoring

An independent data safety monitoring board (DSMB), including 3 members with expertise in statistics and clinical epidemiology, was responsible for the interim monitoring process. The statistician at the DCC was the only person with access to the full database and provided the required information for interim quality assurance and to the DSMB.

All participants began the intervention simultaneously. The first interim monitoring was conducted when the participants in the study had completed 6 weeks of evaluation. The DSMB and the DCC agreed to no additional interim analysis on the basis of the absence of adverse events and the exploratory profile of the trial. Stopping guidelines for futility were not considered.

K. Analysis Plan

Longitudinal data relevant to the study outcomes, with repeated measurements at 0 and 12 weeks, were recorded in data tables in Microsoft Excel 2010; they then were exported to SPSS statistical software version 22. We used the paired *t* test to calculate mean differences between basal and postintervention laboratory values; for independent groups, we used the Student *t* test to compare mean values between the placebo and β-glucan intervention groups. We only performed a per-protocol analysis because of the presence of common infectious diseases, such as the common cold, in 32 children who were excluded from analysis.

III. RESULTS

A. Patient Characteristics

Among 276 potentially eligible children, 167 were randomly assigned to receive β-glucans (n = 85) or placebo (n = 82). Exclusion criteria were the main reason 89 children were not included in the study. Of the 167 participants, 156 (93%) were randomized and completed the 12-week clinical and laboratory follow-up (Fig. 1), but only 124 (74%)—60 in the β-glucans group, and 64 in the placebo group—were considered for statistical analysis because of the presence of common infectious diseases in 32 of the children (21 in the β-glucans group and 11 in the placebo group) (Fig. 1). It is well known that common viral and bacterial infections are associated with changes in the absolute counts and relative percentages

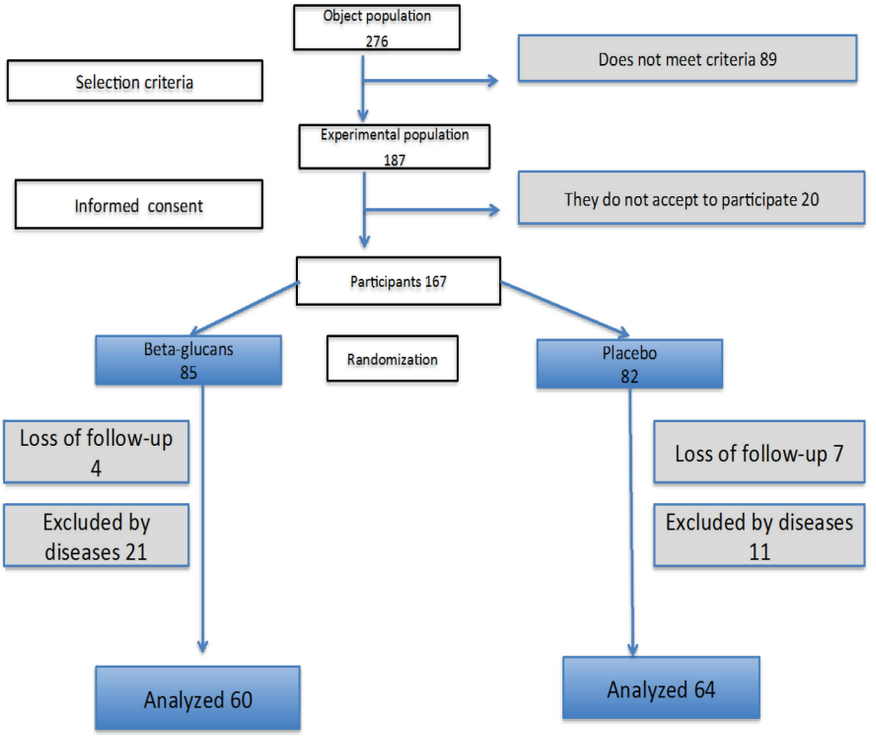

FIG. 1: Diagram showing the flow of the 276 screened participants, the main reasons for refusal to participate and withdrawal from the study, and the final size of the sample with complete clinical and laboratory data

of various circulating leukocyte subpopulations (e.g., lymphopenia or leukocytosis, high granulocyte count, and thrombocytosis), which could threaten the accuracy of modifications in primary and secondary outcomes due to the interventions.

The median ages of the children in the placebo and β-glucans groups were 3.03 years (standard deviation, 1.00 year) and 2.99 years (standard deviation, 0.97 year), respectively. Sex distribution was relatively equal between the intervention groups (60.7% and 57.8% boys in the β-glucan and placebo groups, respectively; $P = 0.89$, χ^2 test). The main baseline characteristics of children in the study groups are shown in Table 1.

B. Primary Outcomes

When we compared the parameters of the primary outcome between the 2 groups after 12 weeks of the intervention, we found that the group receiving the yogurt with β-glucans presented a significantly higher absolute number of circulating $CD8^+$ T cells (1116.3 ± 341.3 cells/μL) than did the group receiving placebo (923.2 ± 344.9 cells/μL; $P = 0.002$) (Table 2). We found no significant difference in the absolute number of NK cells in the peripheral blood between the intervention groups (263.2 ± 216.7 cells/μL in the group receiving β-glucans and 298.1 ± 229.6 cells/μL in the group receiving placebo; $P = 0.385$) (Table 2).

C. Other Outcomes

The group of children receiving yogurt with β-glucans over 12 weeks presented significantly higher values for several parameters than those in the group of children receiving placebo: absolute numbers of peripheral

TABLE 1: Baseline Characteristics by Study Group

Laboratory Parameters	Children Receiving β-Glucans (n = 61)		Children Receiving Placebo (n = 64)		*P* Value*
	Mean	SD	Mean	SD	
ALT (mg/dL)	16.1	8.3	15.1	5	0.406
AST (mg/dL)	32.5	5.8	30.8	6.1	0.099
Creatinine (mg/dL)	0.33	0.06	0.35	0.07	0.278
Serum IgA (mg/dL)	84.7	33.9	96.5	39.5	0.077
Cell counts (cells/μL)					
Total lymphocytes	4154.6	1525.1	4371.8	1534.8	0.431
$CD3^+$ cells	2793.1	1063.7	2860.7	1092.2	0.728
$CD4^+$ T cells	1576.7	655.8	1583	625.4	0.957
$CD8^+$ T cells	1022	399.5	1060.3	536.5	0.654
CD4-to-CD8 ratio	1.59	0.52	1.64	0.56	0.619
NK cells	245.8	168.8	308.5	235.2	0.092

ALT, alanine aminotransferase; AST, aspartate aminotransferase; IgA, immunoglobulin A; NK, natural killer.
*$P < 0.05$ is statistically significant.

TABLE 2: Primary and Secondary Outcomes by Study Group, Before Starting Interventions and at 12 Weeks of Follow-up

Laboratory Parameters	Children Receiving β-Glucans (n = 61)		Children Receiving Placebo (n = 64)		*P* Value*
	Mean	SD	Mean	SD	
ALT (mg/dL)	16.0	5.2	14.9	4.8	0.217
AST (mg/dL)	31.8	4.8	30.4	5.0	0.123
Creatinine (mg/dL)	0.38	0.07	0.39	0.07	0.359
Serum IgA (mg/dL)	88.8	34.8	99.6	40.8	0.113
Cell counts (cells/μL)					
Total lymphocytes	4433.6	1266.5	3876.7	1252.1	0.015
$CD3^+$ cells	3024.3	873.1	2566.1	848.8	0.004
$CD4^+$ T cells	1703.4	607.6	1461.5	554.2	0.022
$CD8^+$ T cells	1116.3	341.3	923.2	344.9	0.002
CD4-to-CD8 ratio	1.58	0.55	1.67	0.55	0.351
NK cells	263.2	216.7	298.1	229.6	0.385

ALT, alanine aminotransferase; AST, aspartate aminotransferase; IgA, immunoglobulin A; NK, natural killer.
*$P < 0.05$ is statistically significant.

blood lymphocytes (4433.6 ± 1266.5 vs. 3876.7 ± 1252.1 cells/μL, respectively; $P = 0.015$), circulating $CD3^+$ cells (3024.3 ± 873.1 vs. 2566.1 ± 848.8 cells/μL, respectively; $P = 0.004$), and circulating $CD4^+$ T cells (1703.4 ± 607.6 vs. 1461.5 ± 554.2 cells/μL, respectively; $P = 0.022$) (Table 2).

After 12 weeks of the intervention, a comparison of the 2 groups showed no significant differences in the following parameters: the CD4-to-CD8 ratio, the serum concentration of IgA (Table 2), various hematological parameters (red blood cell count and volume, hemoglobin, hematocrit, frequency of granulocyte subpopulations, absolute count of platelets), and amounts of cytokines (data not shown).

D. Other Analyses

We also established, by study group, the percentage change (basal vs. 12-week measurements) in the various parameters for the primary and secondary outcomes, and we compared these percentages between both groups (Table 3). The percentage change in several of these parameters was significantly higher in the group receiving β-glucans than in the group-receiving placebo: absolute lymphocyte count (11% ± 28.8% vs. −7.5% ± 24.1%, respectively; $P = 0.00001$); number of circulating $CD3^+$ cells (13.3% ± 29.9% vs. −6.1% ± 22.5%, respectively; $P = 0.00001$); absolute number of peripheral blood $CD4^+$ T cells (12.3% ± 30.6% vs. −5.3% ± 19.4%, respectively; $P = 0.00001$), and absolute number of $CD8^+$ T cells (15.2% ± 33.9% vs. −4.5% ± 30.4%, respectively; $P = 0.001$) (Table 3). In that comparison, no significant differences were found in the percentage change of the CD4-to-CD8 ratio; the serum concentrations of IgA and cytokines (data not shown), and other hematological parameters (such as red blood cell count and volume, hemoglobin, hematocrit, frequency of granulocyte subpopulations, and number of circulating platelets) (Table 3).

E. Safety and Secondary Effects

In general, the interventions were safe and well tolerated. None of the children suspended the intervention because of abnormal effects related to its consumption. Also, as shown in Tables 2 and 3, we found no abnormal or significant increases in the values of serum creatinine, alanine aminotransferase, or aspartate aminotransferase during follow-up. The adherence of children to the interventions was higher than 90% (data not shown).

IV. DISCUSSION

In children, the immune system is immature and has functional activity with a limited capability to control infectious threats.[2] This explains the interest in evaluating substances or drugs that positively modulate immune response in children in order to overcome these immune-related functional limitations.[11]

The discovery of the natural mechanisms required to activate innate and adaptive immune responses motivated research to develop modulatory strategies that could be useful in therapy for a broad spectrum of immune deficiencies.[11] It is well demonstrated that the effector activity of the immune system starts after molecules associated with pathogens (pathogen-associated molecular patterns) or damage signals (damage-associated molecular patterns) are recognized, and this has been fundamental in translating these findings to the field of therapeutics.[11]

Because few initial data are available on the immunomodulatory properties of purified β-glucans (a PRR agonist) in children, we designed a randomized, blinded, placebo-controlled clinical study with a cohort of asymptomatic children from Medellín, Colombia. This study explored the safety, tolerance, and modulatory activity of *G. lucidum*–derived β-glucans, administered in yogurt, in the immune system. Ganogen is a product biotechnologically obtained from mycelia of *G. lucidum*. It contains 70% 1,3-1,6-β-glucan, which has a molecular weight of ~300 kDa; according to analysis by a third-party laboratory (Complex Carbohydrate Research Center, University of Georgia), in addition to glucose it contains other monomers such as rhamnose, xylose, and mannose.

TABLE 3: Percentage Change in Primary and Secondary Outcomes between the Basal and 12-Week Measurements, by Study Group

Laboratory Parameters	Percentage Change				*P* Value*
	Children Receiving β-Glucans (n = 61)		Children Receiving Placebo (n= 64)		
	Mean	SD	Mean	SD	
ALT (mg/dL)	5.8	26.0	2.4	25.2	0.458
AST (mg/dL)	−1.3	12.8	0.4	14.8	0.49
Creatinine (mg/dL)	14.0	13.7	13.5	11.9	0.83
Serum IgA (mg/dL)	6.3	18.1	5.6	27.5	0.87
Cell counts (cells/μL)					
Total lymphocytes	11.0	28.8	−7.5	24.1	0.00001
$CD3^+$ cells	13.3	29.9	−6.1	22.5	0.00001
$CD4^+$ T cells	12.3	30.6	−5.3	19.4	0.00001
$CD8^+$ T cells	15.2	33.9	−4.5	30.4	0.001
CD4-to-CD8 ratio	−1.2	14.4	5.3	26.4	0.096
NK cells	17.3	86.4	28.6	96.6	0.494

ALT, alanine aminotransferase; AST, aspartate aminotransferase; IgA, immunoglobulin A; NK, natural killer.
*$P < 0.05$ is statistically significant.

We selected and appropriately dissolved in yogurt appropriate β-glucans that were biotechnologically obtained from *G. lucidum*. Our results demonstrate that *G. lucidum*–derived β-glucans induce significant expansion of several lymphoid subsets (total lymphocytes and $CD4^+$ and $CD8^+$ T cells) in the peripheral blood. We evaluated the immunomodulatory effect of β-glucans on adaptive humoral immunity by measuring the serum concentration of IgA, but we do not know whether the B-lymphocyte population is also expanded, as observed in other adaptive lymphocyte subsets; thus we cannot support a general expansive effect of *G. lucidum*–derived β-glucans on lymphoid-lineage cells.

β-Glucans can induce proliferation of human mononuclear cells—monocytes, macrophages, dendritic cells, and lymphocytes—in peripheral blood.[27,28] The β-glucan used in this study has a structure similar to that of the β-glucans used in other published studies.[27–30] The difference lies in the fact that the β-glucan in this study was biotechnologically obtained.

Published studies suggest that β-glucans stimulate the innate immune response no matter their source or structure. Our study showed that T lymphocytes (CD4, CD8) are modulated in both the innate and the adaptive immune responses.

This effect was mediated by different receptor-dependent signaling pathways that enhance the activity of transcription factors (e.g., nuclear factor-κB).[28–30] This finding is very interesting because the characteristic functional immaturity of the immune system in children is in part due to the low proliferative response of lymphocytes during the induction of the first adaptive immune responses.[31–33] This altered proliferative response by lymphocytes is usually associated with a higher susceptibility to infections—most of which are of viral origin and are normally controlled by innate and adaptive lymphocytes. It is estimated that functional immune immaturity and absence of immunological memory are responsible for the 10 to 15 viral infections that characteristically occur in a child each year during childhood.[32] Control of viral

infections demands the coordinated activity of several lymphocytes, in particular $CD4^+$ and $CD8^+$ T cells; thus positive modulation of the number and functional activity of lymphoid cells should be important in improving infection control in children with an immature immune system or other secondary affections of the immune system, or who are malnourished.

In this investigation we did not explore the potential molecular mechanisms underlying lymphoid cell expansion in peripheral blood; this expansion was not dependent on increased thymic output or proliferation of effector or memory T cells, nor was it generated by changes in the recirculation of lymphoid-lineage cells. However, it is possible that activation signals and cytokines released in response to stimulation by β-glucans trigger cell proliferation. Signals delivered by β-glucans can promote the proliferation of different hematopoietic cell lines.[27–30,34,35]

Of remarkable interest is that β-glucans have the capability to mediate trained immunity and to support the memory of the innate immune system[21–23]—activities that are responsible for enhanced cross-protection against infection. Through both innate trained immunity and expansion of $CD4^+$ and $CD8^+$ T lymphocytes, β-glucans may overcome immune immaturity in children and confer more effective protection against infection or reinfection. Currently available data provide evidence that β-glucans are potent immunomodulators, with effects on both innate and adaptive immunities, and their activity could be linked to clinical benefits in settings of altered immune response.[36,37]

In this investigation we did not observe a significant increase in the serum concentration of cytokines or IgA in the group of children receiving β-glucans. After most natural and artificial activations of the innate and adaptive immune systems, the production of cytokines is a local event, with only transient increases of some proinflammatory cytokines; rather, chronic inflammation is associated with detectable and sustained levels of serum cytokines. Also, most cytokines are very sensitive to tissue proteases and have a short mean life span. This evidence could help to explain the absence of an increase in amounts of cytokines in serum in response to activation mediated by β-glucans. On the other hand, production of IgA demands a T-cell–dependent, antigen-specific B-cell response, with complex signals that trigger class-switching events. In children, β-glucans may induce PRR-dependent activation of immune cells, but this is not an antigen-specific response that could be associated with a class switch in B-cells and with IgA secretion. In the absence of that specific activation during β-glucan administration, we expect that the serum concentration of IgA remains stable. Furthermore, the main site of IgA production is gut-associated lymphoid tissue, with a minor proportion of body IgA located in serum. However, a phase 1 study with oral administration of a soluble form of β-glucans for 4 days showed a significant increase in IgA production, but the IgA concentration was measured in saliva,[25] a mucosal compartment where β-glucans could activate memory B-cells to switch to the IgA isotype.

Finally, we observed that our treatment (*G. lucidum*–derived β-glucans in yogurt) was safe and well tolerated by the children we evaluated (3 to 5 years old), which confirms previous findings by studies performed with different doses of β-glucans.[24,25] We hope this encourages the development of more clinical studies to evaluate the impact of orally administered β-glucans on immune function and protection against infection, in particular in diseases with lymphopenia and immune dysregulations.

V. CONCLUSION

This randomized clinical study demonstrated that β-glucans from *G. lucidum*, administered for 12 weeks, were safe and well tolerated, and they stimulated the expansion of lymphoid-lineage cells in peripheral blood. Future studies should aim to verify the actual clinical efficacy of *G. lucidum*–derived β-glucans in various clinical scenarios.

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