Exploratory, Randomized, Double-blind, Placebo-controlled Study on the Effects of *Bifidobacterium infantis* Natren Life Start Strain Super Strain in Active Celiac Disease

Edgardo Smecuol, MD,† Hui J. Hwang, MD,* Emilia Sugai, MD,* Laura Corso, MD,‡ Alejandra C. Cherñavsky, MD,§ Franco P. Bellavite, MD,* Andrea González, MD,‡ Florencia Vodánovich, MD,§ María L. Moreno, MD,* Horacio Vázquez, MD,*† Graciela Lozano, MD,* Sonia Niveloni, MD,*† Roberto Mazure, MD,* Jon Meddings, MD,‖ Eduardo Mauriño, MD,* and Julio C. Bai, MD*†#*

Background/Aims: The aim of this exploratory trial was to establish if the probiotic *Bifidobacterium* natren life start (NLS) strain strain may affect the clinical course and pathophysiological features of patients with untreated celiac disease (CD). Positive findings would be helpful in directing future studies.

Methods: Twenty-two adult patients having 2 positives CD-specific tests were enrolled. Patients were randomized to receive 2 capsules before meals for 3 weeks of either *Bifidobacterium infantis* natren life start strain super strain (Lifestart 2) (2×10^9 colony-forming units per capsule) (n = 12) or placebo (n = 10), whereas they also consumed at least 12 g of gluten/day. A biopsy at the end of the trial confirmed CD in all cases. The primary outcome was intestinal permeability changes. Secondary endpoints were changes in symptoms and the Gastrointestinal Symptom Rating Scale, and in immunologic indicators of inflammation.

Results: The abnormal baseline intestinal permeability was not significantly affected by either treatment. In contrast to patients on placebo, those randomized to *B. infantis* experienced a significant improvement in Gastrointestinal Symptom Rating Scale ($P = 0.0035$ for indigestion; $P = 0.0483$ for constipation; $P = 0.0586$ for reflux). Final/baseline IgA tTG and IgA DGP antibody concentration ratios were lower in the *B. infantis* arm ($P = 0.055$ for IgA tTG and $P = 0.181$ for IgA DGP). Final serum macrophage inflammatory protein-1β increased significantly ($P < 0.04$) only in patients receiving *B. infantis*. The administration of *B. infantis* was safe.

Conclusions: The study suggests that *B. infantis* may alleviate symptoms in untreated CD. The probiotic produced some immunologic changes but did not modify abnormal intestinal permeability. Further studies are necessary to confirm and/or expand these observations.

Key Words: celiac disease, probiotics, *Bifidobacterium infantis* NLS super strain, gluten-free diet

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Celiac disease (CD) is an autoimmune intestinal disorder affecting approximately 1% of the general population. The disease is produced by an immune-mediated enteropathy triggered by ingested prolamins present in wheat, barley, and rye (generically called gluten) occurring in predisposal individuals carrying the characteristic HLA haplotype DQ2 and/or DQ8. The disorder is characterized by almost invariable mucosal damage as a consequence of both the innate and adaptive immunologic response to the offensive proteins in the small intestine mucosa.[1]

Until now, the only effective treatment for CD is lifelong compliance with a gluten-free diet (GFD). Adherence to treatment leads to clinical and histologic remission, normalization of biochemical parameters, significant reduction of long-term complications (eg, bone disease), and improvement in quality of life.[2,3] Consensus suggests that the GFD must be absolutely strict to avoid CD-related complications.[2,4] However, most long-term assessments suggest that absolute restriction of offensive proteins is voluntarily achieved in only a variable proportion of cases, usually $< 50\%$.[5,6] Furthermore, assessing strict adherence to dietary measures is complicated because hidden gluten is found in many so-called "gluten-free foods."[7] Considering the risk induced by the continuing consumption of gluten, and the fact that strict dietary restriction is not easy to perform, the identification of new treatment alternatives is

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From the *Small Intestinal Section, Department of Medicine; ‡Department of Alimentation, Hospital de Gastroenterología "Dr. C. Bonorino Udaondo"; §Department of Immunogenetics, Hospital de Clínicas "José de San Martín", Universidad de Buenos Aires; †Consejo de Investigación en Salud, Ministerio de Salud, Ciudad de Buenos Aires; #Department of Gastroenterology, Universidad del Salvador, Buenos Aires, Argentina; and ‖Gastrointestinal Research Group, University of Calgary, Calgary, AB, Canada.
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Reprints: Julio C. Bai, MD, Department of Medicine, Dr. C. Bonorino Udaondo Gastroenterology Hospital, Av. Caseros 2061, Buenos Aires 1264, Argentina (e-mail: jbai@intramed.net).
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justified. Several new approaches targeting different options are being explored as potential complementary or substitutive treatments of CD.[8–10]

The enteric microbiota plays a pivotal role in maintaining health status in normal individuals, modulating the function of the gut-associated lymphoid tissue (GALT).[11] Dysbiosis, the abnormal composition of the gut flora, has been associated with autoimmune inflammatory disorders of the intestines including CD.[12] In this context, reduced concentrations of *Bifidobacterium* species have been demonstrated in duodenal biopsies and feces of active and nonactive CD patients.[13] Furthermore, a very recent molecular study of the microbiota of celiac patients confirms that bifidobacteria is not present in the duodenum and exist in significantly lower levels in feces compared with healthy controls.[14]

Probiotics, live or attenuated bacteria conferring a significant health benefit to the host, are potential candidates to influence pathophysiological mechanisms involved in inflammatory disorders such as postinfective irritable bowel syndrome (IBS)[15] and CD,[16–18] among others. In the context of the CD pathogenesis, several studies have explored the role of *Bifidobacterium* species using in vitro and ex vivo models. Some studies have reported that the use of probiotic bacteria in sourdough fermentation increases the degradation of gluten during the process. Furthermore, bifidobacteria may change the gliadin-derived pattern by in vitro intestinal digestion, attenuating the proinflammatory effect on intestinal epithelial cells.[19] Secreted bioactive factors from *Bifidobacterium infantis* have been shown to normalize permeability defects of the intestinal mucosa.[20] Recently, Lindfors et al[21] have demonstrated that *Bifidobacterium lactis* reduces the toxic effects of wheat-derived gliadin on epithelial cell cultures by inhibiting the gliadin-induced increases in epithelial permeability. Recent publications have shown down regulation of the immune response caused by *Bifidobacterium* species in the proinflammatory milieu of CD.[22–24]

Despite a large volume of preclinical information with regard to the effect of probiotics on underlying mechanisms of CD damage, to our knowledge there are no clinical studies assessing these hypotheses. Thus, we designed an exploratory trial to determine the potential effect of *B. infantis* natren life start strain (NLS) super strain on intestinal permeability, the perception of symptoms, and inflammatory immunologic markers present in patients with active CD before starting treatment and while consuming a regular gluten-containing diet. Results of such exploratory study would help guide future trials with regard to effects, power calculations, and considerations about the potential use of probiotics as a reasonable replacement and/or adjuvant treatment to the well-established GFD.

PATIENTS AND METHODS

Study Population

Patients were recruited at a single center, the Small Intestinal Clinic of the Dr C. Bonorino Udaondo Gastroenterology Hospital. Patients aged between 18 and 75 years initially fulfilling serological criteria suggestive of CD were invited to participate in the screening for the trial. The CD serological protocol included tissue transglutaminase (tTG) and the deamidated gliadin-derived peptide (DGP) antibodies, both of the IgA and IgG subclass, and total IgA serum concentration. If 2 of these tests were positive a potential diagnosis of CD was considered. Additional inclusion criteria were: body mass index between 18.5 and 35.0; patients not taking medications such as non-steroidal anti-inflammatory drugs, aspirin, lactulose, probiotics, and prebiotics in any form of administration (eg, yogurts or other dairy products) or alcohol from the week prior the enrollment to the end of the trial. Patients were excluded if they were diagnosed with refractory CD or severe complications or had other active chronic gastrointestinal (GI) pathologies or comorbidities whose participation, in the investigator's judgment, would be inadvisable. We also excluded patients with symptomatic neurological or psychiatric conditions that could potentially interfere with the study, patients with a clinical severity requiring immediate treatment, subjects not willing to participate, pregnant women, and major alimentary allergies.

Trial Design

We designed a placebo-control, double-blind, randomized study. The trial consisted of a 2-week run-in period, 3 weeks of treatment, and a follow-up visit at day 50 after having initiated treatment with the GFD. A graphical explanation of the general trial design is observed in Figure 1. After written consent, a full clinical history and physical examination was obtained. Clinical laboratory studies during this time included routine blood tests, CD-specific antibodies, and a serum β human chorionic gonadotropin pregnancy test. At the end of the run-in period, patients fulfilling inclusion criteria were blindly randomized to receive one of the following treatments: (a) *B. infantis* NLS super strain, 2 capsules 3 times per day 15 minutes before meals (breakfast, lunch, and dinner) (Lifestart 2; Natren Inc., Westlake Village, CA) (2×10^9 colony-forming units per capsule) or (b) placebo 2 capsules containing rice flour, dehydrated potato powder, cellulose powder, and hydroxypropyl-methylcellulose with the same treatment scheme. Randomization was produced by an external and independent person using the blocked method. Two nutrition experts in CD provided standardized nutritional counseling and assessed patients at baseline and at each follow-up visit to assure intake of a gluten-containing diet (consumption of at least 12 g/d of gluten was required).

Trial visits performed on day 0 (baseline), day 10 and day 21 (end of the drug administration) included the following procedures: report of symptoms, vital signs, clinical safety reports, urine collection for intestinal permeability and nitrite/nitrate measurements, blood draws for tTG and DGP

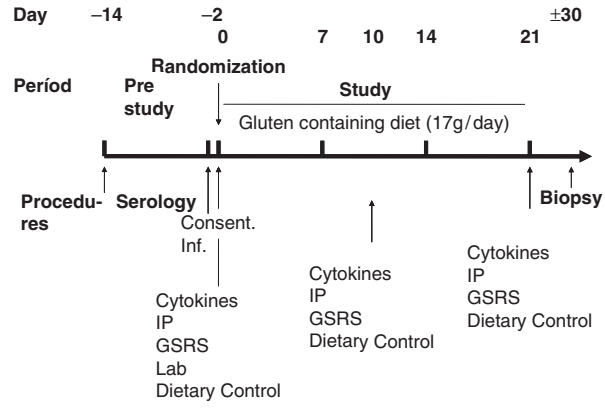

FIGURE 1. A general study design. GSRS indicates Gastrointestinal Syndrome Rate Scale; IP, inflammatory protein.

antibody serum concentrations. At baseline and follow-up visits, patients completed the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire. Blood samples obtained at each time point were tested for cytokine concentrations both in serum and cell-free supernatant from isolated peripheral blood mononuclear cell (PBMC) after 24 hours of culture. At each time point, the count of ingested capsules was recorded. Capsules were preserved refrigerated (4 to 8°C) during transportation and throughout the study period.

Endoscopically procured duodenal biopsies were obtained at the end of the treatment period in all patients. After biopsy, patients started consuming a GFD that was instructed and assessed by the expert dietitians. Final follow-up visit (day 50) included a detailed assessment of adverse events related with the study.

The primary endpoint of the study was to determine the effect of administration of *B. infantis* NLS super strain at a single-dose on intestinal permeability measured by the lactulose/mannitol fractional excretion ratio. Secondary endpoints were: (1) the assessment of the outcome of clinical symptoms measured by both the GSRS questionnaire and the perception of the outcome of the most prevalent symptoms; (2) to evaluate modifications of immunologic indicators of the gluten-driven inflammatory process, by the production of CD-related antibodies and in the secretion to plasma of a series of 17 cytokines, chemokines, and growth factors, and the production of the same immunologic mediators by nonstimulated 24-hour culture of PBMC.

Intestinal Permeability: Lactulose/Mannitol Ratio

After an overnight fast, patients ingested 5 g of lactulose (Technilab, Montreal, Quebec, Canada) and 2 g mannitol (Sigma, St Louis, MO) in 450 mL of water (osmolality approximately 1800 mOsmol/L). Patients collected all urine passed over the ensuing 5 hours into a preweighed container with 5 mL of 10% thymol in isopropanol. The fractional excretion of lactulose and mannitol were calculated from urinary concentrations determined by high pressure liquid chromatography.

Clinical Assessment and GSRS Questionnaire

The presence of GI symptoms was assessed using the GSRS questionnaire, which evaluates common symptoms of GI disorders including CD.[25] It is comprised of 15 items grouped into 5 major GI syndromes: diarrhea, indigestion, constipation, abdominal pain, and GI reflux syndromes. Rating is based on a 7-point Likert scale, from 1 (no discomfort) to 7 (very severe discomfort) where higher scores indicate more severe GI symptoms. The scores for each syndrome were calculated by taking the mean of the items completed within an individual scale.[25] In addition to the analysis of the GSRS questionnaire, we explored patients' perceptions of their most prevalent symptoms and changes produced by the treatment. At each follow-up visit, patients were asked about their perception of diarrhea, distension, gas, and abdominal pain (patients reported symptoms as improved, similar, or impaired).

CD Serology

Determination of CD-related antibodies was performed after 2 different protocols. For the diagnosis of CD, we used anti-tTG IgA and IgA and IgG antibodies against synthetic DGPs (IgA DGP; IgG DGP) detected by enzyme-linked immunosorbent assays (Inova Diagnostic Inc., San Diego, CA) as reported in previous studies.[26] Cut-off values at 20 units (U)/mL were used as recommended by the manufacturer. Because of the limitations of enzyme-linked immunosorbent assay tests for the precision dealing with higher antibody concentrations, analyses for the serologic follow-up at each time point were performed at the Inova Research laboratory using the chemiluminescent immunoassays (Inova Diagnostic Inc.). For this purpose, serum samples were kept frozen at −20°C until the assay was performed. Assays were measured on the BIO-FLASH instrument (Biokit SA, Barcelona, Spain), a fully automated chemiluminescent analyzer.

Blood Samples and Cytokine Detection by Multiplex Microbead Immunoassay

Blood samples were collected from a peripheral vein and kept on ice. Plasma was collected by centrifugation at 800*g* for 15 minutes at 4°C, aliquoted, and stored at −70°C until the analysis. A multiplex biometric immunoassay was used for cytokine measurement according to the manufacturer's instructions (Bio-Plex Human Cytokine Assay; Bio-Rad Inc., Hercules, CA). Cytokines and chemokines measured were: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12 (p70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte monocyte colony-stimulating factor (GM-CSF), monocyte chemoattractive protein (MCP-1/CCL2), macrophage inflammatory protein (MIP-1β/CCL4), and tumor necrosis factor (TNF)-α. Cytokine levels were determined both in cell-free supernatants and plasma using a multiplex array reader from Luminex Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories).

Isolation and Culture of PBMC: Cytokine Detection in Cell-free Supernatants by Multiplex Microbead Immunoassay

Blood samples were collected by venous puncture in heparin. PBMC were prepared by Ficoll-Hypaque density gradient centrifugation at 2000 rpm at room temperature for 20 minutes, washed twice in phosphate-buffered saline, counted, and resuspended in RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 50 mg/mL gentamicin to yield a final concentration of 1×10^6 cells/mL. PBMC were incubated, nonstimulated, for 24 hours at 37°C in a 5% CO_2 humidified atmosphere. Cell-free supernatants were stored frozen at −70°C and analyzed for cytokine levels as above, but without previous dilution.

Duodenal Histology

Seven biopsy samples were obtained from the descending duodenum at different levels distal to the papilla (n = 6) and the duodenal bulb (n = 1) using conventional endoscopic forceps (open cup: 8 mm) during a sedated upper endoscopy. Morphologic and quantitative assessments (intraepithelial lymphocyte density) were performed by an experienced pathologist. Morphology was categorized according to the modified Marsh classification.[27]

Statistical Analyses and Ethical Issues

The protocol was approved by the Research and Ethical Committees of the "Dr. C. Bonorino Udaondo" Gastroenterology Hospital. A written consent was given after serologic screening. Patients rejecting participation in the trial received standard care. The trial was registered at *ClinicalTrial.gov* under the number NCT01257620.

The analysis of results was performed on the intention-to-treat basis. On the basis of data distribution, descriptive data are reported either as mean and 95% of confidence interval (CI) or median and range. Some data (serology and cytokine concentrations) are also reported and analyzed as final/baseline ratios. Data were analyzed using MedCalc version 11.2.1.0. (MedCalc Software bvba, Mariakerke, Belgium). Comparisons within groups were performed using paired *t* test or Wilcoxon test for paired samples according to distribution of data. For comparisons between groups we used unpaired *t* test and Mann-Whitney test.

RESULTS

Demography and Baseline Features

From December 2010 to August 2011, a total of 54 patients had positive serology tests according to the requirements of the protocol, thereby making them candidates for enrollment in the trial. Only 22 of these patients (18 female) fulfilled the strict inclusion and exclusion criteria. Most patients were excluded because they were following some form of gluten restriction at the time of screening (n = 18) or they did not accept the proposed trial (n = 10). After randomization, 12 patients received *B. infantis* NLS super strain and 10 ingested a placebo. Table 1 depicts the demographic and clinical data of patients. Randomization was not stratified according to other variables such as sex or baseline clinical characterization. Thus, there was a sex imbalance between groups with the probiotic arm including the 4 men enrolled. No differences were found in terms of clinical characterization (symptomatic vs. subclinical CD) at diagnosis in both treatment groups. Table 2 reports baseline mean GSRS syndrome scores for patients in the probiotic and placebo arms, respectively. No statistical differences were observed between treatment groups. The most common symptoms for both groups were bloating and abdominal distension (20/22), abdominal pain (19/22), and diarrhea (11/22). Prevalence of symptoms at diagnosis was similar between groups (data not shown).

Lactulose/mannitol ratios determined at study entry were abnormal (ratio >0.025) in 19 patients. The sugar test was within the normal range in 3 other cases, 2 in the *B. infantis* NLS super strain arm and 1 in the placebo group. Mean values and dispersions determined at baseline are reported in Table 1. No statistical differences were observed between groups at baseline ($P = 0.816$).

At diagnosis, all patients were positive for serologic assays. Table 1 shows mean serum concentrations (95% CI) of antibodies at baseline. Using the reported normal physiological levels of human cytokines as reference (Bio-Rad Laboratories Inc., Tech note 6029, 2010), serum immunologic mediators concentrations were increased at baseline, characterized by high mean values of proinflammatory cytokines (TNF-α and IL-6), Th1 cytokine (INF-γ), Th-17 cytokine (IL-17), and chemokines (G-CSF, GM-CSF, MCP-1, MIP-1β), but normal levels for Th2 cytokines (IL-4, IL-5, and IL-10) (data not shown).

TABLE 1. Demography, and Clinical, Serological, and Histologic Characteristics of Patients at Baseline and According to Randomization

Characteristics	Probiotic Arm	Placebo Arm
No. patients (females)	12 (8)	10 (10)
Age, median (range) (y)	46 (29-62)	40 (20-71)
BMI, mean (95% CI) (kg/m^2)	23.2 (19.9-26.4)	22.1 (19.2-25.0)
Clinical characterization (No. patients)		
Symptomatic CD	8	8
Subclinical CD	4	2
Intestinal permeability		
Lactulose/mannitol ratio, median (range)	0.054 (0.015-0.488)	0.110 (0.012-0.503)
Serology, mean (SEM) (U/mL)		
IgA tTG	2121.5 (445.9)	1407.1 (441.2)
IgA DGP	356,8 (189.6)	296.8 (74.4)
IgG DGP	330.8 (83.7)	496.8 (123.4)
Histology		
Marsh categorization (No. patients)		
Marsh 3a	0	0
Marsh 3b	4	2
Marsh 3c	8	8
IELs density, mean (SEM)	42.3 (1.3)	44.9 (2.4)

CD indicates celiac disease; CI, confidence interval; DGP, deamidated gliadin-derived peptide; IEL, intraepithelial lymphocyte.

Effects of Treatment

Primary Endpoint: Intestinal Permeability

The mean lactulose/mannitol fractional urinary ratio at the end of the trial in the placebo group (0.253; 95% CI, 0.098-0.407) was higher than at baseline but the difference was not significant ($P = 0.342$). Similarly, the mean final lactulose/mannitol ratio in the *B. infantis* NLS super strain treated group (0.179; 95% CI, 0.0526-0.306) was non-significantly higher than values determined at baseline ($P = 0.064$). The comparison of final lactulose/mannitol mean ratios for both treatment groups shows comparable results ($P = 0.417$). Table 2 reports that baseline/final ratios were similar for both treatment groups ($P = 0.693$).

Secondary Endpoints

Clinical aspects: Table 2 and Figure 2 summarize the outcome of clinical features as they were assessed using the GSRS questionnaire. Comparing baseline with final scores within groups, patients treated with *B. infantis* NLS super strain experienced a significant reduction in indigestion ($P = 0.0035$) and constipation ($P = 0.0483$) symptoms, but were borderline for reflux symptoms ($P = 0.0586$). Both, abdominal pain and diarrhea symptom scores decreased but did not change significantly over the course of treatment. Figure 2 depicts the time course dynamics of the 5 syndromes expressed as a percentage decline with respect to baseline. In the probiotic group, a continual decline of scores was observed for all syndromes, except diarrhea. Interestingly, the mean diarrhea syndrome score decreased by visit 1 (10 d after randomization) averaging −57% of the baseline estimation; however, this score increased at the final visit to an average of −27% from baseline (Supplementary Table 1, http://links.lww.com/JCG/A60). Analysis of the individual cases showed that one of the patients without diarrhea at entry reported symptoms suggestive of an acute gastroenteritis.

Patients in the placebo group had a different outcome with respect to the GSRS syndrome scores. As shown in Table 2 and Figure 2, placebo did not produce significant changes in any syndrome except the significant improvement

TABLE 2. Mean Scores and 95% Confidence Interval for the 5 Syndromes of the Gastrointestinal Syndrome Rate Scale Questionnaire and the Composite Index (Final/Baseline Ratio) for the Probiotic and Placebo Arms

	Probiotic Arm		Placebo Arm	
Syndromes	**Baseline**	**Final**	**Baseline**	**Final**
Indigestion	4.3 (3.4-5.3)	2.9 (2.1-3.7)*	4.0 (2.7-5.3)	3.6 (2.6-4.6)
Diarrhea	3.3 (2.0-4.7)	2.7 (1.6-3.8)	2.9 (1.7-4.0)	1.6 (0.9-2.3)*
Constipation	3.6 (2.2-5.0)	2.5 (1.4-3.5)ˆ	2.7 (1.5-3.9)	2.4 (1.0-3.7)
Gastroesophageal reflux	2.6 (1.9-3.4)	1.7 (1.1-2.3)°	2.2 (1.0-3.4)	2.0 (0.7-3.4)
Abdominal pain	3.1 (2.1-4.2)	2.4 (1.5-3.4)	3.3 (2.1-4.6)	2.8 (2.1-3.5)
Composite score				
Final/baseline ratio	0.77 (0.56-0.97)		0.90 (0.62-1.18)	

*$P = 0.0035$; ˆ$P = 0.0483$; °$P = 0.0586$.

of diarrhea ($P = 0.0035$). Differences observed between treatment groups for all syndromes were not statistically significant.

At the final visit, patients were asked whether they perceived any change in diarrhea, distension, gas, or abdominal pain. At this time point, diarrhea was reported as improved in 4 of 5 and 5 of 6 patients treated with *B. infantis* NLS super strain or placebo, respectively. Interestingly, subjective improvement of abdominal distension and bloating at the final assessment was higher in

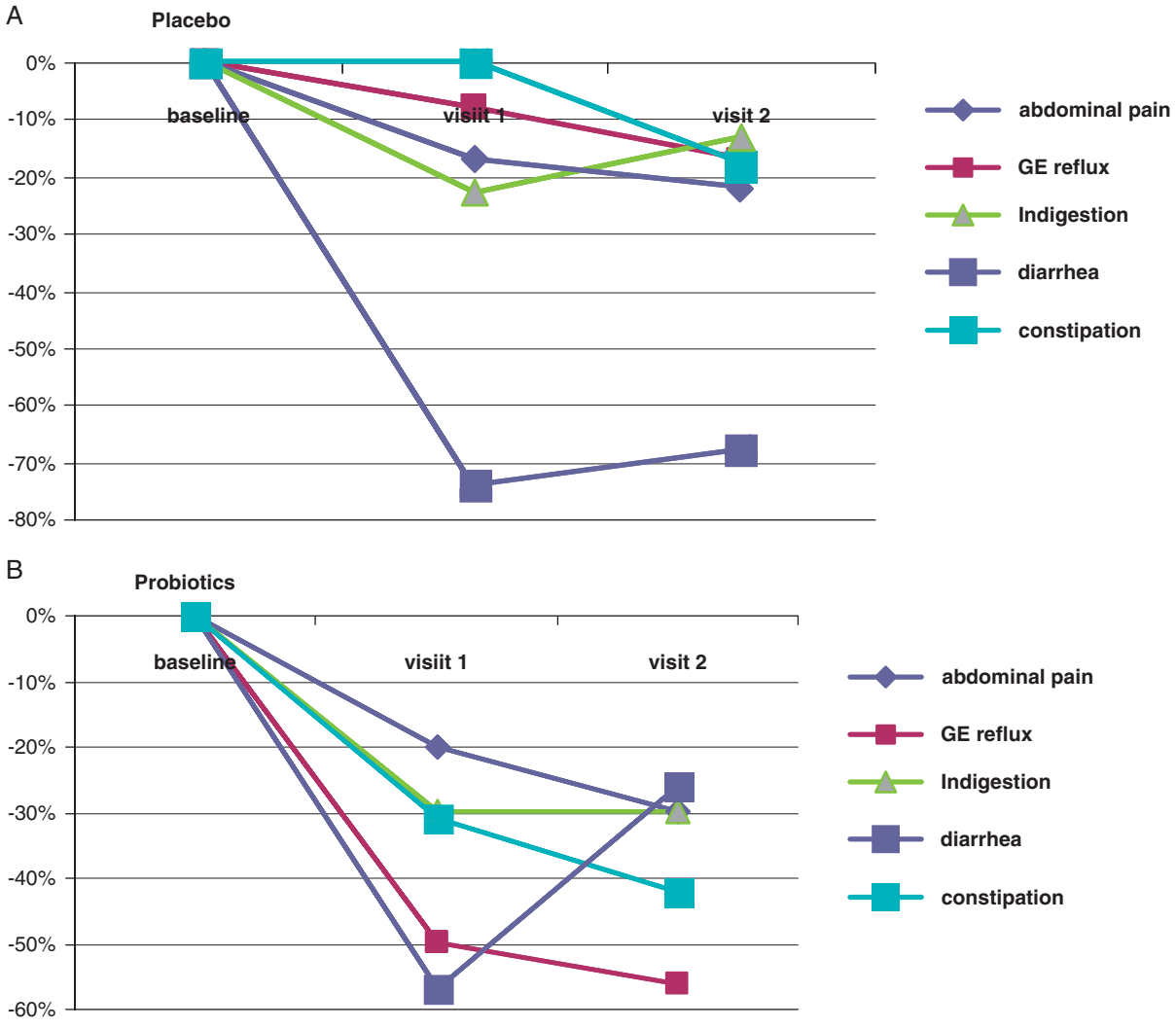

FIGURE 2. Mean percentage (%) of change for scores of the 5 Gastrointestinal Syndrome Rate Scale syndromes in the placebo (A) and probiotic (B) arms.

patients of the probiotic arm (7/10) compared with those with placebo (3/10). These differences were not significant. No serious adverse effects or significant biochemical changes were reported by patients in either treatment arm.

Immunologic markers, serum antibodies: Compared with baseline values, serum antibody concentrations were reduced at the final assessment for patients receiving probiotics (averaging 10% for both IgA tTG and IgA DGP antibodies) (data not shown). In contrast, patients in the placebo arm had 7% and 10% increased antibody serum concentrations at the end of the trial (for IgA tTG and IgA DGP, respectively). Thus, the result expressed as final/baseline ratio for IgA tTG serum concentrations had a borderline significant reduction ($P = 0.0558$) in patients receiving *B. infantis* NLS super strain compared with those on placebo. A similar trend was shown for IgA DGP but, in this case, such reduction was not statistically significant ($P = 0.1809$; Table 3).

Outcome of inflammatory mediators: Globally, the baseline Th1 biased profile of serum cytokines shown in plasma did not change significantly in the analysis within groups after both treatments (Supplementary Table 2, http://links.lww.com/JCG/A61). Similarly, no significant changes were detected in the serum concentration of the chemokines tested. However, we observed that the high baseline serum concentration of MIP-1β increased significantly in patients treated with *B. infantis* NLS super strain ($P < 0.04$), but not in the placebo group (Table 3). No significant differences were observed comparing treatment groups.

No significant changes were detected in the analysis within groups in the production of cytokines and chemokines detected in the supernatant of nonstimulated PBMC culture. The only exception was an increased production of IL-12p70 in the placebo group ($P < 0.02$) as seen by the final/baseline ratio reported in Table 3. No changes were demonstrated in patients treated with the probiotic. We also did not detect significant changes in the final/baseline ratios of proinflammatory cytokines (eg, IL-6) and in the ratio of anti-inflammatory/proinflammatory mediators (IL-10/IL-12p70) within groups (Table 3). No significant differences were observed between groups.

DISCUSSION

This study is the first clinical trial assessing the effect of a probiotic as the sole therapeutic intervention in patients with untreated CD consuming gluten during the study period. We specifically intended to explore the impact of administering the probiotic bacterium *B. infantis*, which was previously found in low concentrations in the intestine of patients with untreated and treated CD.[12–14] Our study aimed to establish the effect of the probiotic independently of the beneficial effect produced by a GFD. Thus, we administered the study drug in the period between serologic testing for CD and confirmation by the diagnostic duodenal biopsy.

Our primary endpoint was to determine changes in intestinal permeability. At baseline, 86% of patients enrolled in the trial had abnormal lactulose/mannitol ratios as evidence of mucosal damage, a proportion similar to that found in our former publication.[28] The present study shows that the impaired barrier function was not significantly modified by the probiotic. Thus, we were not able to confirm former preclinical studies with probiotics of the same family, which showed improvement in permeability defects induced by gliadin administration.[21] Potential explanations for the evident lack of effect of *B. infantis* NLS super strain on intestinal permeability could be related to several factors such as the relatively short course of treatment, the doses of probiotic used (we tested only a single-dose regimen), or that the demonstrated preclinical effects could be strictly dependent on the probiotic strain administered.

In this study, we demonstrated that consumption of *B. infantis* NLS super strain for 3 weeks improves perception of some clinical syndromes (indigestion, constipation, and gastroesophageal reflux) evaluated by the GSRS. However, this effect was not observed with other syndromes (diarrhea and abdominal pain). In contrast, patients in the placebo group did not experience changes in any symptoms except for the diarrhea syndrome, where a significantly improved perception was demonstrated at the end of the trial. However, a more detailed analysis of GSRS for the diarrhea syndrome at the middle of the trial shows that diarrhea improved similarly in both study arms. Although such

TABLE 3. Final/Baseline Ratios for Intestinal Permeability (Lactulose/Mannitol Ratio), Serology (IgA tTG and IgA DGP), and Immunologic Parameters (in Serum and in PBMC 24 h Culture Supernatant) in the Probiotic and Placebo Arms

Parameter	Probiotic Arm	Placebo Arm	*P*
Lactulose/mannitol ratio			
Final/baseline ratio, median (range)	1.11 (0.65-2.13)	0.99 (0.48-6.79)	
Immunologic markers			
Celiac disease serology (final/baseline antibody concentration ratio), median (range)			
IgA tTG	0.90 (0.26-1.19)	1.07 (0.78-2.40)	0.0558
IgA DGP	0.90 (0.57-1.71)	1.10 (0.68-2.07)	0.1809
Inflammatory mediators			
In serum (serum concentrations)			
MIP-1β (pg/mL), median (range)			
Baseline	99.3 (75.5-219.5)	104.8 (81.9-139.5)	
Final	129.9 (78.3-379.2)*	98.8 (52.4-136.5)	
In PBMC 24 h culture supernatant (final/baseline ratio), median (range)			
IL-12p70	0.9 (0.1-4.2)	3.5 (1.2-4.4)	< 0.02
IL-6	0.8 (0.1-1.4)	1.0 (0.2-7.2)	
IL-10/IL-12p70 ratio	1.0 (0.1-14.9)	0.5 (0.3-5.3)	

*$P < 0.04$.
PBMC indicates peripheral blood mononuclear cell.

improvement persisted during the second half in patients receiving placebo, increased diarrhea was perceived between days 10 and the end of the trial in patients treated with probiotics. Whether this was a consequence of a reduced number of patients enrolled in both arms (β error) or is the result of a lack of persistence of the response to the probiotic is not clear. Supporting these perceptions based on the quantitative analysis of the GSRS questionnaire, the qualitative estimation by patients of the outcome of major symptoms also found that probiotic therapy improved distension, bloating, and gas. More than 70% of patients reported that these symptoms had improved with probiotics, whereas improvement occurred in 30% of patients with placebo. Once again, diarrhea was perceived as improved at the end of the trial by 80% of patients in both treatment arms. Interestingly, these observations on distension, bloating, and gas are very similar to a previous study of administration of *B. infantis* 35624 in women with IBS (constipation predominant and diarrhea predominant).[29] Furthermore, studies using the referred *Bifidobacterium* strain (which seems equivalent to that used in the present trial) in IBS also suggested a superior effect on symptoms compared with those obtained with single bacteria or even a blend of probiotics.[15,30,31] If this beneficial effect of *B. infantis* NLS super strain in the management of the most prevalent symptoms in patients with CD is confirmed in further studies, the mechanism(s) by which they induce such improvement need(s) to be identified. A strong body of evidence has explored potentially common pathobiology of these overlapping symptoms affecting IBS and gluten-sensitive patients. It has been suggested that neuroendocrinologic and immunologic factors might activate efferent pathways, increasing acetylcholine release and producing activation of peristaltic and secretory reflexes, which may affect gut function generating symptom.[32] Studies in animal models of IBS and gluten sensitivity suggest that gluten may contain a critical antigen triggering all these abnormalities and symptoms, and that these disturbances could, at least in part, involve innate immunity. It has been hypothesized that the benefits produced by probiotics could be induced by the anti-inflammatory effect of bacteria in the inflammatory milieu.[32]

In this study we observed some changes in regard to antibody production, which might have resulted from the treatment. As expected, all patients had abnormally increased serum concentrations of the 3 antibodies at baseline. After the 3-week trial, patients in the probiotic arm experienced a decrease of serum values of anti-tTG IgA (averaging 10% reduction); contrarily, a 7% increment was observed in the placebo arm. Compared with baseline values, the IgA DGP serum concentration decreased after the probiotic (mean reduction 10%) but not with placebo (10% increase). These differences did not reach statistical significance in the comparison between within groups and between groups. However, the consistent behavior of both antibodies suggests that the effect could be associated with the administration of *B. infantis* NLS super strain. Could probiotic treatment of CD produce changes in antibody concentration only 3 weeks after initiation? In this context, although there is no data on shorter time frames, a previous study by our group has shown that effective treatment with a GFD induces a rapid serum antibody decline over a 3-month time course.[26] Further studies should explore whether these observations represent a genuine therapeutic effect of *B. infantis* NLS super strain or not.

The effect of *Bifidobacterium* strains on the immune response in the proinflammatory milieu of CD has been explored by ex vivo studies and in vivo in animal models of gluten sensitivity. On the basis these data, one of our secondary outcome measurements was to determine the impact of *B. infantis* NLS super strain on the immunologic status of patients by assessing both the serum production of cytokines and chemokines and the release of the immune factors in the supernatant of nonstimulated PMBC cultures from samples obtained at baseline, and after the administration of the probiotic and placebo and the production of CD-related antibodies. At baseline, the assessment of serum samples confirmed former observations on the behavior of immunologic mediators in CD. Thus, our study showed an increased concentration of proinflammatory cytokines (TNF-α and IL-6), Th1 cytokines (INF-γ), Th-17 cytokines (IL-17), and chemokines (G-CSF, GM-CSF, MCP-1, MIP-1β, and IL-8), but normal levels for Th2 cytokines (IL-4, IL-5) and IL-10 (Vázquez H, Nachman FD, Sugai E, unpublished personal observations).[33] At the end of the trial, the baseline proinflammatory status persisted in both the group of patients, with similar concentrations for cytokines and chemokines (data not shown). Furthermore, no significant change was observed for Th2-type mediators mean concentrations after treatment, findings that are similar to those reported after 1 year on a GFD (Vázquez H, Nachman FD, Sugai E, unpublished observations). The only significant change we detected was that the high baseline concentration of the MIP-1β was further significantly increased at the end of the trial in patients of the probiotic arm, but not in those on placebo. The potential importance of the increased chemokine serum concentration after probiotic treatment deserves further consideration. We suggest that this finding in the probiotic treatment arm might be relevant to the clinical response and comparable with the reported down-modulation of CC chemokine receptors recently described in human blood monocytes.[34] MIP-1 proteins are potent chemoattractants of monocytes, but also eosinophils, basophils, and lymphocytes, which are important components of the inflammatory process, enhancing innate immunity and T-cell responses in the GALT.[35] However, the effect of MIP-1 members in the release of cytokines by immunologic cells is different depending on the inflammatory activation. Animal studies have demonstrated a role for CCR5; the receptor for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5, as essential to the induction of oral tolerance, and that the lack of oral tolerance seen in $CCR5^{-/-}$ mice is related to CCL5 regulation of CCL2 expression.[36–38] Two series of observations seem to be of value for interpretation of our findings. Firstly, an experimental animal study showed that CCR2 and CCR5 receptors have an important role inducing symptoms (neuropathic pain associated to inflammation) in rodents and that the agonist chemokine MIP-1β was a modulator of symptoms.[39] This observation could be relevant to our observation of improvement of symptoms in patients of the probiotic arm coincident with the significant increased concentration of MIP-1β. Secondly, it has been suggested that the use of a CCR5 agonist (eg, MIP-1β) in conjunction with high-dose oral antigen might be able to establish the GALT cytokine balance during ongoing autoimmune disease toward anti-inflammatory state, and result in a diminution of autoreactivity.[35] In the context of these observations, we consider that the potential relevance of the probiotic treatment on MIP-1β/CCR5 axis deserves further investigation.

Our study has limitations. First, although the longitudinal design allowed patients to serve as their own control, increasing the statistical power, this does not reduce the importance of the low number of patients enrolled. The length of the study period, the type or species of probiotics to be explored (a single bacterium or a blend), and the doses of probiotics to be used (the present trial was based on single dose) are variables to be considered in further studies. All these observations mean that our results should be carefully considered. Finally, we recommend that, given the results of this trial, further studies should explore surrogates of innate immunity as a potential pathogenic factor involved in symptom generation and as targets for the suggested role of probiotics. If abnormal, this observation could give insight to the symptomatic treatment of both CD and other gluten-related disorders such as non-CD gluten sensitivity.

In summary, data from the present exploratory study suggest that administration of *B. infantis* NLS super strain to untreated CD patients for 3 weeks does not modify inflammatory protein abnormalities, but might improve symptoms and produce some immunologic changes. We conclude that our data suggest that future trials are necessary to confirm whether the use of *B. infantis* NLS super strain in patients with untreated CD is warranted. The design used in this study also provides insights to future clinical research trials designed to generate information independent of the effect of gluten restriction. Whether the chosen probiotic and the doses used are optimal needs additional evaluation. Furthermore, future studies should also consider using a greater number of patients. If further studies confirm the benefit of *B. infantis* NLS super strain, the addition of a probiotic to a GFD might help symptomatic recovery or, alternatively, could provide protection to the small intestinal mucosa against the involuntary consumption of traces of gluten or in voluntary transgressions. Furthermore, the results of this study suggests that use of probiotics should be explored to treat symptoms in other gluten-related disorders.

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