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ORIGINAL ARTICLE

Pilot study on non-celiac gluten sensitivity: effects of *Bifidobacterium longum* ES1 co-administered with a gluten-free diet

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ABSTRACT

BACKGROUND: *Bifidobacterium longum* ES1 is a strain probiotic, colonizing the human gut and capable of a degradative action on gliadin. In an attempt to find new nutritional solutions aimed at improving the quality of life of patients with non-celiac gluten sensitivity (NCGS) we evaluated the effectiveness of this strain, in association with a gluten-free diet, comparing its efficacy *versus* diet therapy alone.
METHODS: The experimental design included a non-randomized, open-label, 1:1 intervention study in parallel groups. Enrolled patients with symptoms attributable to NCGS, and with negative diagnoses of both wheat allergy and celiac disease, were included in this three-month trial divided into four outpatient visits (baseline, T1, T2 and T3). Fifteen patients for each group completed the experimental protocol.
RESULTS: Our results showed that a combination of diet and probiotic determined a more significant reduction in the frequency and intensity of intestinal and extra-intestinal symptoms, and a clear improvement in stool consistency.
CONCLUSIONS: Although the study was carried out on a small number of patients, the results of our pilot trial suggest that a combined strategy of naturally gluten-free diet therapy with administration of the probiotic strain ES1 appears to offer a greater advantage than the dietary regime alone in improving the clinical symptomatic picture and in stabilizing the intestinal microbiota.

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KEY WORDS: Celiac disease; Gliadin; Microbiota; Probiotics; Food hypersensitivity; Wheat hypersensitivity.

If the pathogenesis and diagnosis of celiac disease (CD) and wheat allergy (WA) are both well established, non-celiac gluten sensitivity (NCGS) is still poorly understood[1, 2] and most new cases of NCGS are currently identified *via* the exclusion of CD and WA.[3] Currently, the only proven way to effectively treat CD is by a gluten-free diet (GFD).[4] However, such a diet is not entirely suitable for NCGS treatment because gluten is not always the major or exclusive cause of gastrointestinal disorder.[5] Furthermore, a GFD can be deficient in fiber and in vitamins and minerals.[6] In many cases, a GFD is commercially inaccessible for those who need it most, while strict adherence to the diet is complicated by the presence of small amounts of the gluten components in some foods and even medicines.[7, 8] Last, but not least, persistent intraepithelial lymphocytosis is not always abolished in celiac patients adhering to a GFD and a low-grade tissue inflam-

mation can persist.[9] Therefore, there is an urgent need for new approaches to the GFD to improve both CD and NCGS. It is possible that a gut-colonizing probiotic strain that is able to reduce alterations provoked by a GFD, to enforce tight junctions, to digest gliadin and up-modulate an anti-inflammatory gut response, could have the best chance of success.[10]

Isolated from the feces of a healthy newborn baby while being breastfed, *Bifidobacterium longum* ES1 (CECT 7347) is very well known as regards to its genetic sequence and probiotic features.[11, 12] Colonizing the human gut,[13] the strain is capable of a degradative action on gliadin.[14] Moreover, it shows antagonism towards pathogens derived from the colonic fecal microbiota of patients with a diagnosis of CD where it exerts an anti-inflammatory action mediated by its effects on lymphokine release, enterocyte proteome expression and tight junctions.[15-19] Tested in an experimental model of gliadin-induced enteropathy, *B. longum* ES1 was shown to reduce inflammation and to halt enterotoxicity, as evaluated by histology tests.[20] Finally, when administered along with a GFD to children with a diagnosis of CD, in a randomized and double-blind procedure, the strain significantly improved all signs and symptoms of disease, compared to a GFD plus placebo, with a beneficial effect on the microbiota as well.[21] On this basis, we have therefore tried to highlight the role played by the ES1 strain as an add-on therapy to a GFD in individuals with a diagnosis of NCGS.

Materials and methods

Patient recruitment and inclusion criteria

Thirty-seven participants in the study were enrolled in the study between October 2017 and June 2018 through outpatient visits at the Obesity and Work Center (Occupational Health Protection and Prevention Service) and the Occupational Allergy Center of Clinical del Lavoro Luigi Devoto, in Milan, Italy. The inclusion criteria were: age over 18; a history of symptoms with a clinical picture including both intestinal manifestations such as abdominal swelling, abdominal pain, borborygmi, flatulence, diarrhea, reduced fecal consistency, constipation, urgency to defecate, feeling of incomplete evacuation, nausea, vomiting, heartburn, belching and epigastric pain, and extra-intestinal disorders such as asthenia, general malaise, headache, anxiety, depression, clouded mind, muscle pain and skin rash, which are all symptoms attributable to NCGS in response to the intake of gluten-containing foods. The exclusion criteria were: a diagnosis of CD based on a positive test for serum anti-transglutaminase (anti-TG) class IgA antibodies (EliA method; cut-off: 0.0-7.0 U/mL) and a reduced level of total serum IgA (immunoturbidimetric method or reflex test; cut-off: >15 mg/dL); a diagnosis of IgE-mediated WA, with a positive result for serological determination of specific IgE antibodies using the immune-enzymatic method (CAP System FEIA, Pharmacia & Upjohn – a Pfizer company, Strängnäs, Sweden). A threshold value of 0.35 kUA/L was used for IgE-mediated food allergy screening (a panel of allergens such as wheat flour, gluten, gliadin, rTri a 19.0101 rTri a 14 LTP) and ricombinants for grass pollen and Mux F3 ccd; patients whose serum levels for food were <0.35 kUA/L were included in our study. Subjects with positive recombinants for grass pollens with nMuxF3 ccd and/or the other food allergens >0.35 were excluded.

Design and experimental protocol

A non-randomized, open-label, 1:1 clinical trial was performed in parallel groups. The sample of subjects, which met the pre-set inclusion/exclusion criteria, was assigned to the three-month experimental protocol. This was structured according to four outpatient visits: a baseline visit (T0) plus three other visits over the following three months, with one visit per month (T1, T2 and T3), which were all organized as a one-day outpatient procedure. During enrolment, participants were divided into two groups on the basis of medical doctors' opinions concerning symptoms of gut inflammation or the presence of diarrhea: these were either managed with a GFD approach only (GFD, control group), or with the use of *B. longum* ES1 as an add-on therapy to a GFD in the case of somewhat more severe conditions (GFD+ES1, treated group). As some patients were affected by WA, and other simply dropped out during the course of the study, the

trial was concluded by 30 patients, with 15 for each group. The trial was approved by the Ethical Board of the hospital (Milan) where the study took place (study registration number: 1370).

Bifidobacterium longum ES1

The ES1 strain (deposited with the Spanish Type Culture Collection with the identification code CECT 7347) is, as far as we know, traded only in Spain and in Italy, but only in Italy is the probiotic commercialized as a one-ingredient formula in sachet (Gliadines®; Pharmextracta/Omeopiacenza group, Pontenure, Piacenza, Italy). The finished product was notified to the Italian Health Authority on May 20th, 2016, with the registration number 85464, and was declared to contain not less than 1 billion CFU/dose. The product was taken by patients in the morning after breakfast as 1 dose/day for the duration of the study.

Questionnaire for the evaluation of intestinal and extra-intestinal symptoms

During the four outpatient visits (T0, T1, T2 and T3) patients were given a specific structured questionnaire to be completed in order to monitor the symptom response resulting from the two treatments proposed. In particular, the symptomatic manifestations potentially ascribable to NCGS were evaluated, referring to the position taken by the Italian Ministry of Health on gluten sensitivity and reported in the *"Document of scientific support to the protocol for the diagnosis and follow-up of celiac disease*" of the Italian Ministry of Health.[22] In relation to this document, the spectrum of gut symptoms examined in the study included: abdominal swelling, abdominal pain, borborygmi, flatulence, diarrhea, reduced fecal consistency, constipation, urgency to defecate, feeling of incomplete evacuation, nausea, vomiting, heartburn belching and epigastric pain. The spectrum of extra-intestinal symptoms included: asthenia, general malaise, headache, anxiety, depression, clouded mind, muscle pain and rashes. The questionnaire was divided into three main parts and the symptoms recorded referred to the month preceding the date of compilation of the same. In the first part, there was a list of intestinal and extra-intestinal symptoms, and the patient had to indicate the presence or absence of each symptom. The second part depicted the numerical evaluation scales used to measure the intensity associated with each symptom by means of a score. It was a one-dimensional instrument represented by a horizontal line, along which a numerical range between 0 and 10 was indicated, corresponding respectively to "no pain" and "worst imaginable pain" for each intestinal and extra-intestinal parameter and 11 response possibilities were foreseen.[23] In the third part of the questionnaire, the Bristol Stool Scale was inserted to determine the possible presence of defecation disorders.[24]

Statistical analysis

Data were described using descriptive statistics (mean and standard deviation for continuous variables; percentages for categorical variables) and exploratory comparisons were performed by applying the non-parametric one-way ANOVA on ranks test or Fisher's Exact test, as appropriate. A P value <0.05 was considered statistically significant.

Results

Demographic and clinical features of enrolled patients

After enrolling 37 patients as possible candidates affected by NCGS, four of eight patients with grass pollens were considered to be ineligible due to WA and 3 dropped out of the study for personal and non-health-related reasons. According to the demographic and clinical features reported in Table I, the two groups can be considered to overlap and are therefore comparable.

Frequency of intestinal symptoms

In Table II, the frequency of intestinal symptoms at T0, T1, T2 and T3 in the two study groups is shown. As expected, the GFD approach progressively reduced the frequency of all symptoms, but constipation and incomplete evacuation, in both groups. Furthermore, the adjuvant therapy with the ES1 strain determined significantly better outcomes in particular for symptoms such as abdominal pain, diarrhea, fecal consistency, constipation and the feeling of incomplete evacuation.

TABLE I.—*Distinct demographic and clinical characteristics of the two different treatment groups at baseline.*

Parameter	Treatment group			
	GFD (N.=15; 3 M, 12 F)		*B. longum* ES1 + GFD (N.=15; 3 M, 12 F)	
	Mean±SD	Median	Mean±SD	Median
Age, years	43.53±18.94	42	46.87±17.06	53
Weight, kg	67.61±25.08	58	74.51±18.72	70
Height, cm	163.77±8.46	161	164.27±9.53	161
Waist circumference, cm	84.23±16.80	78	90.33±16.68	88
BMI, kg/m^2	24.7±8.26	21.91	27.56±6.22	26.57
Basal energy expenditure, kcal/day	1363.73±245.78	1267	1384.80±220.69	1322
Waist-to-hip ratio,	0.89±0.08	0.88	0.93±0.08	0.94
Body fat, %	28.64±11.01	28.90	35.25±11.19	34.80
PCR, mg/dL	0.28±0.41	0.05	0.36±0.61	0.15
Leucocytes, 10^3/mm^3	6.04±1.61	6.22	6.18±1.82	5.98
Anti-gliadin IgE, kUA/L	0.10±1.44	0.10	0.11±0.02	0.10
Anti-wheat IgE, kUA/L	0.11±0.006	0.10	0.11±0.03	0.10
Total IgA, mg/dL	226.13±208.10	159	239.53±141.82	222
Anti-TG IgA, UA/mL	1.26±1.30	0.70	1.36±0.72	1.30

GFD: gluten-free diet.

TABLE II.—*Frequency (%) and standard deviation of intestinal symptoms at T0, T1, T2 and T3 in the two study groups.*

Parameter	Treatment group							
	Gluten-free diet (GFD)				*B. longum* ES1 + GFD			
	T0	T1	T2	T3	T0	T1	T2	T3
Abdominal swelling	86.7±0.3	40.0±0.5°°	46.7±0.5°	53.3±0.5°	86.7±0.3	80.0±0.4	66.7±0.5	46.7±0.5°°
Abdominal pain	60.0±0.5	33.0±0.5°°	40.0±0.5°	33.0±0.5°°	73.3±0.5	33.3±0.5°°	40.0±0.5°	20.0±0.4°°
Borborygmi	53.3±0.5	20.0±0.4°°	53.3±0.5	46.7±0.5	73.3±0.5	53.3±0.5°	53.3±0.5°	53.3±0.5°
Flatulence	60.0±0.5	40.0±0.5°	53.3±0.5	46.7±0.5°	80.0±0.5	73.3±0.5	66.7±0.5°	46.7±0.5°°
Diarrhea	46.7±0.5	33.3±0.5°°	40.0±0.5	13.3±0.3°°	46.7±0.5	6.67±0.3^	13.3±0.3^	6.67±0.3^
Reduced fecal consistency	40.0±0.5	20.0±0.4°°	40.0±0.5	26.7±0.5°	60.0±0.5	26.7±0.5°°	33.3±0.5°	20.0±0.4^
Constipation	53.3±0.5	33.3±0.4°	46.7±0.5	53.3±0.5	53.3±0.5	13.3±0.3^	33.3±0.5°	20.0±0.4°°
Urgency of defecation	40.0±0.5	33.3±0.5	26.7±0.5°	20.0±0.4°°	53.3±0.5	26.7±0.3°°	33.3±0.5°	40.0±0.5
Incomplete evacuation	46.7±0.5	53.3±0.5	40.0±0.5	46.7±0.5	67.7±0.5	53.3±0.5	40.0±0.5°	20.0±0.4^
Nausea	46.7±0.5	26.7±0.5°	26.7±0.5°	20.0±0.4°°	20.0±0.4	0.0±0.0^	13.3±0.3°	0.0±0.0^
Vomiting	13.3±0.3	6.7±0.3	6.7±0.3	6.7±0.3	20.0±0.4	0.0±0.0^	0.0±0.0^	0.0±0.0^
Heartburn	53.3±0.5	20.0±0.4°	20.0±0.4°	13.3±0.3°°	46.7±0.5	13.3±0.3^	6.7±0.3^	13.3±0.3^
Belching	53.3±0.5	33.3±0.5°	40.0±0.5	33.3±0.5°	40.0±0.5	40.0±0.5	26.7±0.5°	20.0±0.4°°
Epigastric pain	73.3±0.5	26.7±0.5°°	26.7±0.5°°	26.7±0.5°°	46.7±0.5	20.0±0.4°°	26.7±0.5°	6.67±0.3^

°P<0.05 *vs.* T0; °°P<0.01 *vs.* T0; ^P<0.001 *vs.* T0.

Frequency of extra-intestinal symptoms

In Table III, the frequency of extra-intestinal symptoms at T0, T1, T2 and T3 in the two study groups is shown. The GFD slightly reduced the frequency of 4 out of 8 symptoms, with more robust effect on general malaise, headache and rashes. The adjuvant therapy with the ES1 strain significantly further reduced the frequency of all symptoms except for anxiety.

Evaluation of symptom intensity

A further analysis was carried out to evaluate the trend with respect to the intensity of intestinal and extra-intestinal manifestations to highlight any significant variation. The analysis was conducted both within each study group after one, two and three months from baseline, and between the two different treatments. The latter analysis, unlike the former where a highly posi-

TABLE III.—*Frequency (%) and standard deviation of extra-intestinal symptoms at T0, T1, T2 and T3 in the two study groups.*

Parameter	Treatment group							
	Gluten-free diet (GFD)				*B. longum* ES1 + GFD			
	T0	T1	T2	T3	T0	T1	T2	T3
Asthenia	53.3±0.3	60.0±0.5	53.3±0.5	53.3±0.5	73.3±0.5	60.0±0.5	46.7±0.5°°	46.0±0.5°°
General malaise	73.3±0.5	40.0±0.5°°	53.3±0.5	46.7±0.5°	46.7±0.5	33.3±0.5°°	33.3±0.5°°	20.0±0.4^
Headache	60.0±0.5	26.7±0.4°	20.0±0.5°°	33.3±0.5°	73.3±0.5	40.0±0.5°°	26.7±0.5^	33.3±0.5^
Anxiety	66.7±0.5	46.7±0.5°	46.7±0.5°	46.7±0.5°	66.7±0.5	53.3±0.5°	60.0±0.5	46.7±0.5°°
Depression	33.3±0.5	66.7±0.5	33.3±0.5	33.3±0.5	33.3±0.5	6.67±0.3^	6.67±0.3^	13.3±0.3°°
Clouded mind	20.0±0.4	20.0±0.4	20.0±0.4	20.0±0.4	46.7±0.5	20.0±0.4°°	6.70±0.5^	6.70±0.4^
Muscle pain	53.3±0.5	33.3±0.5°	46.7±0.5	53.3±0.5	73.3±0.5	40.0±0.5°°	53.3±0.5°°	53.3±0.5°°
Rashes	53.3±0.5	13.3±0.3°°	26.7±0.5°	26.7±0.5°	66.7±0.5	26.7±0.5°°	26.7±0.5°°	26.7±0.5°°

°P<0.05 *vs.* T0; °°P<0.01 *vs.* T0; ^P<0.001 *vs.* T0.

tive trend was observed within both groups, did not give clear results, except for abdominal pain, diarrhea, muscle pain and rashes at T2 and T3 where the probiotic add-on therapy reduced the intensity of symptoms by approximately 50% (data not shown).

Evaluation with the Bristol Stool Scale

Regarding the third part of the symptoms questionnaire, related to defecation disorders, a descriptive analysis of how the stools of the subjects were characterized according to the seven categories of the Bristol Stool Scale was performed, both between the two different groups and after one, two and three months from baseline for each study group. Based on how the groups were formed, in the group assigned to the GFD plus ES1 strain, a greater frequency of type 5, 6 and 7 stools were recorded at baseline, representative of a tendency to diarrhea (26.7, 20.0 and 13.3%), while after three months of treatment types 3 and 4 prevailed, indicative of a better stool consistency, with a frequency of 33.3% each. In the GFD group the exact opposite was observed, where at baseline the most frequently occurring category of stool was type 4 (33.3%), while at the final assessment the most prevalent was type 5, with a frequency of 33.3%. As for the evaluations one and two months after baseline, for the test group (GFD+ES1) it is possible to see a prevalence of type 3 and 4 stools after one month of treatment with a frequency of 26.7% for both of these, and of type 3 after two months of therapy (46.7%); for the control group (GFD),

TABLE IV.—*Values represent the percentage of subjects with stools assigned to the different categories of the Bristol Stool Scale (BSS) at T0, T1, T2 and T3 for the two study groups.*

Group	BSS	T0	T1	T2	T3
GFD	1	13.3	0	13.3	0
	2	13.3	26.7	6.7	20.0
	3	13.3	33.3°	20.0	26.7°
	4	33.3	20.0	26.7	13.3°
	5	20.0	6.7°	26.7	33.3
	6	6.7	13.3	6.7	6.7
	7	0	0	0	0
B. longum ES1 + GFD	1	20.0	6.7°	0°°	6.7°
	2	0	20.0°°	20.0°°	20.0°°
	3	13.3	26.7°	46.7^	33.3°°
	4	6.7	26.7°°	13.3°	33.3°°
	5	26.7	13.3°	13.3°	0^
	6	20.0	6.7°°	6.7°°	6.7°°
	7	13.3	0^	0^	0^

°P<0.05 *vs.* T0; °°P<0.01 *vs.* T0; ^P<0.001 *vs.* T0.

a prevalence of type 3 stools was recorded after one month (33.3%) and of types 4 and 5 after two months with a frequency of 26.7% for both (Table IV). These results show that a GFD had a minor impact on stool consistency. In contrast, add-on therapy with the ES1 strain significantly improved this parameter.

Discussion

Our study confirms the beneficial effect of a GFD on symptoms associated with NCGS and allows us to formulate an hypothesis of a therapeutic advantage derived from the combination of a GFD with *B. longum* ES1, a probiotic strain already reported to confer advantages when associated with a GFD in subjects with CD.[21] In particular, dietary therapy alone has proved to be effective in reducing symptoms, especially with regard to intestinal manifestations. The use of the probiotic as an adjuvant therapy to the gluten-free approach has led to a clinical response of greater magnitude than dietary therapy alone, with regard to both intestinal and extra-intestinal symptoms. Moreover, the use of the probiotic has clearly improved the results obtained with the Bristol Stool Scale compared to dietary changes alone, with better stool consistency in most patients at the end of treatment. From this finding too, it can be assumed that in the clinical treatment of NCGS our combined strategy (a GFD and the ES1 strain) is able to offer a greater advantage compared to dietary therapy alone, having a double effect by both improving the clinical picture and symptoms and restoring a condition of equilibrium to the intestinal microbiota.

In our study, we have also observed that the therapeutic action was more evident at the first timepoint after baseline, and there was a slight loss of effectiveness in the following months. This could be due both to the complex nature of NCGS and to patient difficulty in adhering to a GFD for a longer time period. Despite the fact that this was a preliminary pilot study carried out on a limited number of participants, much useful information has been collected. For instance, it was possible to confirm the strong association between NCGS and adulthood (all patients were over 19 years of age, except for one) and the female sex (there were only six male patients). In addition, according to general experience, it was possible to confirm a prevalence of abdominal swelling, abdominal pain and epigastric pain for the intestinal symptoms and a preponderance of asthenia, general malaise, headache and anxiety for the extra-intestinal symptoms.

Limitations of the study

However, due to the non-randomized design, it is likely impossible to attribute with certainty the observed effects to the administered probiotic. We did not take any bio-samples in order to be able to measure intestinal permeability and/or objective inflammatory markers, such as cytokines, zonulin, alpha-1-antitrypin and so on. We just analyzed parameters like C-reactive protein (PCR), white blood cells and monocytes values and no differences at all were seen between the two groups and between the beginning and the end of the study (data not shown). Such a markers are indeed normally low in subjects like the ones we have enrolled and the only parameters we have observed to be different were those concerning with symptoms as described in our work. Moreover, the risk of a possible placebo effect as a main bias does exist. Anyway, since most of the effects have been observed in the last 30 days of the study and not in the first 30 ones, we believe that if a "placebo effect" took place this was not predominant.[25-27]

Conclusions

Despite its main limitations (non-randomized, non-double-blinded, non-placebo-controlled, no bio-samples collected, few subjects per group), our study has addressed an issue that is certainly very current, with NCGS still considered to be somewhat unexplored within the scientific community, both in terms of etiology and in terms of treatment options. We have decided to address this issue by using a dual approach: by removing gluten from the diet and administering a bacterial strain that has been previously described as contributing to the wellness of the gut microbiota and which can apparently digest gluten.[14] We then scored patients by evaluating both intestinal and extra-intestinal parameters, along with stool consistency, and we have observed that the

dual approach is certainly more promising and potentially successful than the GFD diet alone. We consider this an interesting starting point for the evaluation of broader cases. Randomized, double-blind, placebo-controlled studies are now necessary to confirm our results.

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