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Research Article

The Effect of Bioecolians, A Commercial α-Gluco-Oligosaccharide, on Bowel Function in Subjects with IBS-C Symptoms

Yvergnaux Florent, Lassalle Laurent*, Saguet Thibaut

BioEurope-Solabia group, Anet, France

ABSTRACT

Aim: The purpose of the study was to evaluate the effect of the consumption of BioEcolians, a commercial α-gluco-oligosaccharide, on IBS-c symptoms and various strains of the gut microbiome.

Methods: 50 subjects participated in a double blind placebo control study. Subjects consumed 2 gr/day of BioEcolians or placebo capsules for 28 days and were evaluated for IBS-C symptoms (abdominal pain, bloating, discomfort and stool consistency). Fecal exams were performed in order to evaluate changes in the gut microbiom.

Results: BioEcolians treatment resulted in a progressive amelioration of IBS-C symptoms with a statistically significant improvement from day 14 to day 28. Stool frequency showed a progressive and statistically significant improvement with a mean increment of 1.6 complete spontaneous bowel movements (CSBM) in the BioEcolians group. Stool consistency significantly improved in both groups but BioEcolians treatment resulted in higher scores and showed a progressive increment (100% improvement versus 50% in the placebo group). BioEcolians intake resulted in a significant increase in the fecal counts of Bifidobacterium longum and Bifidobacterium animalis and all evaluated Lactobacilli strains. The change was statistically significant compared to baseline. No significant modification of calprotectin and lactoferrin was recorded following BioEcolians or placebo intake. The level of fecal Human Beta-Defensin 2 (HBD-2) increased in both treatment groups but and statistically significant only in the BioEcolians group.

Conclusions: The progressive amelioration of symptoms in IBS-C subjects recorded throughout the 28 days of the study with the modification of the content of fecal Bifidobacteria and Lactobacilli suggests that BioEcolians could serve as a beneficial dietary agent against IBS-C symptoms. Further investigations are required in order to evaluate the improvement of symptoms in healthy population.

Keywords: Bifidobacteria; α-gluco-oligosaccharide; IBS-C symptoms

ABBREVIATIONS: IBS: Irritable Bowel Syndrome; IBS-D: with diarrhea; IBC-C: with constipation; IBS-M: alternating diarrhea and constipation; CSBM: Complete Spontaneous Bowel Movements; HBD-2: Human Beta-Defensin 2; GI: Gastro Intestinal; BMI: Body Mass Index; AB: Abdominal Bloating; AP: worst Abdominal Pain; AD: Abdominal Discomfort; BSFS: Abdominal Discomfort; GLMM: Generalized Linear Mixed Model; RDP: Ribosomal Database Project

INTRODUCTION

Irritable Bowel Syndrome (IBS) is one of the most commonly diagnosed GI conditions, affecting approximately 10-15% of the world's adult population [1]. Symptoms include: frequent abdominal pain, bloating and discomfort, associated with alteration of bowel habits, such as predominant diarrhea (IBS-D), constipation (IBS-C) or alternating diarrhea and constipation (IBS-M) [2,3].

In IBS-C, the abdominal pain, discomfort and bloating are associated with constipation, as defined by the frequency of bowel movements per week and the consistency of the feces. International best practices guidelines promote the diagnosis of IBS based on clinical symptoms by using Rome criteria (now Rome IV) [4-6]. Objective biological markers that could support diagnosis-as well as facilitate the follow-up and the assessment of treatment efficacy of functional gastrointestinal disorders- remain to be clearly identified [7-8].

IBS has a debilitating impact on the quality of life of subjects,

Correspondence to: Lassalle Laurent, BioEurope-Solabia group, Anet, France, E-mail: Laurent.LASSALLE@solabia.fr

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adversely impacting not only their physical health, stamina, and daily functioning but also their social life and professional opportunities [9]. Since there currently is no cure for this disorder, treatment focuses on reducing the symptoms as much as possible. Common treatments include lifestyle modifications, dietary changes and psychosocial therapy. Medications such as Lubiprostone and Linaclotide are prescribed in more severe cases of constipation although they can cause adverse events such as nausea and diarrhea. Linaclotide is not recommended for use under the age of 18.

Although extensive investigations to elucidate the pathophysiology of IBS have been conducted, the condition still is not fully understood, and the roles played by various factors, such as presence of inflammation [10], post-infection low-grade inflammation [11], immunological factors [12], altered microbiome [13], dietary factors and enteroendocrine system involvement remain unresolved [14]. Emerging data support the existence of dysbiosis (imbalance in the intestinal flora) in subjects with IBS [15,16]. These data include a decreased complexity in composition of the gut microbiome, temporary instability [17], and changes in the associated mucosal microbiome, specifically an increase in *Bacteroides* and *Clostridia* as well as a reduction in *Bifidobacteria* [18,19]. Growing evidence suggests that an intentional modification of the intestinal microbiome could represent a beneficial approach to ameliorating IBS symptoms [20]. It is believed to be possible to induce intentional modifications of the intestinal microbiome through the administration of prebiotic carbohydrates, including specific fibers and related compounds. A prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health" [21]. By promoting specific changes in the composition and/or activity of the resident GI microbiome toward a healthy condition, prebiotics represent a highly promising dietary strategy for achieving positive results in ameliorating the symptoms of IBS [22,23].

BioEcolians® is a commercial α-gluco-oligosaccharide produced by Solabia Group, SA, and characterized by an average DP (degree of polymerization) of 5-6, and α1-2, α1-4 and α1-6 bond, obtained by controlled enzymatic synthesis. The glycosidic bond of this α-glucan results in a very high resistance to hydrolysis by digestive enzymes [24]. An *in vitro* fermentation study of BioEcolians on the human fecal microbiome revealed a significant increase of Bifidobacterium sp. compared to the control, suggesting a prebiotic status for such products [25].

The purpose of this double-blind, placebo-controlled study was to evaluate the effect of the consumption of BioEcolians for 28 days on IBS-c symptoms and various strains of the gut microbiome, as compared to both the baseline evaluation and to the placebo treatment arm.

MATERIALS AND METHODS

Materials

The study included two types of capsules (visually identical) containing either 500mg of the active ingredient BioEcolians (an α-gluco-oligosaccharide (GOSα) capsules, or 500mg of 100% glucose capsules. Both products were packed in identically labeled packs, with no visible differences between active and placebo capsules.

Ethical Approval

The study was conducted at Farcoderm, a research facility in Milan, Italy in accordance with the Declaration of Helsinki. The study was approved by an independent ethical committee for non-pharmacological clinical trials.

Participants

A total of 50 subjects (25 subjects per treatment/placebo treatment group) was included in the study. Two subjects from the placebo group withdrew from the study due to personal reasons. Withdrawn subjects were not replaced. The remaining 48 subjects completed the study.

Inclusion Criteria

Male and female subjects, aged 18-65 years with a BMI of 19-30 and IBS associated with constipation (IBS-C), as diagnosed using Rome III criteria, were eligible to participate in the study. The scores of the weekly averaged IBS-C symptoms in the two weeks preceding enrollment were: worst Abdominal Bloating (AB), worst Abdominal Pain (AP) and worst Abdominal Discomfort (AD) ≥ 3 (all evaluated on a 0-10 visual analog scale); fewer than three Complete Spontaneous Bowel Movements (CSBM; defined as a BM occurring in the absence of a laxative and associated with a sense of complete evacuation) per week; and stool consistency of 1-2 on Bristol Stool Form Scale (BSFS) at least twice per week. In addition, only subjects willing to not change their normal daily routine (i.e. lifestyle, physical activity, etc.) or to alter their usual diet or fluid intake during the trial periods were enrolled.

Exclusion Criteria

Subjects with the following medical conditions were excluded from the study: IBS associated with diarrhea (IBS-D); alimentary/eating disorders (e.g. bulimia, psychogenic eating disorders, etc.); Inflammatory Bowel Disease (ulcerative colitis, Crohn's disease, colitis); Metabolic Syndrome or diabetes, severe uncontrolled hypertension; significant uncompensated cardiac or respiratory diseases; impaired immune system due to immunosuppressive diseases such as AIDS and HIV; food allergy or food intolerances; drug abuse, alcohol abuse; prior extensive intestinal resection, diverticular stricture (narrowing of the colon), or celiac disease. Pregnant or breastfeeding women were also excluded; as were any potential subjects who participated in a probiotic, prebiotic and or laxative study within the three months prior to the study.

In addition, use of immunosuppressive medications, pharmacological treatment (topic or systemic) know to interfere with the tested product or having effect on metabolism, or antibiotics within the four weeks prior to commencing the study was prohibited. Dietary restrictions included: use of supplemental fibers within the four weeks prior to commencing the study, chronic use of laxatives, intake of exceptionally high content of plant-based/fiber foods, a strict vegetarian diet, and dietary intake of probiotics.

Study Design

The study was designed in accordance with FDA guidelines for clinical evaluation of IBS-C treatments [26]. This was a randomized, double-blind, placebo-controlled, single-center study. During the screening, eligible subjects were supplied with a daily IBS-C symptoms diary containing the Bristol Stool Form Scale (BSFS), visual analog scales, and a daily alimentary diary to complete for 14 days prior to the next visit. During the second visit, the diaries were reviewed, and IBS-C symptoms were confirmed for eligible subjects. These subjects were instructed to return to the clinic with stool samples prior to randomization and allocation to one of the two treatment arms (25 subjects per group). During the baseline visit, subjects were supplied with BioEcolians or placebo capsules, a stool collection kit, an IBS-C symptoms daily diary containing the BSFS, and an alimentary daily diary to be completed during the 28 days of interventional arm of the study. Subjects were instructed to consume 4 capsules daily in the morning, before breakfast, for 28 days.

Subjects returned for the final visit after 28 days of either product or placebo intake, during which stool samples of the last days were collected, compliance was evaluated by product accountability assessment, and diaries reviewed.

Randomization

Subjects were assigned to treatment groups using a computer-generated, restricted randomization list. The statistical software used was: PASS 11-PROFESSIONAL, vers. 11.0.8 released December 2nd, 2011 and running on Windows Server 2008 R2 Standard.

Efficacy Assessment

All subjects were instructed to fill out the diaries on a daily basis from screening to end of consumption, and rate the following symptoms: worst Abdominal pain, Abdominal bloating, Abdominal discomfort, the number of CSBM and the stool consistency. Abdominal Pain (AP) was defined as the weekly average of the worst daily abdominal pain that was experienced in the previous 24 hours; Abdominal Bloating (AB) was defined as "a sensation of feeling full, tight, or swollen in the abdomen"; Abdominal Discomfort (AD) was defined as "an uncomfortable sensation not described as pain."

AP, AB, and AD were assessed using a 0-10 Visual Assessment Scale (where 0=none; 10=very severe). Stool Consistency was assessed according to the 7-point of Bristol Stool Form Scale (BSFS). Responders were defined in accordance with FDA guidelines for clinical evaluation of drugs for the treatment of IBS-C. Subjects were categorized as "weekly responders" if there was a decrease of at least 30% in the weekly average report of abdominal pain and an increase of at least one CSBM per week as compared to baseline pain and stool frequency.

Fecal Samples

Fecal samples were either kept at room temperature and delivered to the laboratory within 3 hours from collection, or stored at -18°C in a domestic freezer and transported to the laboratory in a freezer pack within 24 hours of collection. Samples were kept at -80°C and analyzed in bulk at the end of the study. Each stool sample was processed using MoBio PowerFecal DNA Isolation Kit. DNA samples were checked by spectrophotometer, PCR and qPCR analyses to evaluate DNA quantity, quality and amplificability. Each DNA sample was archived in an internal bank (Matrix System-Thermo Scientific) for a long storage at -20°C.

Bifidobacteria and Lactobacilli Determination

Changes in the number of Bifidobacteria and Lactobacilli (*B. animalis, B. longum, L. rhamnosus, L. paracasei, L. casei, L. reuteri*) were investigated using qPCR real time.

Each DNA primer couple was tested in terms of its specificity and amplification efficiency with respect to DNA samples isolated from pure bacterial cultures, and at several serial dilutions, in qPCR analyses. By using the following probiotic species –*B. animalis, B. longum, L. casei, L. paracasei, L. reuteri, L. rhamnosus*-a value of efficiency between 1.8 and 2.2 was found, and as such, the result represents a good amplification performance of qPCR analysis. Each sample was analyzed with specific primer couples, in several qPCR analyses, in triplicate. For each DNA sample and for each specific DNA primer couple, the analysis provided a value of CT (comparative Ct) that is descriptive of the quantitative of DNA specific of the bacterial species, and so explanatory of the quantitative of bacterial cells in stool samples.

The relative quantification was applied a Generalized Linear Mixed Model (GLMM) under Poisson-lognormal error to account for higher variation at the lower end of target abundance. MCMC. qPCR R package was used to convert Ct data in bacterial counts. Evaluation of statistically significant difference between T28d and T0d values of both groups was carried out.

Profiling of the Microbiome Composition

Microbial community profile was carried out by a 16S amplicon sequence.

The 16S rRNAs detection was performed with an OTU-picking approach, which consists in assigning sequences to OTUs by clustering the sequences on the basis of a threshold value that the user may modify after the OTU picking step. The most abundant sequences in that OTU was chosen for subsequent analyses in order to reduce the computational power and the analysis time, without losing the frequency information. QIIME was used to perform the taxonomy assignment. Ribosomal Database Project (RDP) classifier 2.2 against the Silva database (2014 release), using the 0.8 as a confident interval was used. After taxonomic assignment, QIIME generates a BIOM file that was used for all the downstream analyses.

Biochemical Markers

Calprotectin and lactoferrin were determined in the stool using respectively Elisa kit Orgentec Diagnostika GmbH ORG280 and Elisa kit Orgentec Diagnostika GmbH ORG284 according the manufacturer instruction. Human Beta-Defensin-2 was determined by ELISA Kit Li StarFish S.r.l. for the in vitro determination of HBD-2 in the stool according to the manufacturer instructions.

Statistics

Sample size was calculated on the expected changes in Bifidobacteria

and Lactobacilli following the BioEcolians treatment, and was based on a 50% margin of non-inferiority with treatment, with the reference mean true ratio of 1 and coefficient of variance of 0.8, using a one-sided, two-sample t-test at the 5% significance level.

Statistical analysis of efficacy was carried out on the Per Protocol Population (PP). The statistical analysis of the safety of the treatment was based on the Intent to Treat Population (ITT).

For IBS-C related symptoms, stool frequency and stool consistency, the analyzed data were the weekly average scores. Comparison to baseline was evaluated on day 7, 14, 21 and 28 days.

Evaluation of microbiome, Bifidobacteria, lactobacilli and biochemical markers was conducted at baseline and at Day 28.

Comparisons Parameters

Intra-group analysis=Tx versus T0 (active and placebo). The non-parametric tests included Friedman test followed by Bonferroni post-test/Wilcoxon signed test. The parametric test (performed on the microbiome data) included student-paired T test. Inter-group analysis included comparison of BioEcolians vs. placebo at all time points. The non-parametric tests included the Mann-Whitney U test.

Results of the safety evaluation were based on AE/SAE listing, and were presented descriptively as absolute and relative frequencies. Statistical analysis was carried out using statistical software (NCSS 10-PROFESSIONAL, vers. 10.0.7, released July 22, 2015 running on Windows Server 2008 R2 Standard).

RESULTS

A total of 50 subjects were enrolled in the study, 25 in each treatment group. All 25 subjects from the BioEcolians group concluded the study; 23 out of 25 subjects from the Placebo group concluded the study, as two subjects dropped out for personal reasons not related to the protocol (Figure 1).

During the trial period, participants did not vary their normal daily routine (lifestyle, physical activity, etc.) nor did they alter their usual diet or fluid intake. No probiotics were taken before or during the study. Participants had a complete adherence (100% compliance) to the study protocol in terms of residual number of capsules and fecal sample collection. No adverse events were reported, and intake of the capsules was well tolerated.

Efficacy Results

IBS-C symptoms, CSBM, BSFS

BioEcolians treatment resulted in a progressive amelioration of IBS-C symptoms (Table 1). A statistically significant improvement was observed from day 14 in subjects treated with BioEcolians, and continued until day 28. No significant improvement of abdominal

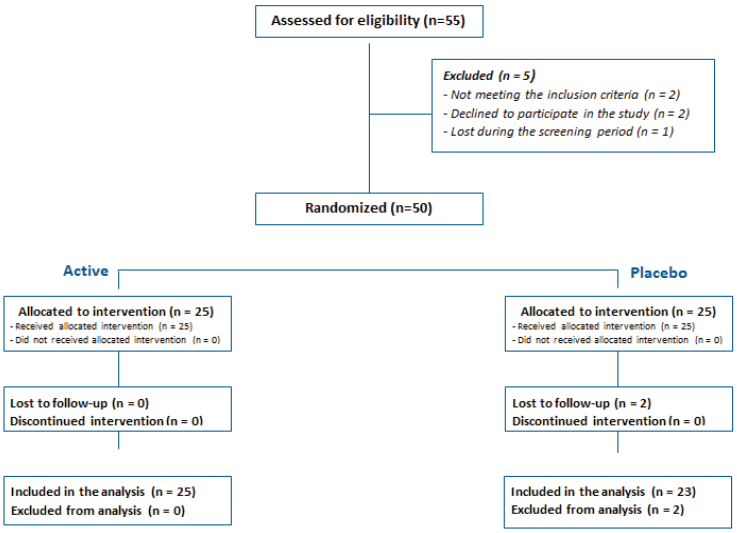


Figure 1: Subjects flow throughout the study.

Table 1: IBS-C symptoms (Abdominal Pain, Abdominal Bloating, and Abdominal Discomfort) The data are presented as mean weekly score ± standard error mean, and as percent change from baseline for each treatment group.

	Abdominal Pain		Abdominal Bloating		Abdominal Discomfort	
	Placebo	BioEcolians (R)	Placebo	BioEcolians (R)	Placebo	BioEcolians (R)
Screening T_{-14-0}	4.3 ± 0.221	4.8 ± 0.331	5.4 ± 0.311	5.9 ± 0.311	5.1 ± 0.302	5.4 ± 0.326
T_{7d}	3.8 ± 0.268 (-11.6%)	3.6 ± 0.408 (-25%)	5.0 ± 0.332 (-7.4%)	4.4 ± 0.348* (-25.4%)	4.5 ± 0.299 (-11.8%)	4.1 ± 0.368 (-24.1%)
T_{14d}	3.6 ± 0.274 (-16.3%)	3.1 ± 0.407* (-35.4%)	4.9 ± 0.279 (-9.3%)	3.7 ± 0.407*# (-37.3%)	4.4 ± 0.307 (-13.7%)	3.5 ± 0.409* (-35.2%)
T_{21d}	3.6 ± 0.281 (-16.3%)	2.8 ± 0.433* (-41.7%)	4.7 ± 0.302 (-13.0%)	3.2 ± 0.391 (-45.8%) * ##	4.1 ± 0.317 (-19.6%)	3.1 ± 0.395* (-42.6%)
T_{28d}	3.7 ± 0.301 (-14.0%)	2.3 ± 0.388* ## (-52.1%)	4.8 ± 0.381 (-14.8%)	2.7 ± 0.373* ### (-54.2%)	4.2 ± 0.364 (-17.6%)	2.7 ± 0.383* ## (-50.0%)

Intra-group comparison (T_X vs T_0)- Bonferroni test $p<0.05$*; Inter-group comparison Mann - Whitney U test T_{14d} $p<0.05$#, T_{21d}/T_{28d} $p<0.01$##, T_{28d} $p<0.001$###

pain, abdominal bloating, and abdominal discomfort was reported in subjects treated with the placebo (Figure 2). Intergroup analysis indicated that scores of IBS-C symptoms were significantly lower in the BioEcolians group compared to the placebo group; a statistical significance between the two groups was found for the abdominal bloating, starting from T_{14d}, and for abdominal pain and abdominal discomfort at T_{28d}.

Stool frequency showed improvement as a progressive and statistically significant increment in the BioEcolians group throughout the entire trial period. Stool frequency also increased in the placebo group, but to a lower extent, and was non-progressive. Also, a significant difference with respect to the baseline level was achieved only at T_{21d} and T_{28d} for the placebo group.

Overall BioEcolians treatment evoked a significant increment of CSBM at T_{21d} and T_{28d} compared to the placebo treatment at the same time points. At the end of the treatment, the mean score increased from 2.2 to 3.8 (+73%) in the BioEcolians treatment group as compared to an increase from 2.4 to 2.9 (+21%) in the placebo group.

Stool consistency significantly improved in both groups, except in the placebo group at T_{14d}. However, BioEcolians treatment resulted in higher scores, and showed a progressive increment. A significant difference was found between the two groups starting from the 2nd week of treatment (Table 2 and Figure 3). At the end of the treatment, the mean score increased from 1.7 to 3.4 (+100%) in the BioEcolians treatment group as compared to an increase from 1.8 to 2.7 in the placebo group. The percent of responders (weekly improvement in both pain and frequency in CSBM) was 44.0% (11 out 25) in the BioEcolians group, and 13.0% (3 out 23) in the placebo group (difference +21%; p=0.020).

Bifidobacteria and Lactobacilli Fecal Count

BioEcolians intake resulted in a significant increase in the fecal counts of *Bifidobacterium longum* and *Bifidobacterium animalis*, whereas no significant increase was achieved following placebo intake. The change from baseline in bacteria count (presented in log units) was statistically significant compared to baseline and compared to the placebo group (Table 3).

BioEcolians intake resulted in increased fecal counts for all evaluated Lactobacilli strains. The change from baseline in bacteria count (presented in log units) was statistically significant compared to baseline for *L. casei*, *L. reuteri* and *L. rhamnosus*.

Intergroup analysis showed a statistically significant difference between BioEcolians and placebo groups for all lactobacilli strains except for *L. paracasei*.

Fecal Microbiome

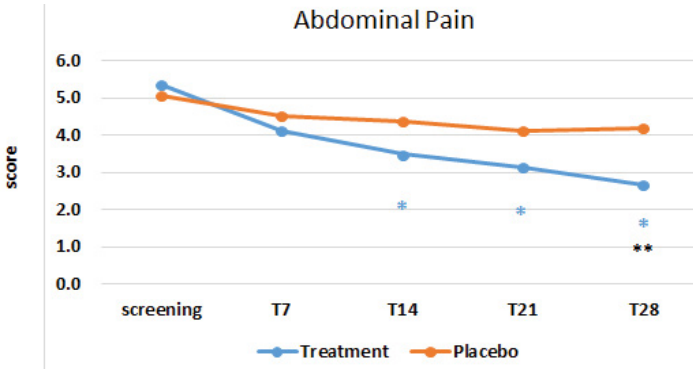
A total of 20,521,998 sequence of reads were obtained, with 416 assigned OTUs (Operational Taxonomic Units)

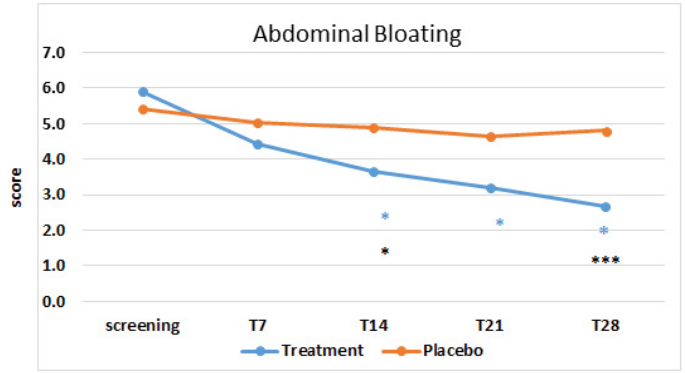
The top 13 genera assigned to the 128 most abundant OTUs (i.e. with a relative abundance more than 0.1%) were found in each subject, describing a core microbiome in agreement with other reports [27,28]. No difference was detected between the prebiotic and placebo groups in terms of presence/absence of genera at baseline and Day 28; all genera were found in every volunteer.

Biochemical Markers

Fecal *calprotectin*, a calcium and zinc binding protein, and Fecal lactoferrin, a glycoprotein expressed by activated neutrophils, are recognized as indicators of intestinal inflammation, proportional to neutrophil migration toward the intestinal tract [29,30]. Human β-defensin-2 (HBD-2), a human antimicrobial peptide produced by (among others) intestinal epithelial cells in response to pathogenic bacteria is an indicator of the host defense against microbes [31]. HBD-2 induces the formation of micropores in the membrane of pathogenic bacteria in the gut leading to loss of the structure, and cell collapse. In addition to its direct effect on the pathogenic bacteria, HBD-2 was shown to induce production of mucins molecules, which are essential part of the mucosal lining of the gut and serve as part of the intestinal barrier [32].

The results of fecal calprotein, lactoferrin and HBD-2 levels at baseline and at Day 28, including the differences between the two time points, are presented in Table 4.





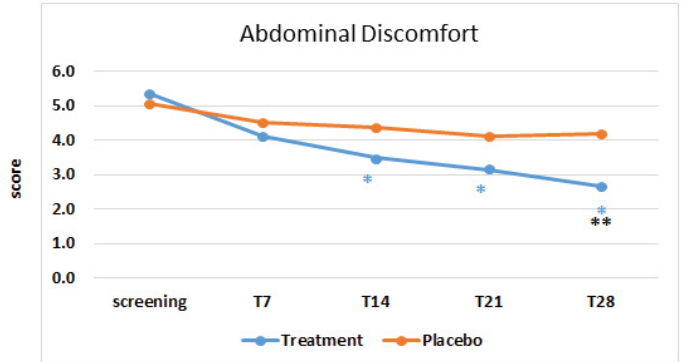


Figure 2a-2b-2c: Mean weekly score of abdominal pain, bloating and discomfort

Intra-group significance * $p<0.05$ versus T_0; Inter-group significance (treatment versus placebo) * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 2: CSBM and Stool Consistency.

	Complete Spontaneous Bowel Movement (CSBM)		Stool Consistency (according to BSFS)	
	Placebo	BioEcolians (R)	Placebo	BioEcolians (R)
Screening	2.4 ± 0.11	2.2 ± 0.12	1.8 ± 0.06	1.7 ± 0.07
T_{7d}	2.9 ± 0.14 (+20.8%)	3.3 ± 0.26* (+50%)	2.4 ± 0.17 * (+33.3%)	2.7 ± 0.19* (+58.8%)
T_{14d}	3.1 ± 0.17 (+29.2%)	3.3 ± 0.26* (+50%)	2.4 ± 0.18 (+33.3%)	3.0 ± 0.24*# (+76.5%)
T_{21d}	3.0 ± 0.11 * (+25%)	3.7 ± 0.29 *# (+68.2%)	2.7 ± 0.22* (+50%)	3.4 ± 0.26 *# (+100%)
T_{28d}	2.9 ± 0.24 * (+20.8%)	3.8 ± 0.29 *# (+72.7%)	2.7 ± 0.20* (+50%)	3.4 ± 0.26*# (+100%)

CSBM: * Bonferroni test p<0.05 vs T_{0d}; # Mann - Whitney U test p<0.05 vs T_{21d}/T_{28d} placebo. Stool Consistency: * Bonferroni test p<0.05 vs T_{0d}; #Mann - Whitney U test p<0.05 vs T_{14d}/ T_{21d}/T_{28d} placebo

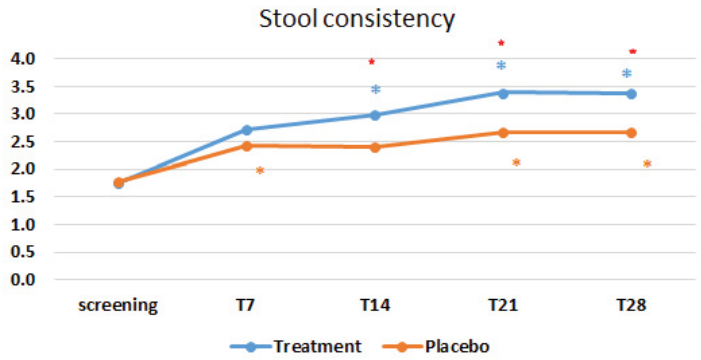


Figure 3: *Comparison of the stool consistency scores* Intra-group significance * p<0.05 treatment versus T_0, * p<0.05 placebo versus T_0 Inter-group significance (treatment versus placebo) ★ p<0.05

Table 3: Fecal content (log units/10 mg) and variation vs t_{0d} of Bifidobacteria and lactobacilli.

	PLACEBO			BioEcolians		
Probiotics	T_{0d}	T_{28d}	% change vs T_{0d}	T_{0d}	T_{28d}	% change VS T_{0d}
Bifidobacterium longum	6,10 ± 0.12	6,11 ± 0.12	+0.16%	5,63 ± 0.24	6,30 ± 0.28**	+11.8%##
Bifidobacterium animalis	2,67 ± 0.26	2,36 ± 0.22	-11.6%	2,16 ± 0.31	2,58 ± 0.24**	+16.2%###
Lactobacillus casei	1,12 ± 0.13	0,97 ± 013*	-13%	1,15 ± 0.14	1,91 ± 0.22***	+65.7%###
Lactobacillus paracasei	0,61 ± 0.20	0,38 ± 0.15	-38%	0,41 ± 0.16	0,76 ± 0.19	+85.2%
Lactobacillus reuteri	1,28 ± 0.23	0,87 ± 0.21	-32.2%	0,78 ± 0.20	1,50 ± 0.19*	+92.7%#
Lactobacillus rhamnosus	2,30 ± 0.29	2,14 ± 0.27	-6.8%	2,25 ± 0.37	3,21 ± 0.33**	+42.9%##

Intragroup (vs T0) analysis Wilcoxon signed test: * p<0.05, ** p<0.01, *** p<0.001, Intergroup analysis on differences T_{28d}-T_{0d} (BioEcolians vs placebo): Mann - Whitney U test: # p<0.05, ## p<0.01, ### p<0.001

Table 4: Fecal levels of Calprotectin, Lactoferrin and Human β-Defensin-2

	Calprotectin (µg/g)			Lactoferrin (µg/g)			Human β-defensin-2 (ng/g)		
	T_{0d}	T_{28d}	Difference T_{28d}-T_{0d}	T_{0d}	T_{28d}	Difference T_{28d}-T_{0d}	T_{0d}	T_{28d}	Difference T_{28d}-T_{0d}
BioEcolians	27,43 ± 7.85	23,76 ± 6.83	-3,67	3,98 ± 1.06	3,43 ± 1.04	-0,55	51,28 ± 8,17	62,33 ± 8,79	+11,05 (*)
Placebo	27,40 ± 6,67	24,23 ± 8,45	-3,17	2,80 ± 0.59	1,70 ± 0.29	-1,10	49,77 ± 7,53	54,52 ± 8,43	+4,75

Data are reported as mean score value ± SEM. Intragroup (vs T0) analysis: Wilcoxon signed test: * p<0.05

Basal calprotectin levels in all subjects were well below the cut-off of 50 µg/g considered as limiting value for attributing the subjects to the IBS condition [29] (59-61), and basal levels of lactoferrin in IBS-C subjects were in agreement with data reported. No significant modification of these two markers was recorded following BioEcolians or placebo intake (Difference T_{28d} vs T_{0d}), and no difference was found between the BioEcolians vs the placebo groups at the end of the treatment.

Basal level of fecal HBD-2 increased following BioEcolians or placebo intake: the increment was statistically significant in the BioEcolians groups, with no intergroup significant difference recorded at T_{28d}.

DISCUSSION

Health and disease can be influenced by the composition and activity of the intestinal microbiome through its involvement in nutrition, host physiology functions and pathogenesis of certain disease conditions [33]. Recognition of the health-promoting properties of certain gut microorganisms has encouraged dietary-

based interventions to provide optimal environment for beneficial microbiome composition and metabolism [34,35].

Prebiotics, probiotics and synbiotics represent an alternative, promising and natural approach for ameliorating IBS-symptoms [20].

Results of the present randomized, placebo-controlled clinical study support the prebiotic status of BioEcolians and its efficacy in the management of IBS-C subjects. BioEcolians administration increased screened levels of fecal Bifidobacteria and Lactobacilli; the increment resulted statistically significant either with respect to the screening levels of *B. longum*, *B. animalis*, *L. casei*, *L. reuteri* and *L. rhamnosus*, but not for *L. paracasei* either with respect to the placebo group.

The prebiotic activity of BioEcolians was also demonstrated in pre-clinical studies.

In a previous *in vitro* fermentation study, incubation of human fecal microbiome with BioEcolians resulted in a significant increase in the number of Bifidobacterium and Lactobacilli sp. compared to the control [25]. Another pre-clinical study that compared the effect of BioEcolians and other oligosaccharides on the intestinal microflora of rats inoculated with a human faecal flora showed substantially higher production of SCAF in the BioEcolians treatment group (Djouzi 1997).

Subjects receiving BioEcolians experienced a progressive and significant amelioration of IBS-C symptoms throughout the treatment period. Specifically, abdominal pain, abdominal bloating and abdominal discomfort were significantly reduced at the end of the treatment, as compared to the placebo treatment group. The BioEcolians treatment resulted in a progressive and significant improvement of CSBMs and stool consistency as compared to the screening period in comparison to the placebo group. Evaluation of the results in terms of weekly responders, as defined by FDA [26], indicated a significantly higher percentage of weekly responders in the BioEcolians group compared to the placebo group.

Similar results for IBS symptoms were obtained in a clinical trial with a *trans-galacto-oligosaccharide* [36], a prebiotic suggested to have a potential beneficial effects in IBS. Stool consistency, flatulence, bloating, composite score of symptoms and subjective global assessment were all significantly improved in subjects treated with trans-galacto-oligosaccharide compared to the placebo in that study, and it was determined that the prebiotic was selective toward the beneficial genus Bifidobacterium (Depeint, Tzortzis, Vulevic, I'Anson, & Gibson, 2008), which is recognized to improve lower gut health. However, the two clinical trials differed in terms of doses and duration of treatment, as BioEcolians achieved an improvement at a lower dose of 2g/day after 4 weeks of treatment, whereas trans-galacto-*oligosaccharide* was administered at 3.5 -7.0 g/day for 12 weeks.

Correlation between amelioration of IBS symptoms and increment of fecal Bifidobacteria content, is further supported by results obtained in clinical studies with subjects administered probiotic Bifidobacteria [37-39]. In the present study, BioEcolians administration did not induce any significant intragroup and intergroup difference in the overall profile of fecal microbiome, expressed as relative abundance of bacterial genera. The use of a different sequencing method, such as the high-resolution shotgun metagenomic sequencing, together with observation of both species and strain level in stool samples, could reveal more accurate differences [16]. However, a modulation of the fecal microbiome was achieved by the prebiotic treatment compared the placebo and resulting in a larger variability of the fecal microbial species in subjects receiving BioEcolians versus the placebo group. The modulation of the fecal microbiome might have contributed to the amelioration of the IBS-C symptoms.

Recently, a panel of eight biomarkers was determined to correlate moderately although significantly with GI symptom severity in IBS, suggesting that a biomarkers panel could be used in addition to symptoms scores to quantify the responsiveness to treatments in addition to symptoms scores [30]. Fecal levels of calprotectin, lactoferrin and HBD-2 measured in the screening period in IBS-C subjects were in the range of data previously reported [30]. BioEcolians treatment did not significantly modify the fecal level of calprotectin and lactoferrin in IBS-C subjects, and no difference was detected compared to the placebo treatment. Fecal HBD-2 levels showed an improvement increment with respect to basal levels in both groups, with the increment statistically significant only in the BioEcolians group, and no significant difference compared to the placebo group calculated.

Although the magnitude of increment could represent a limiting factor in indicating a correlation between such modification and IBS-C symptoms, specifically amelioration by the BioEcolians treatment, nevertheless such result could suggest an activation of the mucosal innate defense system. Increment of HBD-2 levels, an inducible antimicrobial protein, achieved by the BioEcolians treatment, could represent an original way to modulate the gut microbiome by limiting the proliferation of pathogenic bacteria. The way such HBD-2 increment is achieved by BioEcolians treatment has to be further investigated.

In conclusion, modification of the content of fecal Bifidobacteria and Lactobacilli adds more evidence to the prebiotic status of BioEcolians. Moreover, the progressive amelioration of symptoms in IBS-C subjects recorded throughout the 28 days of the study suggests that BioEcolians could serve as a beneficial dietary agent against IBS-C symptoms. The European Food Safety Authority (EFSA) panel of dietetic products, nutrition and allergies indicated that IBS patients or subgroups of IBS patient are generally considered a suitable study group to substantiate claims on GI discomfort intended for the general population, and therefore BioEcolians may improve symptoms of discomfort and bloating in healthy subjects. Further investigations are required in order to define any correlation between such results and other parameters that have been investigated, such as modification of microbiome profile and activation of the mucosa innate defense system.

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