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An Exploratory Study on a Multispecies Probiotic Formulation and Markers of Intestinal Health in Healthy People

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Abstract

This study aimed to explore the efficacy of oral supplementation with 11 probiotic strains, combined in the strain mix Probatech™ (Centro Sperimentale del Latte S.r.l Strada Provinciale per Merlino, 326839 Zelo Buon Persico (LO), Italy) and delivered through the food supplement PROBAFLOR and how it plays a positive role in maintaining normal intestinal function, providing benefits in healthy adult subjects. A 16-week, randomized, double-blind, placebo-controlled clinical study was conducted starting with 60 participants. Participants were randomly assigned to either the probiotic or placebo group. Participants were asked to provide one faecal sample at the beginning of the study, another one after 12 weeks of supplementation and the final one after 16 weeks. Amplicon 16S rRNA gene sequencing and GC-MS Short-Chain Fatty Acid (SCFA) profiling were performed on the faecal samples. Participants filled out questionnaires to assess their gastrointestinal health and psychological well-being. The overall mean GIQLI scores increased in both groups over time. The increases were significant within both groups but not between groups. Following the administration of PROBAFLOR, Shannon and Simpson diversity indices showed a significant increase at day 90 (week 12) ($p < 0.05$), demonstrating that the intervention effectively enhanced gut microbiota diversity. A shift in the intestinal microbiota towards SCFA-producing families and genera was observed. Moreover, the change in total and single SCFAs was significantly different between probiotic and placebo groups at the end of the supplementation period. Once-daily consumption of the PROBAFLOR probiotics formula regulated gut microbiota balance by modulating SCFA production. It may be beneficial for gut health, improving defecation habits and satisfaction, normalizing stool frequency, and promoting bacterial metabolism.

Keywords: gut health; PROBAFLOR; Probatech™; short-chain fatty acids; microbiota; psychological well-being

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1. Introduction

In recent years, it has been highlighted that gastrointestinal microbiota is implicated in the pathophysiology of multifactorial functional intestinal disorders, irritable bowel syndrome (IBS), and constipation. There are many ways to support gut health and gastrointestinal well-being. Current medical research is much more focused on the treatment of defined gastrointestinal diseases than on secondary or even primary prevention; on the other hand, preventive medicine is increasingly perceived as important in medical and

economic terms, particularly in the field of gastroenterology. An innovative approach to support gut health is based on modulating the gut microbiome or the gastrointestinal barrier, such as with probiotics and/or prebiotics [1]; their potential to improve bowel habits in healthy populations has been examined in a limited number of studies [2]. Probiotics are currently defined as "live microorganisms that, when administered in adequate amounts, confer a positive effect on the health of the host" [3–5].

The mechanisms of action by which probiotics may contribute to human health are grouped into three categories; the first concerns the ability of probiotics to exclude or inhibit pathogens and is the best studied and most referenced mechanism. A further mechanism is the enhancement in intestinal barrier function by affecting pathways that lead to mucus secretion, tight junction regulation, and apoptosis modulation. Furthermore, probiotics can modulate the host immune response, exerting both local and systemic effects. In addition to intestinal epithelial cells, probiotics can come into contact with intestinal dendritic cells which play a crucial role in innate and adaptive immunity [6]. Besides modulating immune responses, probiotics also affect some aspects of brain function, as suggested in the field of gut–brain axis research [7]. Multispecies probiotics which may have improved functionality compared to single-strain probiotics are increasingly found on the market. Well-designed multispecies probiotics can benefit from a certain amount of synergism when different probiotic effects of different probiotic species are combined [8].

Based on the above statements, we hypothesized that the combination of 11 probiotic strains, supported through the PROBAFLOR food supplement, may play a positive role in improving or maintaining normal intestinal function in healthy people. The multispecies formulation was designed using a research-driven selection process that identifies strains based on genomic and functional profiles. The formulation approach was to maximize microbial genomic diversity with the aim of optimizing efficacy across a diverse population. From a functional perspective, the strains' activity was supported by previous in vitro studies on single strains [9–12] demonstrating their safety, stability and survival, enabling them to reach the gut alive. The choice to use healthy people in this study lies both in the nature of the probiotic product, which is not a drug and therefore cannot boast curative properties, and in the awareness that gastrointestinal symptoms are frequent in otherwise healthy people [13]. Gastrointestinal symptoms reduce quality of life [14] and often prompt individuals to seek advice to find management strategies. In line with current strategies, we conducted a randomized, double-blind, placebo-controlled trial to study the safety and effect of a multispecies probiotic formulation on gastrointestinal symptoms, quality of life, and gut microbiota composition and activity in healthy adults.

2. Materials and Methods

Clinical study overview: The present clinical trial is a randomized, double-blind, parallel-group, placebo-controlled single-centre study, with a 4-week run-in period, a 12-week intervention period, and a further 4-week follow-up period without intervention to evaluate the potential prolonged effect of the probiotic product. Specifically, we hypothesized that probiotic supplementation should improve parameters related to gut health and the general well-being of subjects. Healthy adult participants were recruited and randomized into two supplementation groups in a 1:1 ratio. The two intervention groups were differentiated by the supplementation that they received: probiotic or placebo, both of which were similar in appearance.

Inclusion and Exclusion criteria:

Inclusion criteria included the following:

- Men and women between 18 and 65 years;
- Healthy based on medical history;

- Commitment to comply with all study procedures;
- Commitment to refrain from using any home remedies for control gastrointestinal problems, particularly if live bacteria are involved;
- Signed informed consent to participate in the study.

This study focused on a healthy adult population; hence, the exclusion criteria ruled out individuals with significant acute or chronic illnesses and/or those in an immunocompromised state, pregnant women who are breastfeeding or planning to become pregnant during the study period, subjects with clinically significant gastrointestinal tract disease, subjects with allergy or sensitivity to the active or inactive ingredients of the investigational product, and subjects who had used antibiotics in the month prior to enrolment or who had used probiotics continuously in the two months prior to enrolment. The included subjects were asked not to modify their diet or physical activity during the study period. Baseline data were collected to assess whether the groups were balanced. In addition, any episodes of gastrointestinal diseases experienced by the participants in the 3 months prior to their inclusion in the study were recorded. Enrolment for the study was conducted through word of mouth and social media. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and national laws and regulations. The study protocol was approved by the Ethics Committee (CERM, Marche, Italy) and registered in ClinicalTrials.gov (Registration Number: NCT06619067, accessed on 27 September 2024). To guide the reporting of this study, the CONSORT checklist is provided in Supplementary Material Table S1. The participants provided written informed consent for enrolment.

Study products: The active and placebo products were manufactured and provided by AIXSWISS B.V. (Kerkrade, The Netherlands). The active product contained 11 probiotic strains (Probatech™): *Bifidobacterium animalis* ssp. *lactis* BLC1, *Lactobacillus delbrueckii* ssp. *lactis* LL82, *Lactobacillus plantarum* 14D, *Lactobacillus acidophilus* LA1, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB2, *Streptococcus thermophilus* Z57, *Bifidobacterium bifidum* SP9, *Bifidobacterium longum* ssp. *longum* SP54, *Lactobacillus salivarius* SP2, *Lactobacillus rhamnosus* SP1, and *Lactobacillus casei* BGP93 at 2×10^{10} CFU/capsule. The placebo product consisted of corn starch. Both the active and placebo powders were encapsulated in hydroxypropyl methyl cellulose capsules with an identical appearance to ensure blinding. Each participant received three bottles, each containing 30 capsules of their randomly assigned product (active or placebo), sufficient for the 12-week study.

Assessment of outcomes: The primary study outcome was the effect of the probiotic product on health-related quality of life, assessed through the GIQLI questionnaire (Gastrointestinal Quality of Life Index). Secondary outcome measures included changes in intestinal microbiota composition assessed through metagenomic analysis; changes in metabolomic biomarkers by determining the concentration of short-chain fatty acids (SCFAs) from faecal water samples using gas chromatography techniques; changes in quality of life, assessed through the PGWBI questionnaire (Psychological General Well-Being Index); changes in intestinal function assessed according to the Bristol Stool Form Scale; changes in gastrointestinal symptoms assessed through the GSRS questionnaire (Gastrointestinal Symptom Rating Scale). For data collection regarding efficacy variables, participants were trained how to fill out a paper diary. Additionally, faecal sample collection tubes were provided for the collection and determination of microbiota composition and SCFA profiling at the beginning of the study, after 12 weeks of supplementation and after 1 month of follow-up. Figure 1 summarizes the development of the PROBAFLOR clinical trial, the actions performed, and the variables collected at each of the data collection time points.

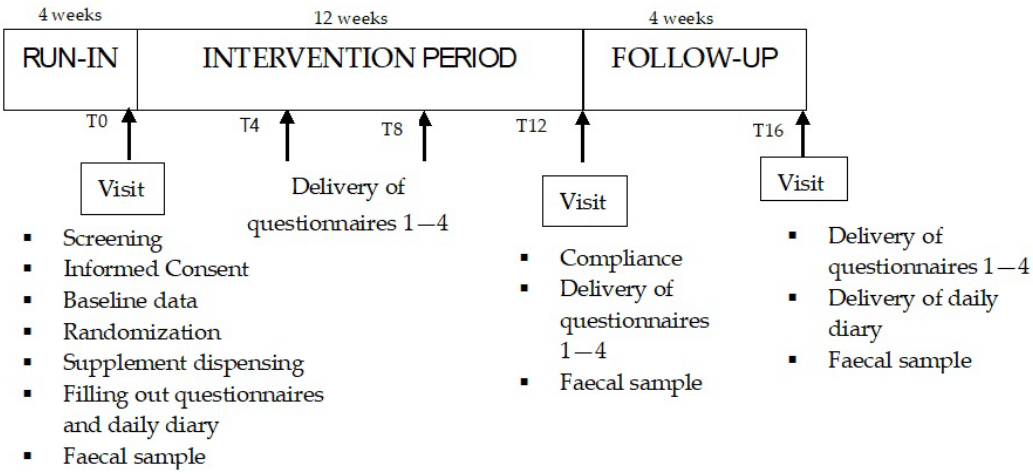


Figure 1. Timeline and activities of clinical study. T0 (Baseline); T4 (Week 4); T8 (Week 8); T12 (Week 12); T16 (Week 16).

Metagenomic analysis of microbiota composition: Faecal samples were collected into individual sterile tubes and quickly transferred into a −80 °C cryogenic freezer until DNA extraction. The microbial DNA was extracted from faecal samples using a Stool DNA Isolation Kit (Norgen Biotek Corp, Thorold, ON, Canada) in accordance with the manufacturer's protocol. The V3—V4 hypervariable regions of 16S rDNA were amplified using universal primers (341F 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC-TACGGGNGGCWGCAG-3′, 805R 5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC -3′) following the 16S Metagenomics Sequencing Library preparation protocol (Part # 15044223 B). Libraries were sequenced using the MiSeq Illumina Platform (Illumina Inc., San Diego, CA, USA) with a 2 × 250 paired end run. Poor quality reads were filtered with Trimmomatic (Version 0.39) [15]; paired-end reads were merged using FLASH (Version 1.2.11) [16] and processed with VSEARCH (Version 2.21.1) [17] to detect potential chimera sequences and to cluster merged amplicons in operational taxonomic units (OTUs), with a minimum pair-wise identity threshold of 97%. The NCBI 16S RefSeq database [18] was employed for taxonomic classification. Evaluation of microbial alpha (Adv, Chao1, Simpson's and Shannon's diversity) and beta (UniFrac distances, Bray–Curtis dissimilarity) diversity measures was performed using an internal pipeline.

SCFA determination: The same faecal samples were used for SCFA detection and quantification. The extraction and quantification procedure using GC-FID (Gas Chromatography–Flame Ionization Detection) was performed as described by Scortichini et al. (2020) [19].

Questionnaires: The GIQLI [20] is a measure of the subjective perception of the patient's well-being that includes 36 items with five response categories for each item; evaluating the answers from 0 to 4, a global score can be estimated that goes from 0 to 144. The higher the score, the better the result. Besides a global mean, the GIQLI provides 5 different dimensions (gastrointestinal symptoms; emotional, physical, and social factors; and a therapeutic component), allowing for a more detailed analysis of the patient's quality of life (QOL).

The GSRS [21] is a 15-item instrument designed to assess the symptoms associated with common gastrointestinal disorders. It is organized into 15 items (symptoms), each rated on a 7-point Likert scale from no discomfort (score 1) to very severe discomfort (score 7). It has 5 subscales (Reflux, Diarrhoea, Constipation, Abdominal Pain, and Indigestion Syndrome). Subscale scores range from 1 to 7, with higher scores indicating greater discomfort.

The PGWBI [22] is a general measurement of psychological well-being composed of 22 items covering six aspects: anxiety, depression, self-control, positive well-being, general health, and vitality. The six multidimensional scores are combined to obtain a global score ranging from 0 (severe stress) to 110 (well-being). Higher scores represent better QOL.

The Bristol stool consistency scale is a visual medical aid, used to classify the shape and consistency of human stool; it includes seven distinct categories, from the first, which indicates a more or less severe degree of constipation, to the sixth and seventh, which describe a diarrheal state. These are the seven groups: Type 1 stool, separate hard lumps, like nuts, hard to pass; Type 2, sausage-shaped, but lumpy; Type 3, like a sausage but with cracks on its surface; Type 4, like a sausage or snake, smooth and soft; Type 5, soft blobs with precise cut edges (passed easily); Type 6, fluffy pieces with ragged edges, a mushy stool; and Type 7, watery, with or without solid pieces.

Daily, each participant had to fill in a table composed of 3 lines; on the first line, they had to indicate whether or not they had taken the capsule; on the second line, they were asked about the frequency of defecation; on the third and last line, they were asked about the morphology of their faeces according to the BSC. Weekly, each participant had to answer the question "How would you rate your general abdominal discomfort in the last 7 days compared to the month before you started using the study product?" by indicating one of the following options: "significantly relieved", "somewhat relieved", "unchanged", "somewhat worse", or "significantly worse".

Sample Size Estimation: The sample size was calculated for the primary outcome (changes in GIQLI) based on the results of a previous study [23], applying a clinically relevant mean difference of 6–8 points and a standard deviation of 17.5 on the GIQLI. Therefore, an effect size of 7/17.5 = 0.4 was assumed. Considering $\alpha = 0.05$ and power = 0.90, 27 patients per group were needed. Assuming an average drop-out of 10%, a sample size of 60 subjects (30 for the Probiotic group and 30 for the Placebo group) was determined.

Statistical Data Analysis: Statistical analyses were performed using GraphPad software (Version 8, Software Inc., San Diego, CA, USA). Shapiro–Wilk statistics were used to evaluate data normality. Demographic and baseline characteristics were summarised by study group. Continuous outcome variables were reported as means ± SD and categorical outcome variables as frequency (%). Differences between study groups were assessed using analysis of variance (ANOVA). Differences between baseline and post-intervention outcomes within each study group were assessed using the *t*-test; when appropriate, a post hoc analysis was performed using the Tukey test. The significance level for statistical tests was set at 0.05. Before starting the data analysis, the level, pattern, and likely causes of missing points in the baseline variables and outcomes were studied by setting up appropriate tables. This information was used to determine whether the level and type of missing data had the potential to introduce bias into the analysis results or substantially reduce the precision of the estimates for the proposed statistical methods. The primary outcome (change in GIQLI) was analysed within each group using pairwise *t*-tests to evaluate whether changes from baseline to 3 months and after 1 month of follow-up were statistically significant. The absolute change (mean value at baseline subtracted from the mean value after intervention for each subject) was estimated with independent samples of *t*-tests. One-way analysis of variance was used to test for differences between changes in the two groups at different time points. Secondary outcome variables were analysed in the same way. Changes in symptoms reported in the bowel diary from baseline were compared between groups each week using the Wilcoxon rank-sum test.

Multiple strategies were employed to reduce attrition, including thorough recruitment, a structured and time-limited protocol, inclusion of a run-in period, limiting the burden and inconvenience of data collection on participants, and fostering a trusting and collaborative

relationship between research units and participants. Sensitivity analyses were undertaken, based on the assumption that missing results are the worst possible or the best possible, in different randomization groups. If these demonstrated that conclusions may differ based on missing values, additional multiple imputations for missing values were performed. These analyses accounted for any resulting losses in relation to differences in the measured variables (i.e., under the assumption of missing at random). For metagenomics and metabolomics data, specific bioinformatics tools were applied to compare the two intervention groups (alpha and beta diversity measures, including Shannon diversity index measures and UniFrac distances). Differences in relative abundances of taxa between time points were evaluated using the Wilcoxon test.

3. Results

3.1. CONSORT Diagram

The complete recruitment and study process was carried out in July 2024. Data from the participants screened for eligibility, those randomised and finally the cases analysed are included in the CONSORT diagram (Figure 2). Data regarding the reasons for exclusion and the number of subjects lost to follow-up are also included for each intervention group. A total of 60 subjects were included and randomised in the study (30 participants in each group). After loss to follow-up during the intervention period, 27 subjects were analysed in the Probiotic group and 24 in the Placebo group.

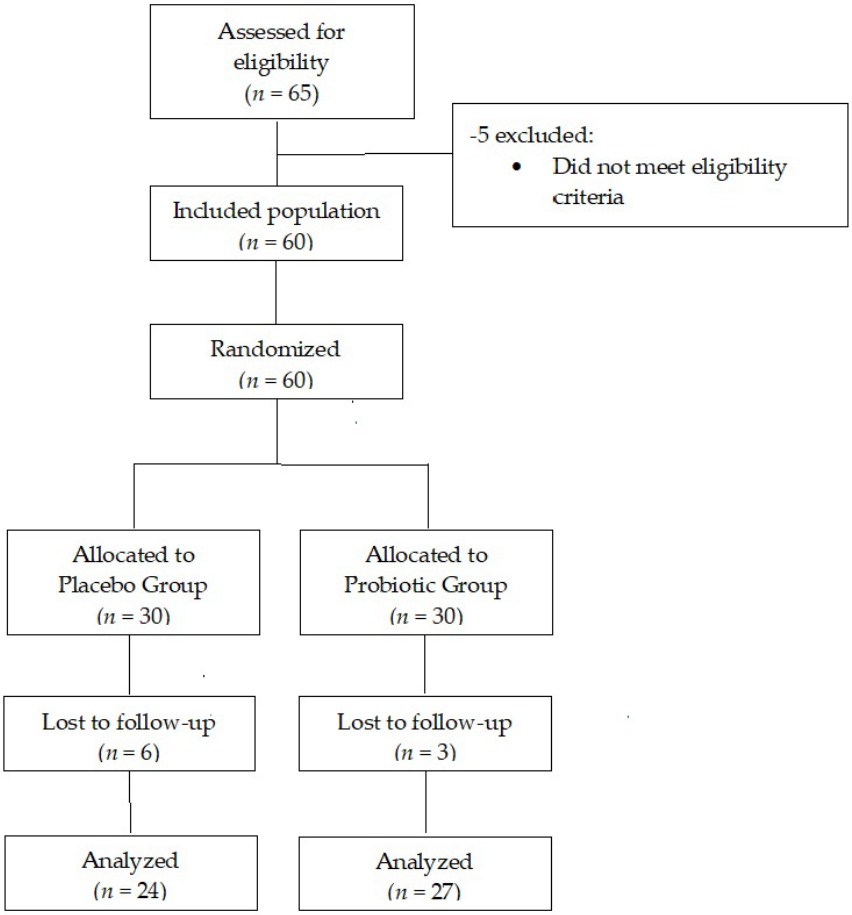


Figure 2. Participant flow.

Description of Study Group: Homogeneity

A homogeneity analysis of the descriptive variables of the population in both groups was conducted to assess the efficacy of the randomisation process for participants (Table 1).

The study groups are considered balanced across all descriptive variables, indicating that randomisation was homogeneous.

Table 1. Baseline demographic data of study participants.

	Placebo (*n* = 30)	**Probiotic (*n* = 30)**	***p*-Value ***
Year	45.3 ± 11.5	47.7 ± 11.5	0.47
Male	48.0 ± 11.4	47.6 ± 10.3	0.93
Female	44.3 ± 11.7	47.9 ± 13.0	0.40
Gender			
Male *n* (%)	8 (27)	16 (53)	0.07
Female *n* (%)	22 (73)	14 (47)	
Height (m)	1.67 ± 0.1	1.70 ± 0.1	0.23
Weight (kg)	67.9 ± 15.5	71.0 ± 15.0	0.24
BMI	24.2 ± 4.2	24.3 ± 3.0	0.67

* *p*-value corresponding to the comparison test of proportions or means between supplementation groups was calculated. For continuous variables, the *t*-test was used, and proportions were compared using the Chi-square test. BMI, Body Mass Index.

3.2. Efficacy Analysis: Primary Variable

The study groups are balanced with respect to GIQLI scores at T0. It can be seen that a normal supposedly "healthy" population is not likely to achieve total GIQLI points of 100% (maximum 144) (Table 2).

Table 2. Mean value, SD and significance of GIQLI scores at T0 for two groups.

	Placebo	**Probiotic**	***p*-Value**
Overall GILQI score	114.6 ± 15.2	112.6 ± 17.5	>0.05
Gastrointestinal symptoms (19 items, range 0–76)	61.8 ± 8.2	61.5 ± 9.7	>0.05
Emotional status (5 items, range 0–20)	15.8 ± 3.1	14.7 ± 3.4	>0.05
Physical condition (7 items, range 0–28)	19.8 ± 3.7	29.4 ± 4.6	>0.05
Social function (5 items, range 0–20)	17.1 ± 2.6	17.0 ± 2.3	>0.05

The scores of GIQLI dimensions over time are reported in Figure 3. The scores increased in different ways in both groups with respect to baseline values. The increases were significant within groups, but not between groups.

Table 3 depicts a gender-wise comparative analysis for the intervention. Both males and females showed significant differences in scores for specific domains at different times compared to baseline. In general, across all domains, scores increase over time in both genders. However, probiotic female scores are significant only in the emotional subdomain, while probiotic male scores are significant at T12 (end of supplementation) in all subdomains except the emotional domain.

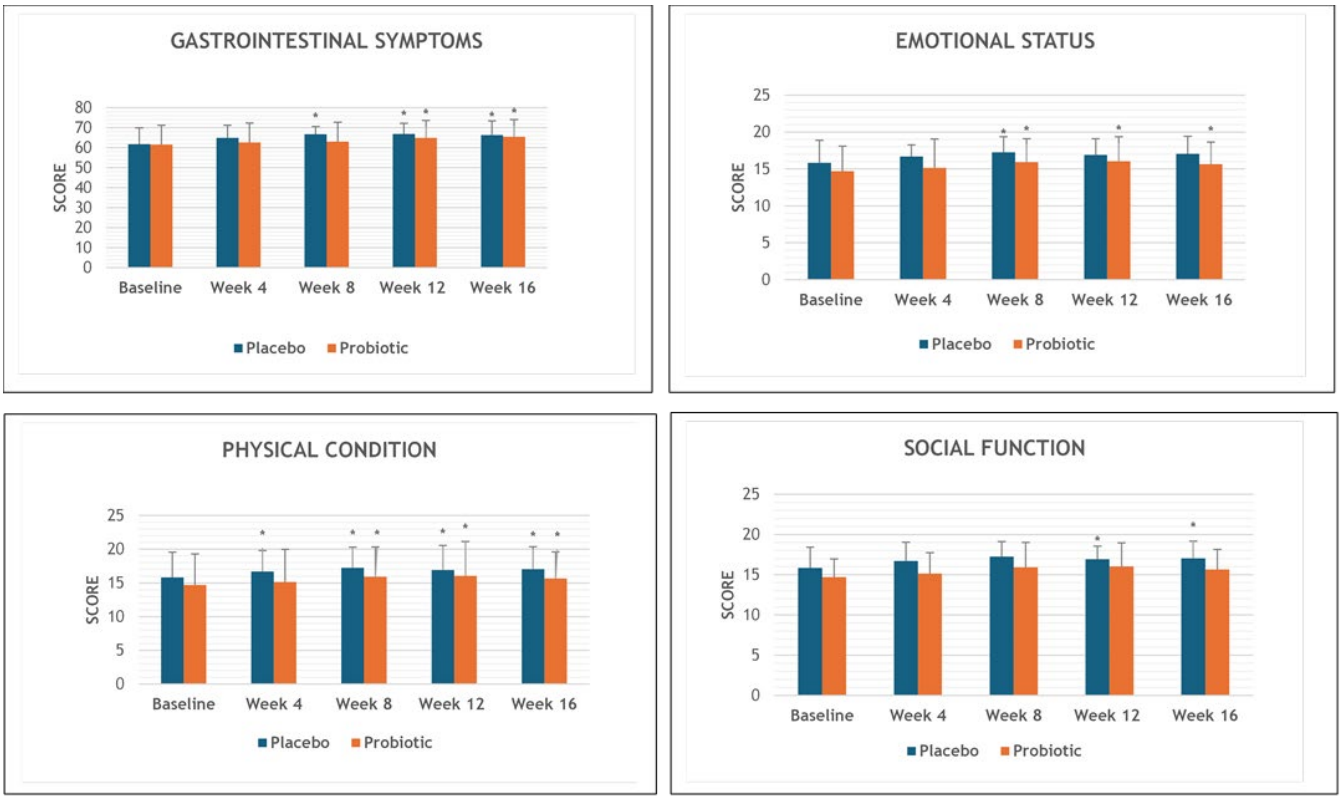


Figure 3. Scores of GIQLI dimensions. The mean values are plotted. Between-group and within-group differences were evaluated using repeated-measures analysis of variance and *t*-tests. * Significant difference compared to baseline. *p*-values < 0.05.

Table 3. Mean values of GIQLI scores categorized by gender.

	Group	Time	Overall GIQLI Score	Gastrointestinal Symptoms	Emotional Status	Physical Condition	Social Function
Male	Placebo	Baseline	118.9 ± 15.4	62.7 ± 9.6	17.0 ± 2.2	20.7 ± 4.1	18.4 ± 1.5
		Week 4	124.6 ± 11.4	65.9 ± 8.8	17.4 ± 1.4	22.6 ± 3.0	18.7 ± 1.1
		Week 8	129.1 ± 6.0	67.1 ± 4.5	18.4 ± 1.3	24.6 ± 2.3 *	19.0 ± 1.5
		Week 12	128.7 ± 6.5	66.9 ± 4.6	18.1 ± 1.3	24.3 ± 2.2 *	19.4 ± 0.8
		Week 16	124.1 ± 15.0	64.7 ± 9.1	17.7 ± 2.3	22.6 ± 3.6	19.1 ± 1.2
	Probiotic	Baseline	123.0 ± 14.9	64.9 ± 7.1	15.5 ± 3.1	19.9 ± 4.2	17.5 ± 2.0
		Week 4	121.9 ± 15.4	63.7 ± 9.9	15.9 ± 3.1	21.4 ± 3.2	18.0 ± 2.0
		Week 8	123.1 ± 13.5	65.5 ± 7.7	17.0 ± 2.2	23.4 ± 2.6 *	17.8 ± 2.3
		Week 12	127.9 ± 16.3 *	67.6 ± 7.2 *	17.2 ± 2.1	23.9 ± 3.3 *	18.4 ± 2.0 *
		Week 16	127.0 ± 15.3 *	68.0 ± 7.9 *	16.4 ± 2.2	22.8 ± 2.9 *	17.6 ± 2.6

Table 3. *Cont.*

	Group	Time	Overall GIQLI Score	Gastrointestinal Symptoms	Emotional Status	Physical Condition	Social Function
Female	Placebo	Baseline	112.8 ± 15.2	61.4 ± 7.8	15.4 ± 3.3	19.5 ± 3.6	16.6 ± 2.8
		Week 4	120.2 ± 9.3	64.5 ± 5.2	16.4 ± 1.5	22.4 ± 3.2 *	16.8 ± 2.5
		Week 8	122.9 ± 8.5 *	66.6 ± 3.7 *	16.8 ± 2.2	22.1 ± 3.1 *	17.5 ± 1.8
		Week 12	124.1 ± 11.3 *	66.9 ± 5.7 *	16.4 ± 2.3	22.6 ± 4.1 *	18.1 ± 1.8 *
		Week 16	124.5 ± 12.4 *	67.0 ± 6.2 *	16.8 ± 2.4	22.8 ± 3.3 *	17.9 ± 2.3
	Probiotic	Baseline	107.0 ± 19.4	57.9 ± 11.1	13.8 ± 3.6	18.8 ± 5.1	16.4 ± 2.5
		Week 4	111.7 ± 22.1	61.4 ± 9.9	14.4 ± 4.7	19.3 ± 6.1	16.6 ± 3.1
		Week 8	112.2 ± 22.1	60.5 ± 11.1	14.8 ± 3.7 *	20.5 ± 5.5	16.4 ± 3.7
		Week 12	113.2 ± 20.7	62.1 ± 9.4	14.8 ± 4.0 *	19.9 ± 6.0	16.5 ± 3.5
		Week 16	114.8 ± 16.9	62.8 ± 8.8	14.9 ± 3.6	20.4 ± 4.6	16.7 ± 2.4

Between-group and within-group differences were evaluated using repeated-measures analysis of variance and *t*-tests. * Significant difference compared to baseline. *p*-values < 0.05.

3.3. Analysis of Secondary Variables

3.3.1. Gastrointestinal Symptoms

The scores of the five syndromes over time are shown in Table 4. The scores decreased in both groups and across all syndromes compared to baseline, even though the values remained within the range 1–2, i.e., in the comfort zone.

Table 4. Gastrointestinal symptoms score according to Gastrointestinal Symptom Rating Scale (GSRS) in two experimental groups at different study times.

Subscales	Placebo					Probiotic				
	Baseline	Week 4	Week 8	Week 12	Week 16	Baseline	Week 4	Week 8	Week 12	Week 16
Abdominal pain	2.0 ± 1.1	1.6 ± 0.7 *	1.4 ± 0.5 *	1.4 ± 0.7 *	1.4 ± 0.9 *	1.9 ± 0.9	1.4 ± 0.7 *	1.6 ± 0.7 *	1.6 ± 0.7 *	1.5 ± 0.7 *
Reflux Syndrome	1.5 ± 1.0	1.3 ± 0.9	1.2 ± 0.3	1.2 ± 0.4	1.5 ± 1.0	1.5 ± 1.0	1.4 ± 0.7	1.6 ± 1.0	1.4 ± 0.9	1.4 ± 0.7
Diarrhoea	1.9 ± 1.2	1.8 ± 1.0	1.4 ± 0.5 *	1.5 ± 0.6	1.4 ± 0.8 *	1.7 ± 0.7	1.7 ± 1.1	1.5 ± 0.6 *	1.4 ± 0.6 *	1.4 ± 0.7 *
Indigestion Syndrome	2.3 ± 1.0	1.8 ± 0.6 *	1.5 ± 0.5 *	1.5 ± 0.4 *	1.7 ± 0.8 *	2.0 ± 0.9	2.0 ± 1.1	1.9 ± 1.0	1.9 ± 1.1	1.7 ± 0.6 *
Constipation	2.1 ± 1.0	2.0 ± 0.9	1.8 ± 0.8	1.7 ± 0.7 *	1.7 ± 1.0 *	2.0 ± 1.2	1.9 ± 1.0	1.9 ± 1.0	1.9 ± 1.2	1.8 ± 0.8

Between-group and within-group differences were evaluated using repeated-measures analysis of variance and *t*-tests. * Significant difference compared to baseline. *p*-values < 0.05.

3.3.2. Quality of Life

The comparison of the overall index of well-being (GWBI) between the groups did not show significant differences. Both groups obtained scores within the no-stress range (70–90). The comparison for group analysis showed that the overall index increased in both groups during the intervention period, with the total score falling again within the no-stress areas. The subscale scores of the PGWBI over time showed significant results only in the placebo group for positive well-being and vitality (Table 5).

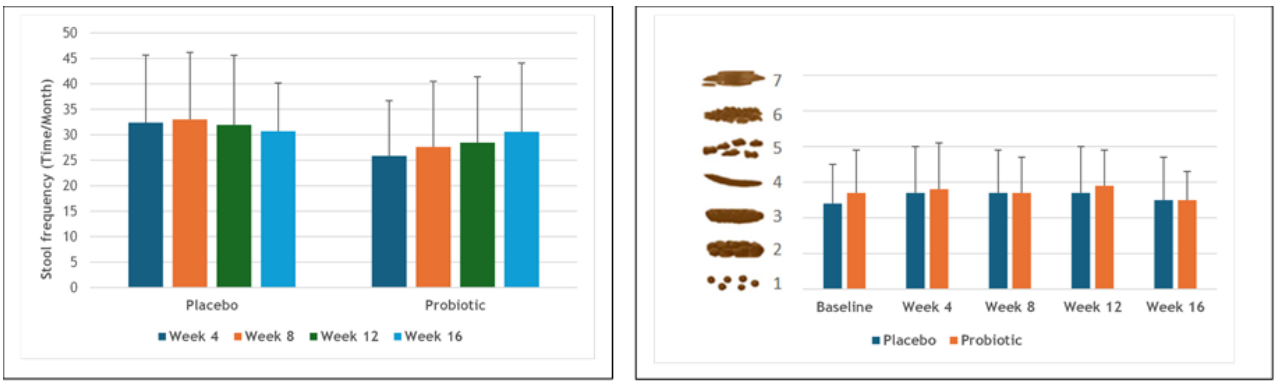
3.3.3. Stool Frequency and Bristol Scores of Stool Characteristics

Weekly stool frequency was recorded. Stool characteristics were evaluated using the Bristol Stool Form Scale. On average, study participants had 25 to 33 bowel movements per month and had normal stool consistency (BSS score 3 or 4). The change in the number of bowel movements over time was not significantly different. The number of bowel movements increased in subsequent months in the probiotic group, but the increase was not significant. The change in stool consistency over time was also not significantly different (Figure 4).

Table 5. PGWBI subscales scores in two experimental groups at different time points.

Subscales	Placebo					Probiotic				
	Baseline	Week 4	Week 8	Week 12	Week 16	Baseline	Week 4	Week 8	Week 12	Week 16
Anxiety	17.4 ± 4.3	18.8 ± 3.4	18.8 ± 3.4	18.3 ± 3.5	19.1 ± 2.6	16.6 ± 5.9	18.0 ± 4.9	17.7 ± 4.3	18.4 ± 4.6	17.6 ± 4.7
Depression	16.6 ± 1.4	13.8 ± 0.9	14.0 ± 1.0	13.9 ± 1.0	14.0 ± 1.0	12.7 ± 1.9	13.2 ± 1.5	13.3 ± 2.0	13.3 ± 2.3	13.1 ± 2.3
Positive Well-being	13.3 ± 3.3	15.4 ± 3.2 *	14.7 ± 2.9 *	14.5 ± 3.5 *	14.8 ± 2.9	12.8 ± 3.7	13.4 ± 3.4	13.4 ± 3.5	13.6 ± 3.8	13.0 ± 3.9
Self-control	12.0 ± 2.6	12.7 ± 2.1	13.0 ± 1.4	12.8 ± 2.1	12.9 ± 2.1	11.5 ± 2.9	12.1 ± 2.4	12.1 ± 2.2	12.4 ± 3.0	11.7 ± 3.3
General health	12.6 ± 1.7	12.6 ± 2.1	12.6 ± 2.1	12.7 ± 1.9	12.9 ± 1.7	11.8 ± 2.0	12.1 ± 1.9	12.1 ± 2.0	12.2 ± 2.0	12.2 ± 2.0
Vitality	12.4 ± 3.5	13.7 ± 3.6 *	15.0 ± 2.7 *	14.8 ± 2.7 *	14.6 ± 3.0 *	12.9 ± 3.9	13.6 ± 3.1	13.8 ± 2.9	13.6 ± 3.5	13.5 ± 3.3

Between-group and within-group differences were evaluated using repeated-measures analysis of variance and *t*-tests. * Significant difference compared to baseline. *p*-values < 0.05.



(**a**) (**b**)

Figure 4. Monthly stool frequency and Bristol scores of stool characteristics. (**a**) Stool frequency. (**b**) Bristol scores of stool consistency. Mean values are plotted.

3.3.4. Abdominal Discomfort

Each week, participants were required to record any abdominal discomfort over the last 7 days in the diary. The degree of this discomfort was ranked on a 5-point scale ranging from significantly relieved (score: 1) to significantly worsened (score: 5). The volunteers did not present any abdominal discomfort problems at the beginning of the study, and there were no significant changes from baseline and between study groups for ratings of discomfort.

3.3.5. Faecal Microbiota

To characterise the effects of probiotic supplementation on the gut microbiota of participants, V3–V4 regions of the 16S *rRNA* genes were sequenced using the Illumina MiSeq platform. The gut microbiota composition of all samples in placebo and probiotic groups at three time points, T0, T90 days and T120 days, was analysed. Shannon, Simpson, Chao1 and Adv indices were used to evaluate the change in alpha diversity before and after the probiotic/placebo was consumed. Then, the overall impact of the probiotic intervention on the macro-diversity of participants' gut microbiota was assessed by analysing Shannon and Simpson diversity indices. Initially, no significant differences were observed between the two groups on day 0, indicating stable gut microbiota diversity before the intervention. Following the administration of PROBAFLOR, Shannon and Simpson diversity indices showed a significant increase at day 90 ($p < 0.05$; Figure 5), demonstrating that the intervention effectively enhanced gut microbiota diversity. However, this increase in alpha diversity was transient, as it remained stable thereafter, suggesting that while 3 months of probiotic intake can enhance diversity, it may not be sufficient to induce persistent changes within the complex gut ecosystem. The Bray–Curtis distance used to reveal beta diversity, that is,

bacterial structural differences before and after the intervention with probiotics, did not show a congruent directional pattern between the two groups.

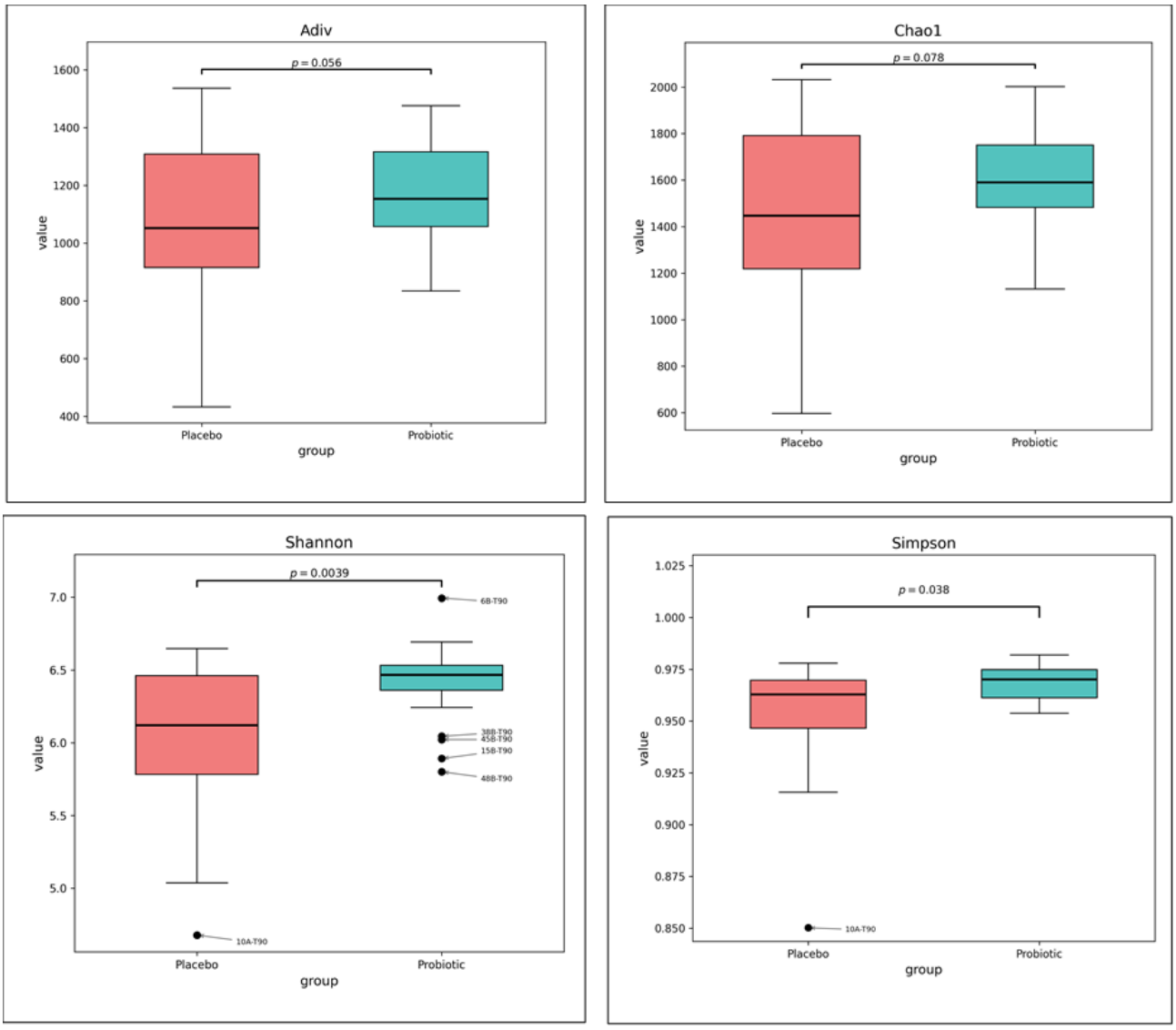


Figure 5. Comparison of alpha diversity indices Adv, Chao1, Shannon and Simpson in faecal samples collected at T90 days in subjects in the Placebo and Probiotic groups. Statistical differences between groups were assessed, with $p < 0.05$ considered significant.

To identify differences in taxonomic levels between the two groups, changes in the relative abundance of the taxonomic groups after the intervention were compared. At the phylum level, about 99% of the sequences belonged to the five most abundant bacterial phyla in both groups, namely Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, and Actinobacteria (Figure 6A). In both the placebo and probiotic groups, there was a reduction in the abundance of the phylum Firmicutes and an increase in Bacteroidetes' abundance at the end of the intervention period (T90). This situation was also maintained at the follow-up (T120). Moreover, a decreasing trend in the relative abundance of Proteobacteria was documented in the probiotic group at T90 and T120 with respect to T0 and when compared to the placebo group.

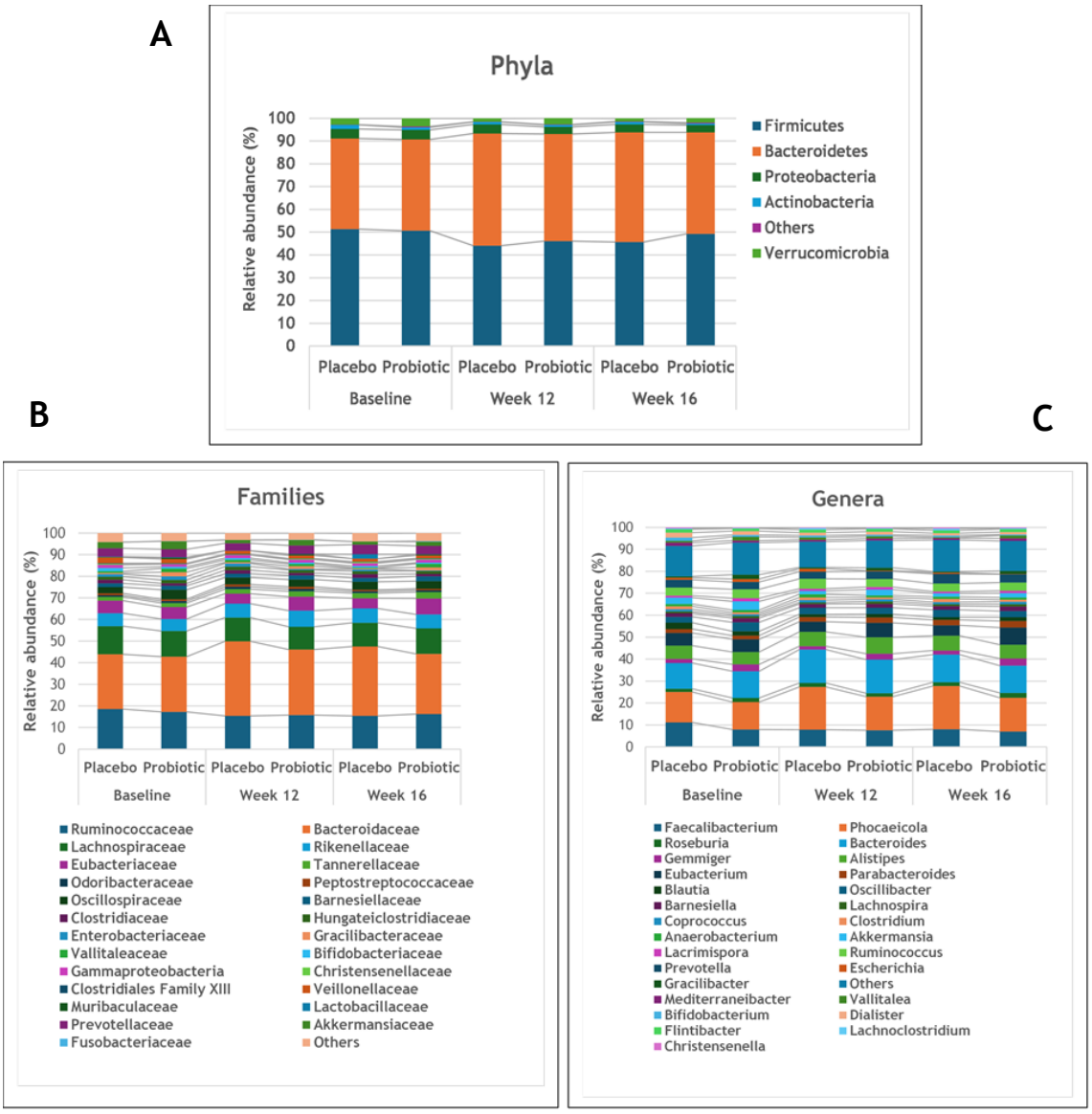


Figure 6. A histogram of the relative abundance of the phyla (**A**), families (**B**), and genera (**C**) found. The *x*-axis represents sampling times and respective groups, and the *y*-axis represents the relative abundance.

At the family and genus level, 25 families and 36 genera were found to be at both time points (relative abundance of >1%). We observed an increase in the abundance of the Eubacteriaceae family in the probiotic group at the end of the intervention (Week 12), an increase that persisted at the end of the follow-up (Week 16) (Figure 6B). The same trend was also observed for the Barnesiellaceae family (genus *Coprobacter*). At Week 12, we also observed an increase in the Rikenellaceae family, which returned to baseline levels following cessation of the probiotic intervention. The results demonstrate a gradually higher relative abundance of Ruminococcaceae within the probiotic group over the study period, while the placebo group showed a gradual decrease in Ruminococcaceae.

Results from an ad hoc analysis, performed to identify taxa that showed variation after the intervention, revealed that probiotic product administration appeared to enhance some genera (Figure 6C). The positive variations observed after the intervention were maintained throughout the follow-up period. Among the taxa enhanced by probiotic intake after 12 and 16 weeks, an increase in *Lachnospira* and *Eubacterium*, members of the Lachnospiraceae and Eubacteriaceae families of the Firmicutes phylum, was evident.

3.3.6. SCFA Profile

Acetate was the major SCFA detected in both groups. Following both placebo and probiotic administration, there was a consistent pattern of increased total and individual faecal SCFA levels in the probiotic group compared to the placebo at Week 12 (end of administration), which did not reach significance. Analysing the change in total SCFAs, acetate, propionate and butyrate, following placebo and probiotic intervention, a significant difference was found between the two supplementations in acetate, propionate and total SCFAs, with a decrease in the concentration of all SCFAs in the placebo group versus an increase in the probiotic group (Figure 7).

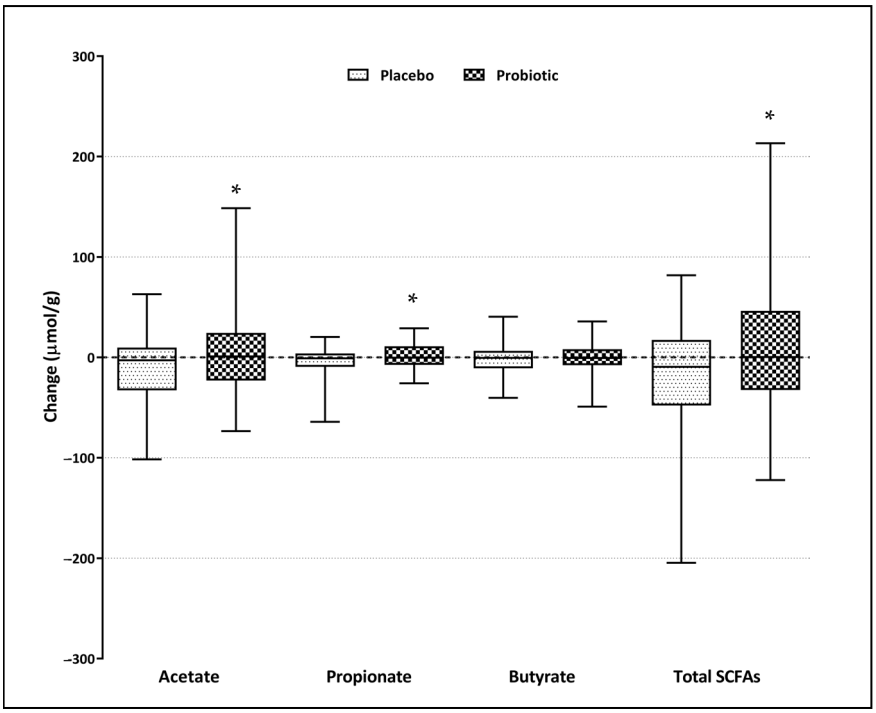


Figure 7. Changes in total SCFAs, acetate, propionate and butyrate in the placebo and probiotic groups after the intervention (Week 12). Data are reported as box and whisker plots, where the box represents the median (IQR) and the whiskers represent the range. * A significant difference compared to placebo. *p*-values < 0.05.

3.3.7. Intervention/Supplementation Adherence

The percentage of participants who adhered to the intervention was calculated by counting the number of capsules returned at week 12. The overall mean adherence in the 51 volunteers analysed was over 95% in both supplementation groups (99.2% in the probiotic group and 98.5% in the placebo group), which is considered an excellent rate of compliance by the participants.

3.3.8. Safety Analysis

All adverse events (AEs) reported by the 51 enrolled participants in the diary were collected. In total, five AEs were registered, but only three were attributed to the study product (possible/probable relationship)—two in the probiotic group and one in the placebo group—all affecting the digestive system (abdominal swelling).

4. Discussion

This study evaluated the ability of a commercially available multispecies probiotic formulation to maintain and/or improve general well-being in a healthy population. Con-

sistent with the prevalence of GI symptoms among the general population, there remains a need to study the effects of probiotics on GI symptoms in healthy participants [24], although this is quite challenging because no biomarkers exist and they rely solely on patient-reported symptoms. Moreover, it is currently not clear which outcomes are most valid for use. Clinical trials are further complicated by a potentially very high placebo response rate, and studying healthy subjects with mild GI symptoms entails the additional difficulty of measuring improvements in already low symptom scores [25–27]. There is an increasing tendency in the market to work with multistrain probiotics, in particular, products with a high number of different strains. Multistrain probiotics might be more helpful because of synergy and additive effects among the individual isolates [28,29]. Multistrain probiotic applications include alleviation of disease conditions [30,31], inhibition of pathogens [32] and restoration of the gastrointestinal microbiome [33]. So, for general daily maintenance in a healthy person, multispecies probiotic could be considered favourable due to their broader and synergistic approach.

Regarding the primary efficacy variable of this study, the overall mean GIQLI scores increased in both groups over time. The increases were significant within groups but not between groups. In the sample size estimation, we assumed that an increase of 6 to 8 points on the GIQLI scale was clinically relevant [23]. This result was achieved in both groups but, clearly, the primary endpoint was not achieved compared to placebo. It is important to keep in mind that in a clinical study involving healthy people, placebo response can be high, potentially leading to altered outcomes reported by the volunteer, and the power calculation for the current study may have underestimated this response. Focusing on a gender-comparative analysis, although there was a general increase in scores across all domains for both genders over time, the statistical significance is well established. In fact, a significant increase in the scores of all domains (except for emotional status) was highlighted among males of the probiotic group, both at the end of the supplementation period and after the follow-up compared to the beginning of the study. Conversely, females in the probiotic group showed a statistically significant increase in the emotional status domain only, compared to baseline, both after 8 weeks and at the end of the intervention. This is in line with other research that has highlighted how gender differences could be attributable to the way men and women report their health status [34]. Other researchers have also found differences between men and women in different situations, even those related to gastrointestinal problems. The fact that women more frequently report a worse health status than men is a common finding; indeed, we also found that before the intervention women had worse scores than men in all GIQLI domains [35].

Regarding the secondary efficacy variables of this study, no statistically significant differences were observed between the study groups in the GSRS and PGWBI results, both of which improved in both groups after the intervention. From this, we can deduce first of all that the multispecies probiotic formulation was well tolerated by study participants and also that daily consumption of the probiotic, assessed via scheduled questionnaire completion and behavioural analysis to identify any improvement in overall well-being, had positive impacts on mood, irrespective of whether the probiotic or placebo was consumed [36]. Probiotics have been extensively studied for their ability to modify the gut microbiota, and despite the uncertainty surrounding probiotics' effects on the gut microbiome in healthy individuals, these supplements are often recommended to promote overall gut health and well-being [37]. Gut microbial diversity and abundance are key determinants of host health, and a decrease in gut microbial diversity has been linked to intestinal dysbiosis in several human diseases [38]. Following the administration of PROBAFLOR, Shannon and Simpson diversity indices showed a significant increase at week 12 ($p < 0.05$), demonstrating that the intervention enhanced gut microbiota diversity.

At the phylum level, the decreasing trend in the relative abundance of Proteobacteria in the probiotic group is interesting, mainly because members of the phylum Proteobacteria have a low abundance in the gut of healthy humans, and Proteobacterial load is suggested as a potential diagnostic criterion for dysbiosis and disease [39]. At the family and genus level, an increase in the abundance of the Barnesiellaceae family (genus *Coprobacter*) was observed. In the gut microbiome, this taxon plays a protective role in moderating inflammation and gut permeability. The genus *Coprobacter* produces propionic and acetic acids [40]. Both propionic [41] and acetic acids [42] also exhibit protective effects on the intestinal barrier. Consequently, these taxa may contribute to gut integrity in individuals with high environmental sensitivity.

A gradually higher relative abundance of Ruminococcaceae within the probiotic group over the study period was recorded, while the placebo group showed a gradual decrease in Ruminococcaceae. The Ruminococcaceae family is commonly associated with *Clostridium* clusters IV/XIVa, essential bacteria that produce SCFAs. Further, Ruminococcaceae abundance has been shown to be positively correlated with Bristol stool scale scores and faster intestinal transit [43]. Based on this, we might hypothesize that the increase in Ruminococcaceae abundance in the probiotic group could be one of the factors that contributed to the gradual increase observed over time in the number of bowel movements registered in the probiotic group. Obviously, this is speculation that will need to be confirmed by correlation studies. Two of the bacterial strains present in the formulation (BLC1 and BGP93) were included in another study with the aim of evaluating the efficacy of a multistrain probiotic product on functional constipation in elderly people living in a nursing home [44]. Again, the results showed a gradual increase in stool frequency after probiotic treatment. Moreover, in the probiotic group, they observed an increase in the two genera *Lachnospira* and *Eubacterium*, members of the Lachnospiraceae and Eubacteriaceae families of the Firmicutes phylum, which are very well known to be SCFA producers. The present results show that the probiotic cocktail containing 11 strains can modulate the composition of the intestinal microbiota towards SCFA-producing species. In fact, a significant change was registered in the concentration of acetic, propionic and total SCFAs in the probiotic group compared to placebo after the intervention. SCFAs are the primary energy substrate for colonocytes, synthesised in the gut, by gut microbes that ferment dietary fibres [45,46]. SCFAs play crucial roles in maintaining gut homeostasis and exerting systemic effects beyond the gastrointestinal tract [47–49]. The most common SCFAs which the intestinal tract can absorb are acetate, butyrate, and propionate present in the faecal matter in a molar ratio of 3:1:1 (acetate/propionate/butyrate). The balance of these SCFAs is crucial for maintaining a healthy gut microbiome, systemic metabolic functions, and immune homeostasis.

This study has some limitations. First of all, the very high placebo response rates observed are considered a limitation, although similar placebo effects have previously been reported in both healthy subjects and IBS patients [50]. In fact, this study may have been statistically underpowered to detect clinical differences. Conducting clinical studies in populations with minor gastrointestinal symptoms, as in the present study, is not only challenged by the underlying heterogeneity but also by fluctuations in symptom presentation, which tends to regress towards the mean during the course of a clinical study. In addition, efficacy measures still rely on patient-rated outcomes. The use of self-reported questionnaires is vulnerable to individual subjective perceptions, which may have been influenced by their psychological beliefs and viewpoints regarding the intervention. Secondly, no correlation analysis was performed because the primary objective of this study was to establish the efficacy of probiotic treatment, compared to placebo, in maintaining and/or improving the well-being of healthy subjects. The focus was therefore on the difference between groups, not the correlation. Future research is needed, particularly in individ-

uals with GI symptoms, to determine whether the increased microbiome diversity and increased beneficial metabolites demonstrated in this study lead to clinical improvements in GI outcomes.

5. Conclusions

In conclusion, our exploratory study suggests that once-daily consumption for 12 weeks of a specifically designed multispecies probiotic product (PROBAFLOR) regulates the balance of gut microbiota and seems to be beneficial for gut health in healthy people, improving defecation habits and satisfaction, normalizing stool frequency and promoting the production of beneficial bacterial metabolites (SCFAs). The presumed correlations between the observed balance of the intestinal microbiota and a significant increase in positive metabolites (SCFAs) with intestinal health will need to be confirmed by further study with an adequate number of subjects to mask the high placebo effect typical of studies on healthy subjects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals6020022/s1, Table S1: CONSORT 2025 checklist of information to include when reporting a randomised trial [51].

Author Contributions: Conceptualization, M.C.V. and M.M.C.; methodology, M.C.V., M.M.C., F.M.C. and S.S.; validation, M.M.C. and S.S.; formal analysis, M.C.V., M.M.C., S.S. and F.M.C.; data curation, M.C.V. and F.M.C.; supervision, S.S. and F.M.C.; writing—original draft preparation, M.C.V.; writing—review and editing, M.C.V., M.M.C., F.M.C. and S.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: The data presented in this study are only available on request from the corresponding author due to legal restrictions.

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Abbreviations

The following abbreviations are used in this manuscript:

SCFAs	Short-Chain Fatty Acids
GIQLI	Gastrointestinal Quality of Life Index
PGWBI	Psychological General Well-Being Index
GSRS	Gastrointestinal Symptom Rating Scale
QOL	Quality of life
BSS	Bristol Stool Scale

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