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Randomized Control Trials

Supplementation with *Bifidobacterium breve* BR03 and B632 strains improved insulin sensitivity in children and adolescents with obesity in a cross-over, randomized double-blind placebo-controlled trial

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SUMMARY

Background & aims: Variations in gut microbiota might impact metabolism leading to body weight excess. We assessed the impact of a probiotic supplementation in pediatric obesity on weight, metabolic alterations, selected gut microbial groups, and functionality.
Methods: Cross-over, double-blind, randomized control trial (BIFI-OBESE trial; NCT03261466). 101 youths (6–18 years, Tanner stage ≥2) with obesity and insulin-resistance on diet were randomized to 2×10^9 CFU/AFU/day of *Bifidobacterium breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) (51) or placebo (50) for 8 weeks with a 4-weeks wash-out period.
Results: All subjects (M/F 54/47) completed the first 8 weeks, and 82 (M/F 43/39) the last part without adverse events. Mixed-effects models revealed a carry-over effect on many variables in the entire study, narrowing the analysis to the first 8 weeks before the wash-out periods. All subjects improved metabolic parameters, and decreased weight and *Escherichia coli* counts. Probiotics improved insulin sensitivity at fasting (QUICKI, 0.013 CI95%0.0–0.03) and during OGTT (ISI, 0.654 CI95%-0.11–1.41). Cytokines, GLP1, and target microbial counts did not vary. Of 25 SCFAs, acetic acid and acetic acid pentyl-ester relative abundance remained stable in the probiotics, while increased in the placebo ($p < 0.02$). A signature of five butanoic esters identified three clusters, one of them had better glucose responses during probiotics.
Conclusion: An 8 weeks treatment with *B. breve* BR03 and B632 had beneficial effects on insulin sensitivity in youths with obesity. Microbiota functionality could influence metabolic answers to probiotics. Long-term studies to confirm and enrich our findings are justified. Tailored probiotic treatments could be an additional strategy for obesity.
Trial registration: NCT03261466.

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1. Introduction

Obesity is one of the most serious public health challenges associated with multiple and severe comorbidities such as metabolic, cardiovascular, respiratory disorders and cancers. Globally, the prevalence of obesity has rapidly increased, affecting in 2017 603.7 million of adults and 107.7 million of children [1]. Besides genetic, behavioral, and environmental factors, gut microbiota has been proposed to be another cause of obesity [2]. Variations in gut microbiota and their end-products seem to have an important impact on the caloric intake and energy expenditure, altering the set up to extract calories from the daily food intake, and modulating the gut entero-immune-endocrine system, acting on insulin sensitivity, glucose production, lipogenesis, fatty acid oxidation, then leading to body weight excess [2,3].

Metagenomics studies revealed a large variability among individuals in terms of gut microbiota composition [4,5]. The link between obesity and the microbiota is likely to be more sophisticated than the simple phylum-level Bacteroidetes:Firmicutes ratio that was initially identified. Individuals with obesity, including children, present a different microbiota composition with decreased biodiversity and altered proportion of specific populations such as reduced levels of *Bifidobacterium* and increased levels of *Prevotella*. The complexity of microbiota–diet interactions is accentuated by drugs altering gut homeostasis, including antibiotics, indicated as players in determining critical changes in infant gut microbiota resulting in overweight later in life [6].

Since intestinal dysbiosis following unhealthy diets has been associated with obesity and its comorbidities, a probiotic supplementation could be an approach for these conditions. At present, few randomized clinical trials have been conducted in obese adults [7], and even less in pediatric patients [8]. These studies used foods fermented with selected bacterial strains (as yogurt) or single/mixed strain formulations of *Lactobacillus* and *Bifidobacterium* [7–9], which are considered beneficial bacteria for the human health.

Bifidobacteria are present in abundance in the intestine of newborns due to the ability to digest specific oligosaccharides present in the human milk. Their annihilation by early antibiotic therapy in the first months or years of life has been hypothesized to be one of the factors implicated in the pediatric obesity onset. Within this microbial group, *Bifidobacterium breve* has been largely investigated for its beneficial effects especially in pediatric subjects [10,11]. Interestingly, several findings, mainly in animal models, evidenced a potential anti-obesity property associated to this species [12–15]. For this study, we selected *B. breve* BR03 and *B. breve* B632 because they present an anti-inflammatory activity, capability of colonizing the human intestine and protect the gut epithelium integrity, of stimulating the immune response and competing against pathogens including some *Escherichia coli* strains, an action of amensalism implicated in the protection from obesity, inflammation, and related diseases [13,16]. In particular, we evaluated the efficacy of the selected dose of these probiotics to improve glucose and insulin homeostasis, reduce chronic inflammation and modulate gut microbiota composition and SCFA production at the start of a weight-loss nutritional program.

2. Research design and methods

2.1. Study design and participants

This was a randomized, double-blind, placebo-controlled, crossover trial (Ethical Committee protocol 165/13; NCT03261466). The second part was designed as a sub-study (Supplemental Fig. 1). The protocol was conducted in accordance with the declaration of Helsinki. Informed consent was obtained from all parents and children prior to the evaluations after careful explanations to each one. Subjects were recruited from November 2013 to August 2017.

Eligible patients were between 6 and 18 years of age, obese, with HOMA-IR > 2.5 or insulin >15 μU/ml and pubertal stage ≥2. Exclusion criteria were genetic or secondary obesity; chronic diseases; medical treatment for chronic diseases; probiotic, prebiotic or antibiotic therapy during the previous 3 months; any adverse reaction to the product or components of the product.

Patients were randomized into two groups. One group received a supplementation with probiotic sachets containing $> 2 \times 10^9$ CFU/AFU/day *B. breve* B632 (DSM 24706) and *B. breve* BR03 (DSM 16604) and the other group a placebo (PLC) formulation, containing the same excipients without the *Bifidobacterium* strains, for 8 weeks (T0, T1). Probiotic (PRB) formulation was a 1:1 mixture of the 2 strains.

All participants were educated to an isocaloric Mediterranean diet according to basal metabolism. They received general physical activity recommendations: 30–60 min of daily aerobic exercise and practicing sport at least 1–2 times per week. At the end of the first 8 weeks, after a wash-out period of 4 weeks (T3) patients could continue the study or stopped it. Then, each patient crossed over to the other treatment for the next 8 weeks (T4). Dietary and physical activity recommendations were once more delivered to all.

2.2. Randomization and masking

Patients were randomly assigned (Lehmer random generator), in a 1:1 ratio, to receive PLC or PRB. PLC was prepared with the same excipients (maltodextrin) without PRB strains using the same package. Both participants and investigators were blinded to the treatment group. Randomization procedures were performed by an investigator not involved in the clinical part. The study personnel would have been unblinded in case of any adverse event.

2.3. Clinical and adherence monitoring

All the analyses were performed at the recruitment (T0), after the first 8 weeks of treatment (T1), at the beginning of the second phase (T2) and after the last 8 weeks of treatment (T3). Case report forms were completed. Patients were asked to report any adverse reaction, antibiotic therapy or other drug administration occurred. Stool characteristics were assessed using the Bristol Stool Scale. The adherence was monitored counting the returned sachets.

2.4. Anthropometric measurements

The following evaluations were performed: height, weight, waist circumference, Tanner stage, and arterial blood pressure.

2.5. Sample collections

Blood samples were collected after 12 h of night fasting to assess: total cholesterol, HDL cholesterol, triglycerides; liver enzymes; IL6, IL10, TNF-α; LPS; GLP1. LDL-cholesterol was calculated (Friedwald formula). Subjects also underwent an oral glucose tolerance test (OGTT; 1.75 g of glucose solution per kg, maximum 75 g) with measurement of glucose and insulin at fasting and every 30 min.

For the assessment of insulin resistance, the functionality of pancreatic beta cells, and the insulin-sensitivity the HOMA-IR, HOMA-B, QUICKI and the Matsuda Insulin Sensitivity Index (ISI) were calculated [17].

2.6. DNA extraction and quantitative PCR (qPCR)

Stool samples of the same morning or the day before were collected and preserved at −80 °C.

DNA was extracted from 200 mg of faeces using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) with a slight modification of the standard protocol [18].

Quantification of selected microbial groups, i.e. *Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis* group (comprising *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*), *B. breve* and *E. coli* was performed with qPCR on DNA extracted from faeces. Primers and PCR conditions have been described elsewhere [13].

2.7. Analysis of fecal SCFAs

SCFAs of fecal samples were detected through solid-phase microextraction–gas chromatography–mass spectrometry (SPME/GC–MS) analysis according to published protocols [19].

2.8. Outcomes

Our primary objective was to evaluate if the selected doses of *Bifidobacterium* strains improve glucose metabolism (fasting or glucose stimulated secretion, or insulin-resistance/sensitivity) during a diet-weight loss treatment.

Secondary endpoints include changes in gut microbiota composition and stool SCFA abundance.

To investigate the persistency of the probiotic effects after stopping the administration, we crossed the treatments after 4 weeks of washout.

2.9. Statistical analysis

A sample of 34 individuals was estimated enough to demonstrate a difference of 10 mg/dl in the basal glucose concentration or 1.4 points in HOMA-IR or a reduction of BMISDS $\geq$10% with 90% power and a significance level of 95% after 8 weeks of treatment [20]. The total sample of each group was increased to 50 subjects by considering a drop-out rate of 20%.

The success of the randomization procedure for each variable was evaluated through Wilcoxon or $\chi 2$ tests.

To display the relationships between treatment, dietary training and each variable in the crossover design a mixed-effects linear regression framework was employed. Gender, age, and pubertal stage were considered the major confounding variables. A log-Likelihood Ratio Test (LRT) was performed to compare the likelihood of the model with the likelihood of the nested one (p-value < 0.1). Since the carry-over interactions were found as statistically significant for many variables (see Results), a simpler mixed-effects regression model framework was estimated only in the first period (T0-T1). The same procedures were performed for SCFA concentrations or when SCFA profile clusters were introduced.

A compositional approach was used for the analysis of SCFAs, by using Centered Log-ratio (CLR) values. A PCA dimensionality reduction was performed using the baseline SCFA abundances, and a hierarchical clustering procedure was done at T0. To characterize each cluster SCFA profile, a sPLS-DA analysis was performed (mixOmics R package).

For all analyses, 2-sided 95% Ci and p-values were calculated. P-values of less than 0.05 were considered statistically significant. All the analyses were performed using R.

2.10. Further information

More details on diet, anthropometrics, biochemical, qPCR and fecal SCFA analyses, and statistical methods are available in Supplement 1.

3. Results

We recruited 101 individuals (M/F 54/47). The enrollment was from November 2013 to August 2017; the follow-up ended in December 2017. All of them completed the first part (T0-T1) and 82 (M/F 43/39) the second part of the study (T2-T3; Supplemental Fig. 1).

No adverse events were reported in any part of the study. Stool consistency and frequency were similar in any arm for all the time (data not shown). The compliance was high; only one patient returned 9 PLC sachets.

Baseline characteristics were similar between groups apart from *E. coli* spp. counts that were higher in subjects allocated to PLC (Table 1). Three patients had impaired fasting glucose, and 9 impaired glucose tolerance, and 2 both conditions. Nobody had type 2 diabetes. Moreover, 48 subjects had hypertension.

3.1. Carry over effect in the cross over study

Several mixed-effects model analyses (Fig. 1) were performed: for the clinical, metabolic, and microbial data of the patients on the entire crossover (T0-T3; Fig. 1a), for the first period of it (T0-T1; Fig. 1b), and for the SCFA concentrations on a subset of 45 patients for the first period of the crossover too (T0-T1; Fig. 1c). The first model revealed a carry-over effect (from the statistically significant interactions between the crossover arm and the treatment type, which is the first order carry over, and also by both of them with the time point, which is the second order carry over) on most of the variables (BMI, BMISDS, WC, blood pressure, fasting glucose and insulin levels, post-OGTT glucose levels, HOMA-B, HOMA-IR, ISI, QUICKI, triglycerides, liver enzymes, HDL and LDL cholesterol, IL10, TNF-α, and LPS), suggesting that it was not possible to split the effect of the PRB from the dietary training using the entire crossover (from T0 to T3) because there no way to re-establish the starting conditions. That is why only the results of the first phase of 8 weeks (T0-T1) were more deeply analyzed (Fig. 1b).

3.2. Clinical, metabolic, and microbial data after the first 8 weeks of treatment

For all the subjects, independently from the arm, we observed a significant decrease in BMI, BMISDS, WC, systolic and diastolic blood pressure, HOMA-IR ($p < 0.01$), insulin after OGTT ($p = 0.02$), and *E. Coli* spp. counts ($p = 0.03$) at T1 compared to T0. Post-OGTT glucose ($p = 0.09$) levels and IL6 concentration ($p = 0.08$) decreased, and TNF-α ($p = 0.07$) and HDL cholesterol levels ($p = 0.09$) increased nearly to significance.

The randomization was successful, even though age, puberty, and patient effects were observed for most variables (Fig. 1b); indeed, after cleaning from all these effects and considered time point, PRB cohort patient mean values were lower than the PLC ones in WC (−3.41 cm, $p < 0.05$), BMISDS (−0.17 kg/m^2, $p = 0.07$), fasting insulin (−4.57 mcUI/ml, $p = 0.06$), ALT (−3.64, $p < 0.05$), and *E. coli* concentrations (−0.4 LogCFU/g of feces, $p < 0.02$). No differences were observed in inflammatory cytokines and GLP1 levels at fasting.

Interestingly, the interaction between PRB treatment with time was statistically significant, for the QUICKI (0.013, $p = 0.05$) and ISI (0.654, $p = 0.097$) indexes after OGTT (Supplemental Fig. 2).

Table 1
Clinical and metabolic characteristics of the probiotic (PRB) and placebo (PLC) groups at T0. Proportion is reported for categorical variables while average values, standard deviations, and number of patients per group is reported for numerical variables. Chi-square test p-values are reported for categorical variables while Wilcoxon Rank Sum test p-values are reported for numerical ones.

Variable	PRB, N = 51[a]	PLC, N = 50[a]	p-value[b]
Gender			>0.9
Male	28 (55%)	26 (52%)	
Female	23 (45%)	24 (48%)	
Puberty			>0.9
pubertal	35 (69%)	34 (68%)	
pre-pubertal	16 (31%)	16 (32%)	
Age	12.07 (2.57) [n = 51]	11.76 (2.86) [n = 50]	0.7
BMI	30.5 (4.7) [n = 51]	30.9 (5.4) [n = 50]	0.8
BMISDS	2.32 (0.48) [n = 51]	2.43 (0.54) [n = 50]	0.4
Waist	95 (11) [n = 49]	96 (16) [n = 48]	>0.9
SBP	131 (17) [n = 50]	129 (15) [n = 48]	0.5
DBP	81 (10) [n = 50]	80 (9) [n = 47]	0.4
Fasting_Glucose	87.9 (7.1) [n = 51]	88.8 (6.5) [n = 50]	0.2
Glucose_120	112 (20) [n = 51]	109 (20) [n = 49]	0.6
AUC_Glucose	13825 (2131) [n = 50]	13674 (1947) [n = 46]	>0.9
Fasting_Insulin	24 (11) [n = 51]	25 (13) [n = 50]	>0.9
Insuline_120	110 (85) [n = 47]	125 (160) [n = 45]	0.6
AUC_Insulin	12357 (7744) [n = 40]	13608 (11208) [n = 35]	>0.9
HOMA_IR	2.78 (1.24) [n = 51]	2.79 (1.43) [n = 50]	0.6
HOMA_beta	361 (186) [n = 51]	369 (198) [n = 50]	0.8
ISI	2.81 (1.59) [n = 42]	2.71 (1.35) [n = 36]	>0.9
QUICKI	0.306 (0.017) [n = 51]	0.304 (0.019) [n = 50]	0.8
DI	1.4 (21.1) [n = 41]	5.0 (6.4) [n = 36]	0.3
Total_Cholesterol	142 (29) [n = 51]	145 (27) [n = 48]	0.8
HDL_Cholesterol	40 (9) [n = 51]	42 (8) [n = 48]	0.2
LDL_Cholesterol	84 (25) [n = 51]	87 (23) [n = 48]	0.8
Triglycerides	88 (50) [n = 51]	83 (36) [n = 48]	>0.9
AST	24 (7) [n = 50]	26 (7) [n = 47]	0.2
ALT	24 (11) [n = 51]	27 (12) [n = 47]	0.11
GLP1	2.87 (0.53) [n = 48]	2.91 (0.49) [n = 47]	0.6
IL6	2.32 (1.50) [n = 47]	2.00 (0.90) [n = 47]	0.4
IL10	4.3 (4.6) [n = 47]	4.5 (4.7) [n = 47]	0.7
TNF_alpha	10.7 (4.6) [n = 47]	11.0 (4.5) [n = 47]	0.8
LPS	1.94 (0.67) [n = 48]	1.96 (0.69) [n = 47]	0.9
E. coli	6.09 (1.06) [n = 46]	6.62 (0.84) [n = 47]	0.021
B. fragilis	8.89 (0.84) [n = 49]	9.07 (0.96) [n = 49]	0.2
B. breve	4.09 (1.06) [n = 39]	4.37 (0.97) [n = 38]	0.3
***Bifidobacterium* spp.**	8.17 (1.17) [n = 49]	8.18 (0.98) [n = 49]	>0.9
***Lactobacillus* spp.**	5.91 (0.87) [n = 48]	5.72 (1.17) [n = 47]	0.6

[a] Statistics presented: n (%); mean (SD) [n = non missing data].
[b] Statistical tests performed: chi-square test of independence; Wilcoxon rank-sum test.

Supplemental Table 1 summarizes estimates, 95% confidence intervals, and corresponding p-values for QUICKI and ISI mixed effects regression models. For more detailed explanation on differences between PRB and PLC supplementation during dietary training see Supplement 2.

3.3. Metabolomic data of stool samples after the first 8 weeks of treatment

Forty-five stool samples at T0 and T1 were adequate to the measurement of 25 SCFAs. For the 25 SCFAs concentrations, CLR were computed. The mixed effects regression models for the SCFA CLR values (Fig. 1c) disclosed that after the 8 weeks of dietary training and treatment supplementation, the acetic acid and acetic acid pentyl-ester CLR values remained stable while they underwent a significant increase of 0.16 (0.01, 0.3) and 0.14 (0, 0.27) in PLC. More details are in Supplement 2.

Overall, the SCFA concentration profiles at T0 and T1 were very similar between the PRB cohort and PLC one (Fig. 2a). To deeply analyze SCFA patient profiles a dimensionality reduction approach was performed through PCA analysis (Fig. 2b). The first two components were able to summarize more than the 60% of the total variance, with a 51.2% for the first alone. From the PCA ordination plot two patients were considered outliers because they were located far from all the other patients and the hierarchical clustering procedure confirmed it (Fig. 2c). They were removed and 3 clusters of patients were clearly visible across the first principal component values (Supplemental Table 2). From the sPLS-DA analysis (Fig. 2d), 5 out of the total 25 SCFA CLR values were considered sufficient to precisely discriminate between clusters (butanoic acid 2-methyl methyl-ester, butanoic acid 3-methyl methyl-ester, butanoic acid 3-methyl ethyl-ester, butanoic acid 3-methyl butyl-ester, butanoic acid ethyl-ester). In the middle, as already observed in Fig. 2b, cluster 2 patients were characterized by average CLR values for all the 5 discriminant SCFAs. See Supplement 2.

3.4. Clinical, metabolic, and microbial findings in relation to SCFA clusters

SCFA profile clusters and their interactions with the other independent variables disclosed some interesting findings. Post-OGTT glucose and insulin levels, GLP1, IL6, *B. fragilis, and B. breve* values in PRB treated patients cluster-specifically changed if compared to PLC ones (Fig. 2e). Table 2 and Supplement 2 reported an easier interpretation for the differences. These results should be evaluated with great caution as the cohort division into clusters produced unbalanced and low numerosity groups of patients.

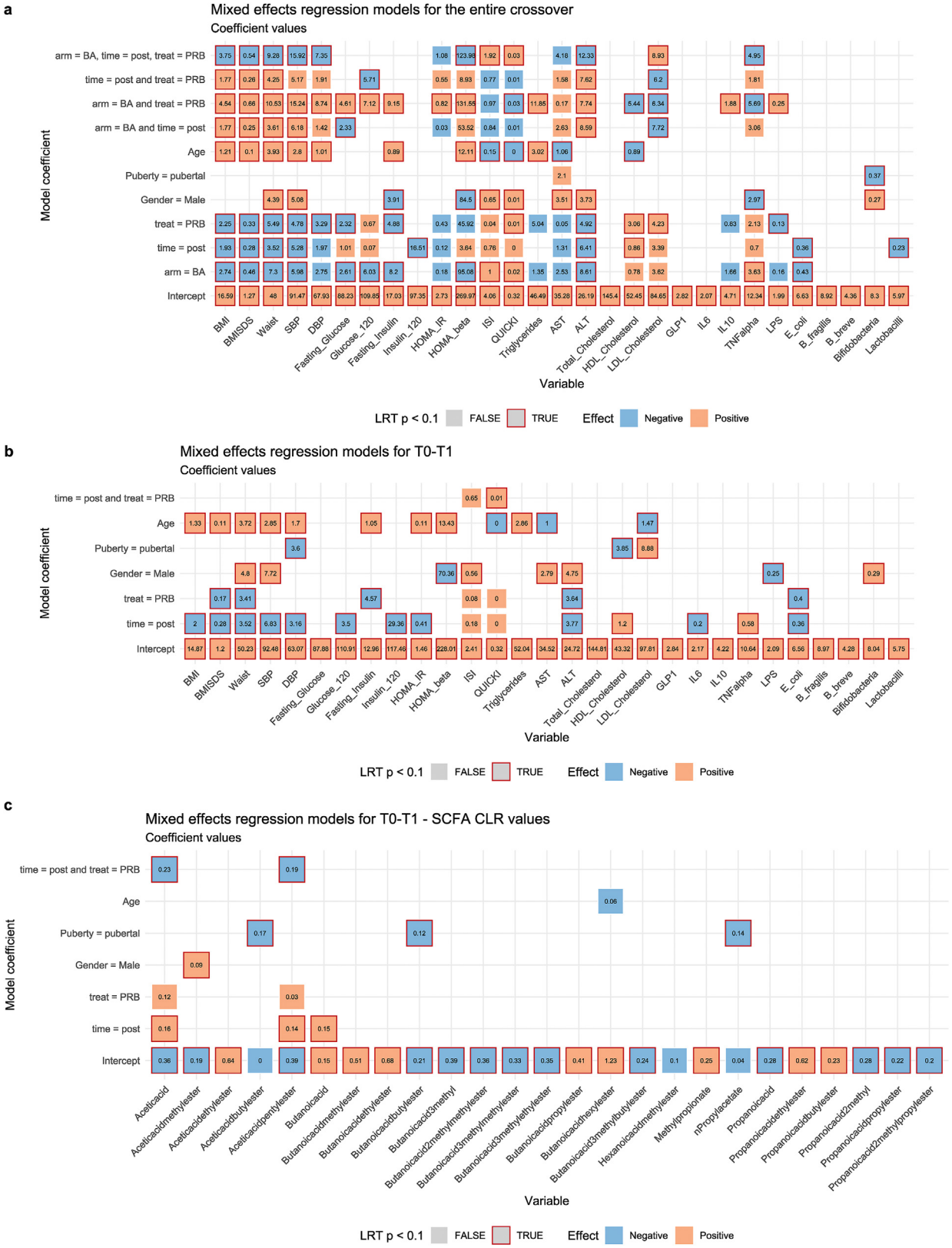

Fig. 1. Graphical representation of the mixed effects regression models. The independent variables and their interactions are on the rows, while the dependent variables are on the columns. The value inside each tile represents the estimated coefficient value while the color corresponds to its sign. A red square around a tile represents a value lesser than 0.1 for the log likelihood ratio test (LRT): the model without the corresponding independent variable is compared to the model with it and a low p-value indicates a significant increase of the information explained by the corresponding variable. **a**. Mixed effects regression models for the entire crossover. **b**. Mixed effects regression models for the first period of the crossover, from T0 to T1. **c**. Mixed effects regression models for the first period of the crossover from T0 to T1, for the 45 samples' SCFA CLR values.

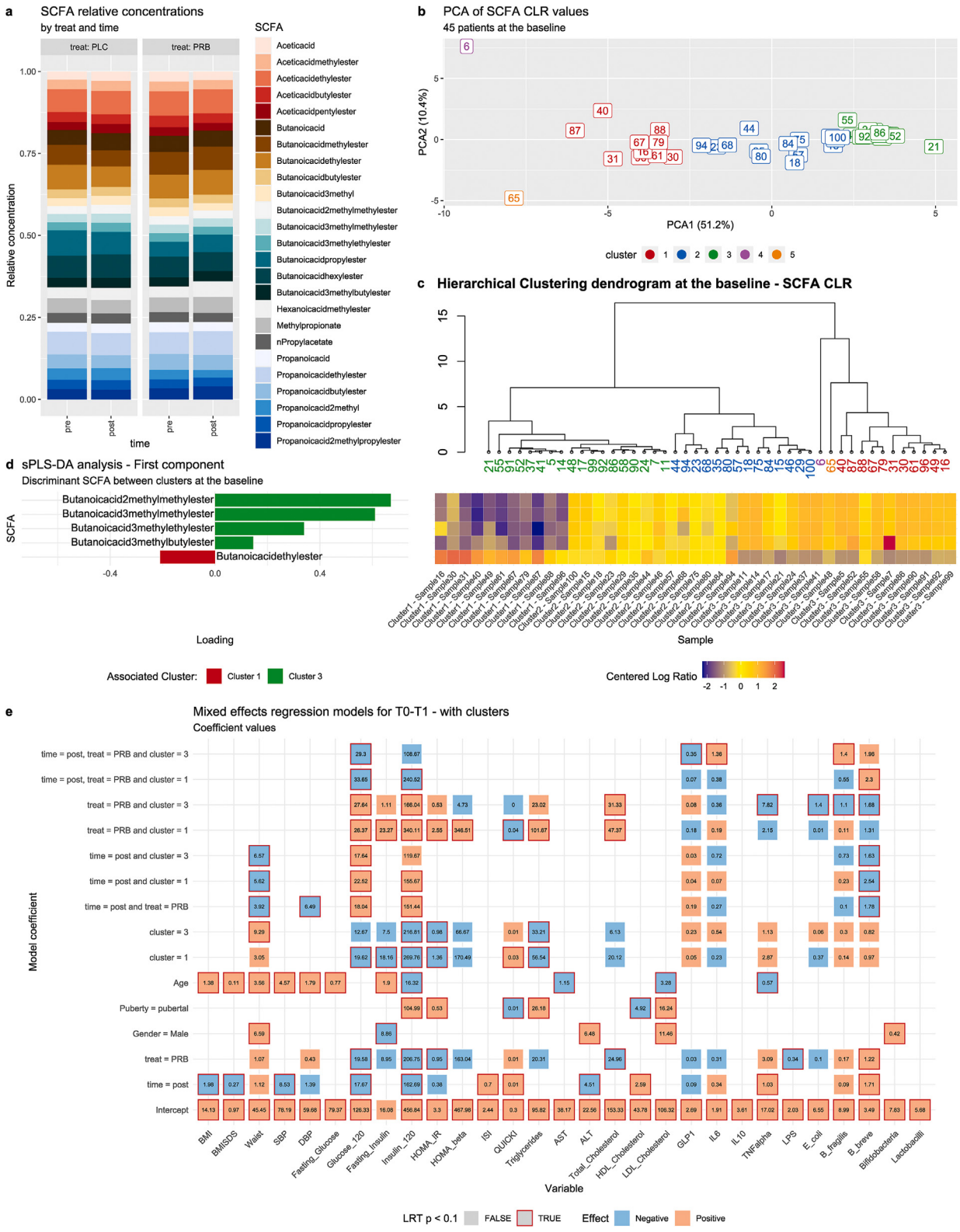

Fig. 2. **a**. SCFA relative abundance stacked bar plots, before (pre) and after (post) the first 8 weeks of the study. **b**. PCA for the SCFA CLR values at the baseline (pre-treatment and dietary training). **c**. Dendrogram for the complete linkage hierarchical clustering based on the first 2 principal components of the PCA in panel b. **d**. Sparse Partial Least Square - Discriminant Analysis (sPLS-DA) and related heatmap to inspect the most discriminant SCFAs for clusters 1, 2, and 3 (clusters are built using the SCFA configuration at the baseline). **e**. Graphical representation of the mixed effects regression models in the first 8 weeks of the study using the cluster membership as an additional independent variable. The independent variables and their interactions are on the rows, while the dependent variables are on the columns. The value inside each tile represents the estimated coefficient value while the color corresponds to its sign. A red square around a tile represents a value lesser than 0.1 for the log likelihood ratio test (LRT): the model without the corresponding independent variable is compared to the model with it and a low p-value indicates a significant increase of the information explained by the corresponding variable.

In this sub-cohort of 45 individuals, PRB effect after the first 8 weeks was not observed for the ISI and QUICKI variables. However, the PRB group showed a more marked reduction of diastolic blood pressure (−6.5 mmHg; CI -12.13, −0.86 mmHg), with respect to the PLC group where the reduction was not significant.

4. Discussion

This double-blind, placebo-controlled clinical trial revealed that a short-term live biotherapeutic supplementation with *B. breve* BR03 and B632 determined beneficial effects on insulin metabolism in obese children and adolescents undergoing dietary training.

Pediatric obesity is characterized by the same comorbidities as adults, requiring more urgent strategies to lose weight and improve metabolism for the long-term health. Education to healthy habits is the first and undeniable step, but in some cases, approved pharmacotherapeutic agents are suggested [22]. The human gut microbiota is characterized by a certain grade of resilience [23], however, its composition in early life is associated with the risk of obesity later in pediatric life [24] also in relation to dietary habits [25]. Intestinal microbiota would attain an adult-like structure at 3 years of age, but recent findings suggested that its development may take longer and, therefore, deviations in this phase may have consequences later in life [26].

Several authors investigated gut microbiota manipulation by probiotic supplementation for the management of obesity following evidence which showed a crosstalk among microbiota, metabolic and hormonal regulation of the host, diet, and drugs [27]. In this scenario, we aimed to evaluate the efficacy of two *B. breve* strains that are typical of the neonatal gut, mainly in modulating glucose metabolism.

First, we observed that *B. breve* supplementation is positively associated to insulin sensitivity at fasting and after an OGTT. This was achieved even though subjects in the probiotic arm were less metabolically compromised at baseline. The loss of insulin sensitivity is one of the first pathological events resulting in the development of glucose alterations up to type 2 diabetes [28], but also in other metabolic impairments [29]. The improvement of insulin sensitivity is one of the main targets of obesity management in the attempt to maintain a healthy phenotype and reduce cardiovascular risk. The adverse effects of alterations in insulin metabolism in the condition of weight excess joined with puberty, a physiological age of life in which insulin-resistance increases, seem to increase the risk of development of some obesity related-diseases [30]. One of these is non-alcoholic fatty liver disease (NAFLD), the most common liver disease in children that results from obesity, insulin resistance, oxidative stress and gut dysbiosis, and is close related to deterioration of glucose metabolism [31,32]. Therefore, early interventions to prevent or delay a deterioration of glucose levels are fundamental. Supplementations with probiotics seem to reveal considerable effectiveness in the management of both insulin resistance and delay or improvement of NAFLD could be one of the implicated mechanisms [31,32].

The findings on insulin agree with reports on bifidobacteria in mice models. Until now, most attention has been focused on *B. breve* B-3 that in a mice model of high-fat diet-induced obesity at 10^8 or 10^9 CFU/day dose-dependently blunted the weight and epididymal fat accrual, resulting in an improvement of metabolic syndrome features, including total cholesterol, fasting glucose, insulin and NAFLD [14,15]. The same probiotic treatment reduced the fat mass of 44 adults with obesity, but the metabolic effects were less prominent than in mice models, likely due to many more determinants that are not standardized in humans [12]. An amelioration of glucose metabolism has been reported also in high-fat diet mice models inoculated with *B. pseudocatenulatum* C95, a strain induced by a diet rich in fibers, as well as in animal models and obese humans treated with pasteurized *Akkermansia muciniphila* [21,34].

We also observed that, although SCFA fecal abundance remained stable, acetic acid and acetic acid pentyl-ester increased in the placebo arm, diversely from the probiotics. Since *B. breve* strains produce acetate, we can speculate that acetate by-products were more used by other acetate dependent species involved in metabolic diseases, through complex cross-feeding mechanisms [33,35]. Importantly, acetate is used as fuel by peripheral tissues, including the liver, muscle, and pancreas, suggesting an increased colonic absorption and transition to the systemic circulation to use it as an alternative source of energy during extended calorie restrictions apart from a regulatory action on glucose metabolism, insulin sensitivity and secretion, muscle function, adipose tissue metabolism and inflammation, and satiety [36].

In humans, the enterotypes are mostly driven by species composition, but prevalent molecular functions are not necessarily provided by abundant species [37]. The functional analysis of microbial communities in relation to stressors is thus critical. In line with this view, we were able to identify at baseline three main clusters of patients according to fecal SCFAs. Five esters of butanoic acid made the signature capable to differentiate them. Intriguingly, cluster 3, characterized by four higher butanoic esters (see Fig. 1d, Table 2), had a more pronounced decrease in glucose levels after the OGTT, in presence of lower GLP1 levels at fasting, and higher IL6 concentrations and *B. fragilis* count in probiotics than placebo arm. Cluster 1 remained stable in both the arms; while cluster 2 presented increased *B. breve* counts and lower glucose and insulin levels after OGTT in placebo arm. We can speculate that diet changes had a role in this unexpected increase in *B. breve* counts in this subset of patients, and that its abundance is associated with a better glucose metabolism. Although all these results should be considered with caution due to low numerosity groups of patients, this is in line with recent findings demonstrating that bloodstream metabolome signatures identify insulin-resistant phenotypes [38]. Indeed, an altered gut microbiota composition impacts both the serum and the gut metabolome contributing to obesity-related comorbidities, including insulin resistance. If a compromised gut is an obesity-related trait, the functional microbial signature could predispose to a more deleterious phenotype or a higher ability to counteract stressors or response to treatments including also living microorganisms. Furthermore, a signature in butanoic esters confirms the role of butyrate in metabolic diseases [39,40].

Our study has some limitations. First, we limited our results to the first 8 weeks of the trial because we observed a carry-over effect. However, following this, we suggest that the crosstalk between diet, probiotics administration and obesity is complex. Probiotics being live organisms, they could not be treated in trials as classical drugs [41]. When we designed the study, many papers suggested that the effects of probiotics were limited to the window of supplementation [42]. Our study shows that, at least for the metabolic effects and relation to the diet, this axiom should be reconsidered. The effect of *B. breve* strains after stopping the supplementation could persist as a promoting factor for the growth of protective bacteria for the host and/or inhibition of detrimental microbial groups, with effects perduring over a month [11].

Table 2
a. Average differences and 95% C.I. between the ending and the beginning of the 8 weeks of dietary training in the PLC cohort patients for the levels for the listed dependent variables from the mixed effects regression models using the SCFA profiles. **b**. Average differences and 95% C.I. between the ending and the beginning of the 8 weeks of dietary training in the PRB cohort patients for the levels for the listed dependent variables from the mixed effects regression models using the SCFA profiles.

a. PLC cohort (T1-T0)			
Formula	Cluster 1 (95% CI)	Cluster 2 (95% CI)	Cluster 3 (95% CI)
	(time = post) + (time = post and cluster = 1)	*(time = post)*	*(time = post) + (time = post and cluster = 3)*
Glucose_120	4.86 (−7.5, 17.21)	−17.67 (−31.01, −4.32)	−0.02 (−10.22, 10.18)
Insulin_120	−7.02 (−101.76, 87.72)	−162.69 (−265.6, −59.78)	−43.02 (−123.07, 37.03)
GLP1	−0.05 (−0.2, 0.11)	−0.09 (−0.29, 0.11)	−0.05 (−0.17, 0.07)
IL6	0.41 (−0.21, 1.03)	0.34 (−0.45, 1.14)	−0.38 (−0.87, 0.12)
B. fragilis	0.32 (−0.36, 1)	0.1 (−0.65, 0.84)	−0.64 (−1.19, −0.09)
B. breve	−0.83 (−1.87, 0.21)	1.71 (0.58, 2.84)	0.08 (−0.75, 0.91)
b. PRB cohort (T1-T0)			
Formula	*(time = post) + (time = post and cluster = 1) + (time = post and treat = PRB) + (time = post, treat = PRB and cluster = 1)*	*(time = post) + (time = post and treat = PRB)*	*(time = post) + (time = post and cluster = 3) + (time = post and treat = PRB) + (time = post, treat = PRB and cluster = 3)*
Glucose_120	−10.75 (−27.09, 5.59)	0.38 (−11.18, 11.93)	−11.29 (−23.64, 1.07)[a]
Insulin_120	−96.11 (−229.97, 37.76)	−11.25 (−94.7, 72.19)	−0.25 (−89.43, 88.92)
GLP1	0.077 (−0.12, 0.28)	0.1 (−0.04, 0.24)	−0.22 (−0.38, −0.06)
IL6	−0.23 (−1.05, 0.59)	0.07 (−0.55, 0.69)	0.72 (0.05 1.38)
B. fragilis	−0.32 (−1.23, 0.59)	0.01 (−0.62, 0.65)	0.67 (−0.02, 1.36)[a]
B. breve	−0.31 (−1.49, 0.87)	−0.07 (−0.98, 0.84)	0.26 (−0.78, 1.30)

[a] PRB cohort (T1-T0) Cluster 3 90% C.I.: −21·65, −0·91 for Glucose_120, 0·09 1·25 for *B. fragilis* (calculated due to low sample size).

Second, although the randomization was correct, at baseline some variables were lower in the probiotics arm and could have mitigated or hidden some effects. Third, the improvement in insulin sensitivity was not reached in the subgroup analysis of 45 subjects, mainly due to the group size, but we observed a higher decrease in diastolic blood pressure in probiotics than placebo arm. These results could depend on a different representation of the clusters in the main group or other unidentified confounders. The hierarchical clustering of the SCFA signature suggested that some effects of *B. breve* administration could be dependent on abundant molecular functions of the resident microbial communities. Most of the probiotic RCTs reported limited effect sizes that may be due to several reasons, including the heterogeneity in gut microbiota composition of different individuals, the presence of non-responders, and the continuous interaction with the environment in an unstoppable crosstalk which is part of a complex mechanism of action [42].

5. Conclusion

To our knowledge, this is the first RCT administering two strains of *B. breve* to children and adolescents with obesity. The study demonstrated that 8 weeks of this intervention was safe, well-tolerated, and efficacious in improving insulin sensitivity and supporting weight loss. The functionality of microbiota seems to influence the metabolic answer to the probiotics, suggesting that tailored probiotic supplementations could optimize responses. The limited sample size and inter-subject variability justify future studies designed to confirm and expand our findings to pave the way for more successful interventions for obesity and comorbidities.

Ethics approval and consent to participate

Ethical Committee protocol 165/13; NCT03261466.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Probiotical S.p.A. designed the probiotics, collaborated with Authors to decide the dose and the final formulation, and supplied furniture of the placebo and probiotics free of charge.

Role of the funding source

The funding and sponsoring organizations had no role in the design and conduction of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. In addition, they had no right to veto publication or to control the decision regarding to which journal the paper was submitted.

Author contributions

Prof. Flavia Prodam and Prof. Nicola Vitulo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Additional contributions

In addition to the authors, the BIFI-OBESE trial team comprised the following: Tiziana Cena, Irene Aloisio, Stefania Moia, Marta Roccio, Silvia Parlamento, Erika Pozzi, Giulia Genoni, Alice Monzani, Roberta Rolla, Anna Rapa.

These individuals involved in the study provided their assistance without any compensation beyond their usual salary.

Conflict of interests

Dr. Prodam reported grants from the Muscular Dystrophy Association, EuroTransBio Program, Cariplo Foundation, Novo Nordisk during the conduct of the study, and personal fees from Eli Lilly (speaker) and Difass International (advisory board) outside the submitted work.

Dr. Bellone reported during the conduct of the study personal fees from Difass International (advisory board) outside the submitted work.

Dr. Bona reported grants from Cariplo Foundation, Comunità Novarese Foundation, AIFA during the conduct of the study, and personal fees Pfizer, Consumer Healthcare, Guna, and Vox (advisory boards and speaker) outside the submitted work. He also participated in the GQM05, V59-66 (Novartis), MMRV063 (OKAH), V 118_05, and Boostrix 048 (Glaxo SmithKline Biologicals) multicenter studies on vaccines during the conduct of the study.

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Marco Pane is employee of Probiotical Research Srl.

Angela Amoruso is employee of Probiotical Research Srl.

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Abbreviations

QUICKI	quantitative insulin sensitivity check index
SCFA	short chain fatty acids
PLC	placebo formulation
PRB	probiotic formulation
OGTT	oral glucose tolerance test
ISI	Matsuda Insulin Sensitivity Index
BMI	Body Mass Index
BMISDS	Body Mass Index Standard Deviation Score
WC	waist circumference
HDL	high density lipoprotein
LDL	low density lipoprotein
LPS	lipopolysaccharide
ALT	Alanine transaminase

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.06.002.

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