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Metabarcoding analysis of gut microbiota of healthy individuals reveals impact of probiotic and maltodextrin consumption

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RESEARCH ARTICLE

Abstract

In a previously published double-blind, placebo-controlled study, we showed that probiotics intake exerted a positive effect on sleep quality and a general improvement across time in different aspects of the profile of mood state, like sadness, anger, and fatigue in 33 healthy individuals. The present work investigates the impact of the probiotic product, constituted of *Limosilactobacillus fermentum* LF16, *Lacticaseibacillus rhamnosus* LR06, *Lactiplantibacillus plantarum* LP01 (all former members of *Lactobacillus* genus), and *Bifidobacterium longum* 04, on the gut microbiota composition of the same cohort through a metabarcoding analysis. Both the placebo and probiotic treatments had a significant impact on the microbiota composition. Statistical analysis showed that the microbiota of the individuals could be clustered into three groups, or bacteriotypes, at the baseline, and, inherently, bacterial compositions were linked to different responses to probiotic and placebo intakes. Interestingly, *L. rhamnosus* and *L. fermentum* were retrieved in the probiotic-treated cohort, while a bifidogenic effect of maltodextrin, used as placebo, was observed. The present study shed light on the importance of defining bacteriotypes to assess the impact of interventions on the gut microbiota and allowed to reveal microbial components which could be related to positive effects (i.e. sleep quality improvement) to be verified in further studies.

Keywords: gut microbiota, metabarcoding analysis, lactobacilli, *Bifidobacterium*, maltodextrin

1. Introduction

A great interest has emerged in the last years on the impact of the gut-brain axis in psychiatric disorders, pointing to stressed and unhealthy conditions of the microbial communities inside the human gut as possible causes for psychological diseases and conditions, such as depression, anxiety and schizophrenia (Bastiaansen *et al.*, 2020; Kelly *et al.*, 2019). It has been observed that the gut microbiota communicates with the brain, exerting effects over several neurobiological mechanisms and related systems; among these the hypothalamic-pituitary-adrenal axis, the immune system, the tryptophan metabolism and the production of various neuroactive compounds (Kelly *et al.*, 2019).

For those reasons, the gut microbiota has become a new target to obtain antidepressant effects; remarkably, the diversity of studies performed and the functional redundancy of the microbiome make it difficult to understand if specific microbial components are more related than others to psychiatric symptoms (Cheung *et al.*, 2019).

Since microbiota composition can be modified in a variety of ways, such as through the use of probiotics, prebiotics and dietary changes (Butler *et al.*, 2019; Cheung *et al.*, 2019), several clinical and translational studies have been published over the years, showing that the prolonged prebiotic and probiotic consumption can positively affect

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aspects of mood, anxiety, and cognition in both healthy individuals as well as in patients diagnosed with clinical psychiatric disorders (Butler *et al.*, 2019; Kelly *et al.*, 2019; Marotta *et al.*, 2019). However, in some clinical trials, lack of evidence of an effect on depression and related symptoms have also been reported either in depressed (Romijn *et al.*, 2017) as well as in healthy individuals (in particular in older adults) (summarised in Butler *et al.*, 2019) even though probiotic strains used were also previously successfully applied.

Probiotic supplements used in clinical trials for the treatment of depression, either alone (Akkasheh *et al.*, 2016; Chahwan *et al.*, 2019; Pinto-Sanchez *et al.*, 2017), in combination with prebiotics (i.e. galactooligosaccharides) (Kazemi *et al.*, 2019), or as adjunctive therapy with antidepressants (i.e. sertraline) (Eskandarzadeh *et al.*, 2019) mainly include *Lactobacillus* (*L. acidophilus*, *L. helveticus*, *L. brevis*, *L. casei*, and *L. salivarius*), *Lactococcus* (*L. lactis*) and *Bifidobacterium* species (*B. bifidum*, *B. lactis*, and *B. longum*). Generally speaking, such treatments led to (1) a significant reduction of depression scores on Hospital Anxiety and Depression Scale and improvement of the cognitive reactivity scores in mild/moderate depression patients (Chahwan *et al.*, 2019; Pinto-Sanchez *et al.*, 2017), (2) a decrease of the anxiety symptoms in individuals with anxiety disorders (Eskandarzadeh *et al.*, 2019) and (3) an improvement of the depression scores on Beck Depression Inventory in patients with a diagnosis of major depressive disorder (MDD) (Akkasheh *et al.*, 2016; Kazemi *et al.*, 2019).

In healthy subjects, administration of probiotics (*L. casei* Shirota, *B. bifidum* W23, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, and *L. lactis* W19 and W58) improved the mood of subjects having lowest baseline mood levels and in general reduced the cognitive reactivity to sadness (Benton *et al.*, 2007; Steenbergen *et al.*, 2015).

Moreover, in other two studies led by Messaoudi *et al.* (2011) and Mohammadi *et al.* (2016), a significant reduction in overall anxiety and depression scores was shown after the treatment with *L. helveticus* R0052 and *B. longum* R0175 as well as with a polybiotic combination of various *Lactobacillus* strains (*L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, and *L. rhamnosus*), *Bifidobacterium* (*B. breve*, *B. longum*) and *Streptococcus thermophilus* strains. Besides mood, anxiety and depression scores, it has also been shown that the short-term administration of *L. gasseri* improved stress-associated symptoms in terms of sleep disturbance (Nishida *et al.*, 2019).

Within the framework of the impact of probiotics on mood, we have previously reported (Marotta *et al.*, 2019) on a double-blind, placebo-controlled study on 33 healthy volunteers who received daily either a probiotic mixture containing *Limosilactobacillus fermentum* LF16, *Lacticaseibacillus rhamnosus* LR06, *Lactiplantibacillus plantarum* LP01 (former members of *Lactobacillus* genus, Zheng *et al.*, 2020), and *B. longum* 04 in maltodextrin, or a maltodextrin-only placebo, for 6 weeks, followed by a 3-weeks washout (Figure 1). Data obtained showed that the probiotics exerted a general improvement and persistence over time in different aspects of the mood state, including sadness, anger, and fatigue, accompanied by improvement in the sleep quality, which indicates that probiotics may increase the production of neuroactive precursors involved in emotional modulation, brain functions and circadian

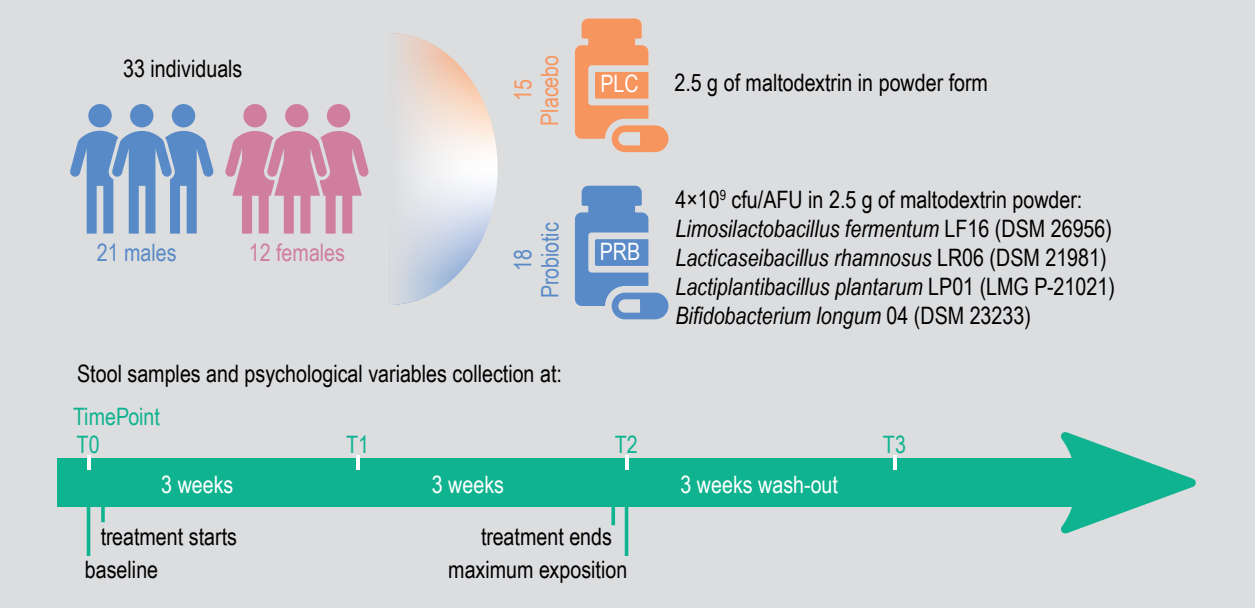

Figure 1. Experimental design of the study.

rhythms. These findings corroborated the positive effect of probiotics on mental well-being, possibly determining changes in cognitive strategies to deal with problems by reducing sensitivity to negative situations.

The aim of the present study was to apply metabarcoding analysis of the faecal microbiota to determine (1) the microbial arrangement at baseline in the same cohort of healthy adults, which were randomised based on other characteristics, and (2) determine the effects of the probiotic and placebo consumption during and after the administration.

2. Materials and methods

Sample collection

The samples analysed derives from 33 healthy subjects enrolled in the study. Stool samples and psychological variables were collected at four time points (see Supplementary Table S1): before the intake of probiotic/placebo (T0), at 3 (T1) and 6 (T2) weeks after the first intake and at the end of the third week of washout (T3).

The experimental group received 42 sachets of the product (one for each day), each containing 4×10^9 cfu/active fluorescent units (AFU) of four probiotic species: *L. fermentum* LF16 (DSM 26956), *L. rhamnosus* LR06 (DSM 21981), *L. plantarum* LP01 (LMG P-21021), and *B. longum* 04 (DSM 23233) in 2.5 g of freeze-dried powder mixture containing maltodextrin (around 85% of the total weight) (Probiotical S.p.A., Novara, Italy). The control group received 42 sachets of placebo, each containing 2.5 g of maltodextrin in powder form. The placebo powder was indistinguishable from the probiotics powder in colour, taste, and smell. Participants were instructed to dissolve the powder in water or milk and drink it in the morning with breakfast. The probiotic sachets were analysed by Biolab Research S.r.l. (Novara, Italy), via flow cytometry (ISO 19344:2015 IDF 232:2015, $\geq4\times10^9$ AFU) and plate count method (Biolab Research Method 014-06, $\geq4\times10^9$ cfu) to confirm target cell count. Product stability was monitored to ensure minimum cell counts were maintained. The study was approved by the Ethical committee of Verona Hospital (Azienda Ospedaliera Universitaria Integrata, AOUI Verona, 766CESC) and it is registered in ClinicalTrials.gov with the number ID: NCT03539263.

Library preparation and sequencing

The 132 collected samples from 33 healthy subjects were stored at -20 °C until analysis. DNA extraction and sequencing were performed at BMR Genomics S.r.l. (Padua, Italy). DNA was isolated with the Mobio Powerfecal kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) adapted for QIAcube HT extractor (Qiagen, Hilden, Germany). V3-V4 regions of 16S rRNA gene were amplified with previously described primers (Takahashi *et al.*, 2014), modified with forward and reverse overhangs necessary for dual index library preparation (Illumina protocol (https://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf) generating amplicons of ~460 bp. The paired-end sequencing of the 16S rRNA gene amplicons was performed using the MiSeq Illumina platform (dual-indexing approach, 2×300 bp) (Illumina, San Diego, CA, USA). A mock community was included as control. The resulting output was a set of 264 raw files in FASTQ format. All the reads have been submitted to SRA archive and are available under the bioproject PRJNA644097.

Bioinformatics data analysis

The whole analysis was performed on R (v3.6.1, R Core Team, 2019). Primarily, the FASTQ sequences were analysed using DADA2 (v1.13) (Callahan *et al.*, 2016), a tool that implements an error correction model and allows to identify exact sample sequences that differ as little as a single nucleotide. The final output of DADA2 was an amplicon sequence variant (ASV) table which recorded the number of times each ASV was observed in each sample. DADA2 was run as described in https://benjjneb.github.io/dada2/bigdata.html using default parameters. In order to improve the overall quality of the sequences, the reads were filtered and trimmed using *filterAndTrim* function implemented in DADA2. Consequently, to remove low quality bases at the end of reads, the *truncLen* option was set to (280, 220) for the forward and reverse FASTQ files respectively. Similarly, to remove adapter sequences at the 5' end, the *trimLeft* option was set to (17, 21), for forward and reverse reads respectively. The *removeBimeraDenovo function* was used to remove chimeras, via consensus method, and then *collapseNoMismatch* function collapsed together all the reads that are identical up to shifts or length variation. Finally, the taxonomic assignment was performed using the naïve Bayesian classifier method implemented in DADA2 (*assignTaxonomy* and *addSpecies* functions) using as reference the EzBioCloud 16S database for QIIME pipeline (version 2018.05, https://www.ezbiocloud.net/resources/16s_download), correctly formatted to work with the taxonomic classifier implemented within DADA2 (https://benjjneb.github.io/dada2/assign.html). A phylogenetic tree of the ASVs was obtained using the function *AlignSeq* implemented in DECIPHER (v2.12) (Wright, 2016), an R package to create multiple sequence alignments. FastTree (v2.1.10) (Price *et al.*, 2010) was used to create the final tree.

Data quality assessment and filtering

Rarefaction curves on raw data were evaluated to assess the species richness among samples as a function of the sequencing depth. Data were pre-processed filtering taxa

(ASVs) with low prevalence (where prevalence is the fraction of total samples in which an ASV is observed), setting a threshold of 0.5% for the cumulative relative abundance across all the samples; furthermore, *taxa* present in less than 2 samples were discarded. *Synergistetes* phylum members *taxa* (cumulative relative abundance = 0.34%) and *Lentisphaerae* phylum members *taxa* (cumulative relative abundance = 0.03%) were discarded by this filter. The pre-processing output data were then transformed to their relative abundances, and the 10 most present genera were plotted to phylum level. Mann-Whitney tests were performed on ASVs detected in these genera and the Benjamini & Hochberg correction was applied to adjust the p-values because of multiple testing.

In order to investigate the presence of probiotic related *taxa*, a further taxonomy classification was performed. The softwares Kraken2 (Wood *et al.*, 2019) and Bracken (Lu *et al.*, 2017) were used to check both raw .fastq data and DADA2 inferred list of ASV, using two different pre-built Kraken2/Bracken databases (minikraken2_v2_8GB_201904, k2_standard_16gb_20200919) and a custom database containing bacteria, archaea, virus, fungi and plants sequences, built using RefSeq (O'Leary *et al.*, 2016) sequences.

Biodiversity measurements

Shannon-Wiener index was used to calculate α-diversity, which was plotted stratifying the samples according to time points, gender and treatment type; the Kruskal-Wallis tests were performed to verify statistical differences in the α-diversity among the samples. To measure β-diversity, data were normalised by three different methods (Cumulative Sum Scaling (CSS), Total Sum Scaling (TSS), Rarefaction) through the *phyloseq_transform_css*, *phyloseq_standardize_otu_abundance* and *rarefy_even_depth* functions respectively. The first two functions are part of the vmikk/metagMisc package (github.com/vmikk/metagMisc) while the latter belongs to the phyloseq package (v1.30.0) (McMurdie and Holmes, 2013). Each type of normalised data was inspected using four different distance metrics (Unweighted UniFrac, Weighted UniFrac, Bray-Curtis, Jaccard) and ordinated using the Principal Coordinates Analysis (PCoA) and Detrended Correspondence Analysis (DCA) ordination methods, through the *ordinate* function of the Vegan package (v2.5-5) (Oksanen *et al.*, 2019).

A rigorous procedure was applied to evaluate the best combination of normalisation, distance metric, and ordination method. Normalization based on rarefaction was not considered as it performs very similarly to TSS due to the similar library sizes between samples.

At first, a hierarchical clustering was applied to the β-diversity bidimensional plot at the baseline grouping the samples in 3 and 4 groups. To test which of the two clustering methods performed better, the homogeneity of the cluster dispersions were tested using ANOVA F-test on *betadisper* function's output. A significant *P*-value indicated that the cluster dispersions were not homogeneous and that data needed to be taken with care. Secondly, the silhouette value was calculated, that is a measure of how similar an object is to its own cluster (cohesion) compared to other clusters (separation). The silhouette ranged from -1 to +1, where a high value indicated that the object was well matched to its own cluster and poorly matched to neighbouring clusters. If most objects had a high value, then the clustering configuration was appropriate. On the other hand, if many samples had a low or negative value, then the clustering configuration might have too many or too few clusters. Finally, the cluster memberships found at the baseline were extended to all the other time points; cluster dispersions and silhouette indexes were computed again to verify the performances of the clustering on the whole dataset.

Mixed effects regression models statistical analysis

Amongst all the tested combinations, the TSS-normalised data, ordinated using the PCoA method and the unweighted-UniFrac distance metric, showed the most consistent results in cluster dispersions homogeneity and silhouettes, hence it was chosen for deeper exploration. To investigate the biological meaning of each PCoA coordinate, mixed-effects regression model analysis was performed on each, using the *lme* function of the nlme package (v3.1-140) (Pinheiro *et al.*, 2020).

Firstly, the model formulation involved the Sample variable as a random component for each individual, and several categorical variables as fixed effects, such as TimePoint, Gender, Treatment and their interactions. Since all the variables were categorical, the regression framework set a baseline formed by *TimePoint = T0*, *Treatment = Placebo* and *Gender = Female* samples. Variable significance was guaranteed through an iterative process. Starting from the complete model, nonsignificant variables were dropped one by one. Every time a variable was dropped a log-likelihood ratio test (LRT) was performed in order to compare the likelihood of the model with the likelihood of the nested one (*P*-value <0.1). This procedure allowed us to reach the most informative as well as parsimonious formulation of the model. Moreover, two versions of each model were compared: the first, where no correlation structure was specified, and the second, where the type of correlation was specified as an AR(1) process through the option *correlation = corAR1(form = ~1|Sample)* of *lme* function.

Secondly, mixed-effects regression models were used to study correlation between sample variables and the PCoA components, with the new information about cluster

memberships. The TimePoint, Gender, Treatment and Cluster variables were tested in the model, together with the interactions between TimePoint and Gender, TimePoint and Treatment, TimePoint and Cluster, Treatment and Cluster, and TimePoint, Treatment and Cluster. The already described model selection procedure was performed to choose the best model.

Biomarkers investigation

To retrieve information about the most discriminant features (Amplicon Sequence Variants, ASVs) of the clusters identified with the hierarchical clustering procedure, a discriminant analysis was computed using PLS-DA and sPLS-DA methods. Following the default mixOmics (v6.8) (Lê Cao *et al.*, 2016; Rohart *et al.*, 2017) pipeline (http://mixomics.org/case-studies/splsda-srbct/), a pseudo-count value of 1 was added to the counts table, which was then normalized with TSS and centered log-ratio (CLR) transformed. At first, the pipeline was performed on the clusters at baseline T0 to identify the most discriminant ASVs of each group. The discriminant analysis was then applied to each significant interaction resulted from the mixed-effects models, to investigate the effect of treatments. For each interaction a summary image was plotted using the *HotLoadings* function of the homonym package (github.com/mcalgaro93/HotLoadings), displaying the discriminant ASVs loadings and the related heatmap.

Psychological variables analysis

To find significant associations between psychological variables, treatments and clusters, Wilcoxon Rank Sum tests were performed between time points T0 and T1, T0 and T2, and T0 and T3 for placebo and probiotics groups. The *P*-values were also corrected for multiple testing using the Benjamini-Hochberg correction method.

3. Results

16S metabarcoding sequencing depth and taxonomy classification

A total of 5,382,700 paired-end sequences (an average of 40,778 reads per sample) with a read length of 300 bp were obtained from the samples of the 33 subjects summarised in Figure 1. After read quality assessment, denoising and chimera filtering, 1,728 different ASVs were obtained. ASVs artefacts were removed with several filters and a total of 730 unique ASVs were obtained (Supplementary Figure S1). The taxonomy classification allowed to identify 10 phyla, 20 classes (730 ASVs), 27 orders (728 ASVs), 46 families (727 ASVs), 170 genera (720 ASVs) and 263 species (273 ASVs). The comparison of rarefaction curves (Supplementary Figure S2) as a function of sampling depth showed that all curves are close to saturation, therefore the richness of the samples has been fully observed or sequenced. The only exception was for subject number 8 at time point T2 that had a library size of 538, while the second lower had a value of 8,848; for this reason, the former was discarded from the analysis.

The most abundant phylum was *Firmicutes*, with a relative frequency of 62.3% followed by *Bacteroidetes* 17.9%, *Proteobacteria* 9.1%, *Verrucomicrobia* 4.9%, and *Actinobacteria* 4.7%. The remaining ~1% accounted for *Euryarchaeota*, *Tenericutes*, *Saccharibacteria*, *Fusobacteria* and *Cyanobacteria*. At genus level, the most abundant populations were *Agathobacter*, *Blautia*, *Dialister*, *Faecalibacterium*, *Ruminococcus*, *Subdoligranulum* (*Firmicutes*), *Bacteroides* (*Bacteroidetes*), *Escherichia* (*Proteobacteria*), *Akkermansia* (*Verrucomicrobia*), and *Bifidobacterium* (*Actinobacteria*) (Supplementary Figure S3).

α-diversity analysis confirmed that the subjects of the cohort were comparable

Samples were stratified according to *TimePoint*, *Treatment* (placebo or probiotics) and *Gender* using Shannon-Wiener index, as shown in Supplementary Figure S4. No significant differences among samples were observed, neither in the experimental nor in the control group (Kruskal-Wallis tests had $P>0.05$). This finding was in line with expectations, as the subjects enrolled in the study were comparable when related to their internal diversity; neither alterations nor major shifts were expected on gut microbiota species richness or evenness regarding probiotic consumers.

β-diversity analysis revealed three clusters and a strong sample-specific effect

The flow chart in Supplementary Figure S5 summarizes the following steps of the analysis. All the β-diversity plots are shown in Supplementary Figure S6, while the homogeneity of cluster dispersions and silhouettes are presented in the Supplementary Results S1. The choice of the number of clusters was performed using only the samples at T0, which represents a snapshot of the microbiome composition before any type of treatment and allows to stratify the samples according to different bacteriotypes.

The identification of the best combination of number of clusters, normalisations, distances, and type of ordinations was then chosen. Specifically, the metrics that performed better in terms of homogeneity of cluster dispersions and silhouette values, when the cluster membership was extended also to all the other timepoints, were selected. Three clusters grouping with PCoA ordination method, based on unweighted UniFrac distances and TSS normalisation (Figure 2A-C), produced the most consistent results (see Materials and methods 'Biodiversity

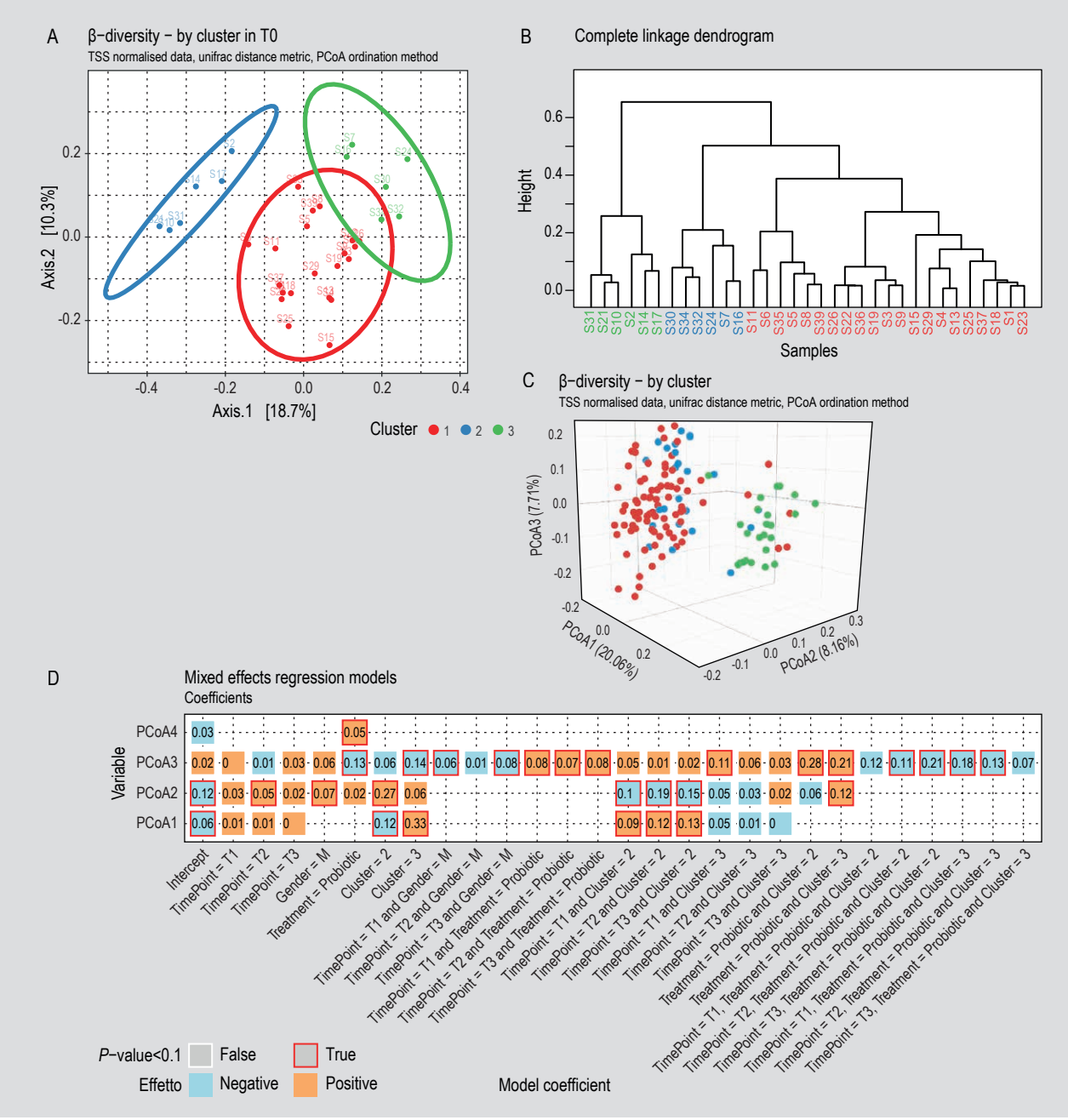

Figure 2. (A) Bidimensional representation of β-diversity for the samples in TimePoint = T0 (PCoA ordination method on UniFrac distance matrix of TSS normalized count data). Coloured by cluster membership obtained cutting the dendrogram in (B) in order to obtain 3 groups of individuals (see Methods for details of normalization, distance, and ordination's choice). (B) Hierarchical clustering dendrogram built with the complete linkage method on the euclidean distance matrix. Distance matrix based on PCoA1 and PCoA2 coordinates of the β-diversity for the samples in TimePoint = T0 (PCoA ordination method on UniFrac distance matrix of TSS normalised count data). (C) Tridimensional representation of β-diversity coloured by cluster membership for the samples in all time points (PCoA ordination method on UniFrac distance matrix of TSS normalised count data). (D) Linear mixed effects regression model coefficients. Blue (orange) coloured tiles represent a negative (positive) effect of the variable referred to the model baseline (TimePoint = T0, Gender = F, Treatment = Placebo, Cluster = 1). Statistically significant (*P*<0.1) effects are represented by red squared tiles.

measurements' and Supplementary Results S1 for details). Indeed, using these combinations of metrics, the stability of the clusters was maximised over time. In other words, the clusters identified at T0 tended to be the most consistent when the information of cluster membership is extended also to the other time points. The underlying idea was that the microbial signatures of the bacteriotype we identified at T0 should be stable over time, even though individual hosts may switch between enterotypes over long time periods (Moeller *et al.*, 2012).

The composition of each cluster is reported in Table 1. As expected, samples of the same subject tended to form close subclusters, regardless the considered time point or treatment (Supplementary Figure S7). This suggests that the differences among subjects are stronger than the effects determined by the treatment.

Mixed-effects regression models found associations between β-diversity and sample variables

To inspect the variability held by the first four coordinates of the PCoA, four mixed-effect regression models were at first estimated without considering cluster membership (see Materials and methods). This regression framework allowed us to find significant correlations between PCoA coordinates and metadata such as Gender, Treatment and TimePoint and to remove sample-specific effects. In this context, we implicitly considered the *Treatment=Placebo*, *Gender=Female*, and *TimePoint=T0* as the baseline level. As shown in Supplementary Figure S8, a correlation between the Treatment variable and the fourth component of the PCoA was found, while the third component showed a statistically significant difference in Gender at time point T3 compared to the baseline. This first analysis did not allow us to identify any statistically significant effects for the interactions between time points and treatments.

To further investigate if adding bacteryotype information would help in identifying significant effects for the interactions between time points and treatments, new mixed effects regression models were estimated adding the cluster membership variable as a fixed effect to the framework. Several significant interactions between time points and treatments were found for the third coordinate (Figure 2D): (1) *TimePoint = T1*, *Treatment = Probiotic* and *Cluster = 3*; (2) *TimePoint = T2*, *Treatment = Probiotic* and *Cluster = 2*; (3) *TimePoint = T2*, *Treatment = Probiotic* and *Cluster = 3*; (4) *TimePoint = T3*, *Treatment = Probiotic* and *Cluster = 2*. Clusters 2 and 3 were commonly affected by the variable *TimePoint = T2* compared to the baseline: Cluster 2 responded later in the treatment (time points T2 and T3) while Cluster 3 responded at the beginning (time points T1 and T2). Although each interaction should be interpreted very carefully, these results highlighted a difference between the considered variable categories and the baseline (T0, Placebo, Cluster 1). Biologically speaking, the identified interactions could be an indicator of a distinct effect of the treatment considering different groups/bacteriotypes.

Table 1. Cluster membership for individuals at the baseline (*TimePoint = T0*).

Cluster	Placebo treated individuals	Probiotic treated individuals
1	9	12
2	3	3
3	3	3

sPLS-DA analysis showed that clusters were characterised by a specific bacteriotype

A sPLS-DA analysis was performed to identify the most discriminant ASVs at the baseline T0. This multivariate approach identified two main components which were able to discriminate the clusters. The first component highlighted 5 *taxa* associated with Cluster 3 (Figure 3A,B): all the members of this group were characterised by the presence of SV33, assigned to *Methanobrevibacter smithii,* while 66% of them also displayed *Sporobacter*, *Eubacterium* and *Oscillibacter* spp. (SV168, SV256, SV37).

The second component highlighted the top 30 *taxa* associated with Clusters 1, 2 and 3 which created two different patterns as shown in the heatmap (Figure 3C,D). Cluster 1 individuals showed the general presence of *Faecalibacterium* spp. (SV3, SV14), while Cluster 3 were also characterised by *Faecalibacterium* spp. and *Alistipes putredinis* (SV4 and SV39); in Cluster 2, the second component revealed the presence of SV94-*Eubacterium* and SV77-*Lachnospiraceae* in almost all the members; 50% of them were also characterised by *Blautia* spp. (SV562).

Lacticaseibacillus rhamnosus is the only probiotic SV that increases significantly in the probiotic cohort in Cluster 1 and 2

The sPLS-DA analysis revealed that SV232, associated with *L. rhamnosus,* was present in the probiotic cohort at time point T1 and T2 in Cluster 1 and Cluster 2, respectively (Supplementary Results S2 a, g). As for bifidobacteria, SV34 associated with *B. longum* was found to increase in Cluster 1 at T2 (where it was abundant also in the placebo individuals) and T3 Supplementary Results S2 e, k). Interestingly, other SVs associated with *Bifidobacterium* spp. displayed a different behaviour: SV228 was found to increase in the placebo cohort in Cluster 1 at T1 (Supplementary Results S2 b), while the relative abundance of SV121 and SV15 decreased in Cluster 1 and 2 at T3 (Supplementary Results S2 l, m). These observations showed that *L. rhamnosus* is

Figure 3. (A) sPLS-DA analysis at the baseline (*TimePoint = T0*). Loading values represent the 5 discriminant *taxa* of the first component, associated with Cluster 3. Bigger the loading absolute value, stronger the association. (B) Heatmap shows the CLR values of the discriminant *taxa* in all the samples. (C) sPLS-DA analysis at the baseline (*TimePoint = T0*). Loading values represent the first 30 (out of 135) most discriminant *taxa* of the second component, associated with Cluster 2 and Cluster 1. Bigger the loading absolute value, stronger the association. (D) Heatmap shows the CLR values of the discriminant *taxa* in all the samples.

the only probiotic SV that increases during the probiotic administration until T1 and T2 in Cluster 1 and Cluster 2, respectively.

Bacteriotypes changed distinctly in the probiotic and placebo cohorts

The probiotic intake in Cluster 1 was associated with an increase of *Coprociproducens leptum* (*Clostridium letpum*), *Romboutsia timonensis* and *Mogibacterium* spp. (SV264, SV25 and SV664) from T1 to T3, respectively; the same cohort displayed a decrease of SVs related to *Butyricimonas* (SV785), *Lachnospira* (SV144) and *Faecalibacterium* spp. (SV4) at the same time points (Supplementary Results S2 a, e, k). The placebo individuals featured a decrease of *Butyricimonas*, *Alistipes*, and *Ruthenibacterium lactatiformans* (SV774, SV465 and SV90) and a higher abundance of *Anaerotignum* (SV370), *S. thermophilus* (ST32) and *Turicibacter* spp. (SV56) from T1 to T3 (Supplementary Results S2 b, f, l).

Individuals who took probiotics in Cluster 2 showed a significant decrease of *Ruminococcaceae* (SV348) in T2 (Supplementary Results S2 g) while the placebo group were characterised by an increment of *Alistipes onderdonkii* (SV73) and *Lachnospiraceae* spp. (SV144) in T2 and T3, respectively, and a drop of *Blautia* spp. (SV562) and *Clostridium* spp. (SV512) in the same time points (Supplementary Results S2 h, n).

In Cluster 3, *Phascolarctobacterium faecium* (SV97) and *Subdoligranulum* spp. (SV16) distinguished the probiotic cohort at T1 and T2 which, conversely, showed negative CLR values for *Dialister invisus* (SV5) and *Eubacterium* spp. (SV256) at the same time points (Supplementary Results S2 d, j); this latter species (SV94) increased together with *Roseburia hominis* (SV161) in the placebo subjects, which also showed a decrease of *Intestinibacter bartlettii* (SV63) and *Bacteroides* (SV47) at T1 and T2, respectively (Supplementary Results S2 c, i).

Maltodextrin exerted an effect on the bacteriotype of each cluster

Since maltodextrins are included both in the placebo and in the probiotic products, their impact on each cluster's bacteriotype (included Cluster 1) was investigated (Supplementary Results S3). Focusing on SVs related to probiotics, SV34-*B. longum* generally increased in members of Cluster 1 at T2 and T3 and in Cluster 2 at T1 (Supplementary Results S3 b, d, g); as for SVs related to other *Bifidobacterium* spp., a general reduction of SV121 and SV15 was observed in both probiotic and placebo groups in Cluster 1 at T2 and in Cluster 2 at T3 (Supplementary Results S3 g, h).

Considering other *taxa*, Cluster 1 was characterised by a general increase in relative abundance of *S. thermophilus* (SV32), *R. timonensis* (SV25), *Turicibacter* spp. (SV56), and a decrease of *Butyricimonas* spp. (SV774) and *Lachnospira* spp. (SV144) from T1 to T3 (Supplementary Results S3 a, d, g).

Cluster 2 individuals were characterised by higher levels of *Faecalibacterium* (SV14), and *Roseburia inulinivorans* (SV54) at T1 and T3; while SVs related to *Escherichia* (SV1), *Agathobaculum* (SV87), *Blautia* (SV17), and *Eubacterium* (SV94) decreased from T1 to T3 (Supplementary Results S3 b, e, h).

Finally, in Cluster 3 positive CRL values were associated to *Anaerotignum*, *Pseudoflavonifractor* and *Sporobacter* (SV282, SV428, SV145) while negative values were related to *D. invisus* (SV5), *Bacteroides* (SV140) and *Blautia obeum* (SV29) (Supplementary Results S3 c, f, i).

Sequence variants related to *Limosilactobacillus fermentum* were detected in only one individual treated with probiotics

ASVs associated with *L. fermentum* and *L. plantarum*, included in the probiotic product, were investigated and checked through the 16S-based ID tool of EzBioCloud.net, https://www.ezbiocloud.net/identify; database version 2020.10.12). SV1273 associated with *L. fermentum* was detected only in one probiotic cohort sample at T1, while conflicting results were obtained using different databases related to *L. plantarum*, confirming that the V3-V4 region for this species is not informative (Figure 4).

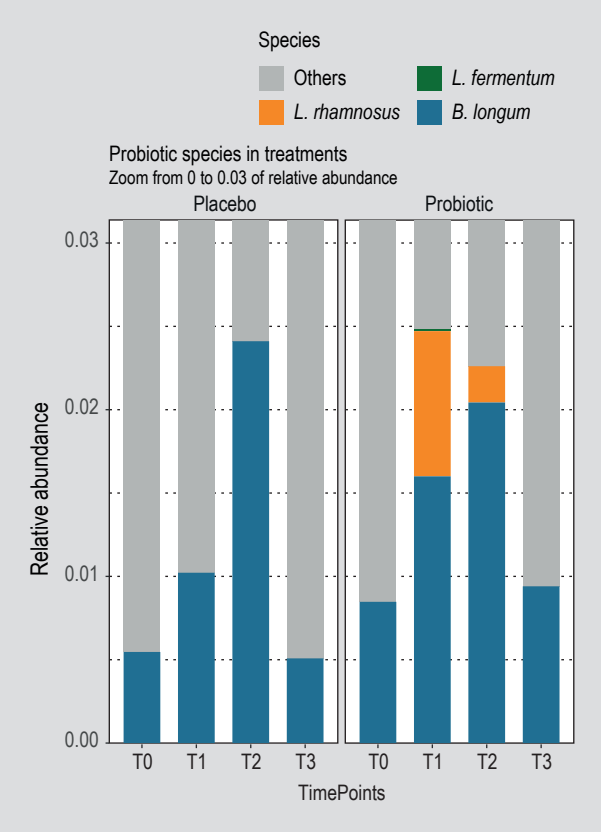

Figure 4. The species related to the probiotic compound were isolated and plotted in this barplot. The relative abundances percentages were zoomed to visualize the portion from 0 to 0.3 and stratified by time points and treatment type. The *Bifidobacterium longum* species is present in each time point for both the treatment types, showing a shared increasing trend. *Limosilactobacillus fermentum* was detected only for the second time point (T1) in the probiotic cohort. *Lacticaseibacillus rhamnosus taxa* were detected for both the second and the third time points, relative to the probiotic cohort.

Sleep quality and mood changes were detected in probiotics treated individuals of Cluster 1

As shown in Figure 5A, a significant reduction (P=0.03) was detected between time points T0-T1 and confirmed for T0-T2 and T0-T3 for the Pittsburgh Sleep Quality Index (PSQI). The PSQI global score is inversely correlated to the sleep quality (the lower the score, the better the sleep quality). The identified reduction indicates a sleep quality improvement for the probiotics treated individuals of Cluster 1.

Other significant changes were detected for the depression, anger, and fatigue subscales of the Profile and Mood State (POMS) psychological variables. Specifically, between T0-T1 and T0-T3 for anger (P=0.08, 0.02; Figure 5B) and depression (P=0.08, 0.06; Figure 5C) indexes, and between T0-T1 (P=0.04), T0-T2 (P=0.02), and T0-T3 (P=0.03) for the fatigue subscale (Figure 5D). It is noteworthy a clear descending trend for all mentioned psychological variables also in Cluster 3, even though these differences were not significant, probably due to the low sample size of the cluster. A similar pattern was not visible in Cluster 2.

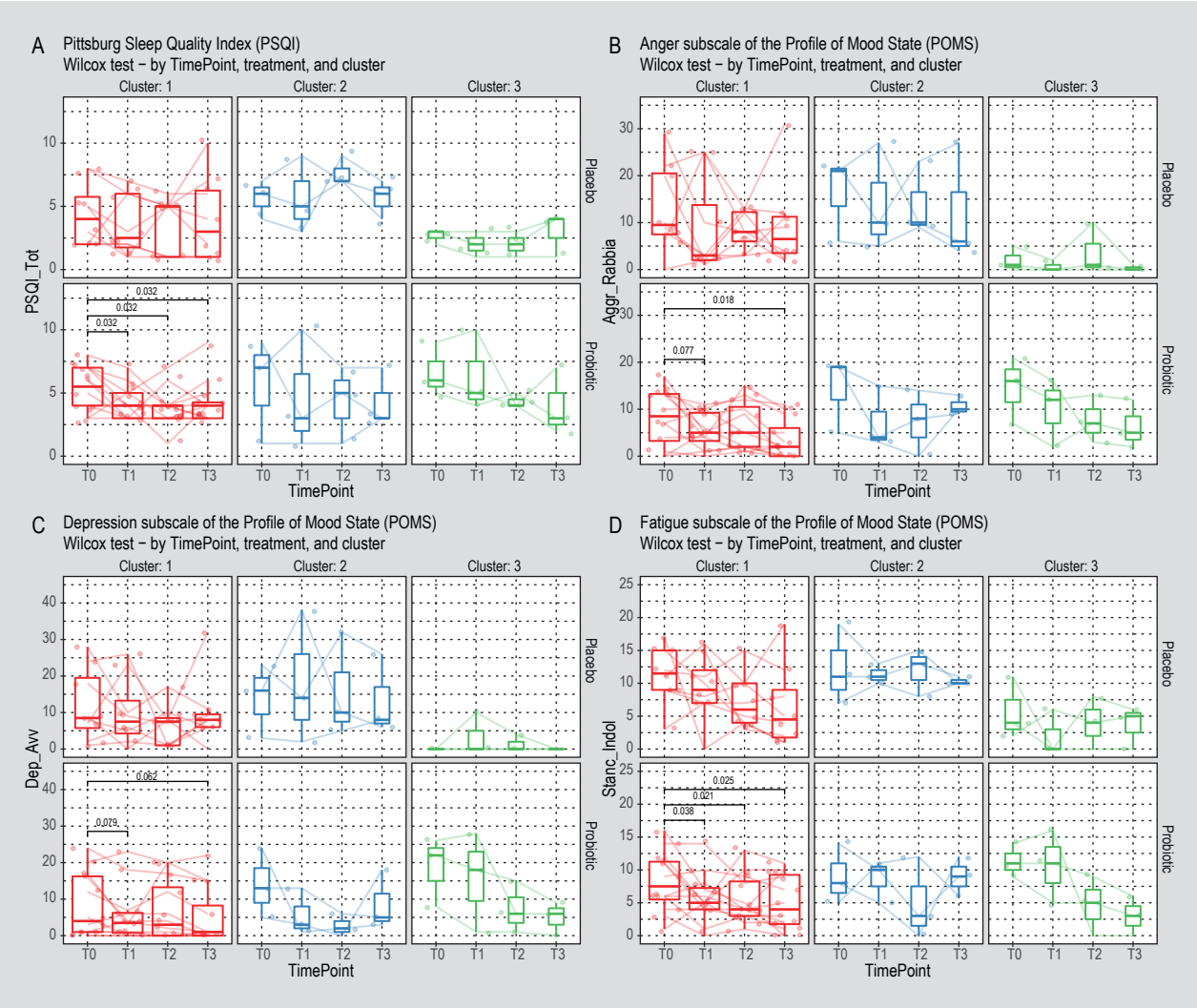

Figure 5. Wilcoxon Rank Sum tests between time points T0-T1, T0-T2, T0-T3. *P*-values are corrected for multiple testing using the Benjamini-Hochberg correction method and only adjusted *P*-values lower than 0.1 are reported. (A) Pittsburgh Sleep Quality Index (PSQI), stratified by cluster and treatment. (B) Anger subscale for the Profile of Mood State (POMS) psychological variable, stratified by cluster and treatment. (C) Depression subscale for the Profile of Mood State (POMS) psychological variable, stratified by cluster and treatment. (D) Fatigue subscale for the Profile of Mood State (POMS) psychological variable, stratified by cluster and treatment.

4. Discussion

Biodiversity measures stratified individuals in three clusters related to their microbiota

In the present work the possible effect caused by the intake of *B. longum*, *L. fermentum*, *L. rhamnosus* and *L. plantarum* strains for 6 weeks (followed by a 3 week-washout) on the gut microbiota composition of a cohort of 33 healthy subjects was investigated.

A robust bioinformatic pipeline was implemented to analyse and characterise the metabarcoding data; a series of exploratory analyses were performed targeting particular effects with a possible biological correspondence, which could be related to the cognitive and emotional improvements we assessed in our previous study (Marotta *et al.*, 2019). Biodiversity measures did not detect a significant diversity within the samples (α-diversity) but a sample-specific effect was found between samples (β-diversity). This finding led to perform a statistical analysis using a mixed-effects model, through which several minor significant effects were found. The 33 individuals were clustered into three groups/bacteriotypes at the baseline; each one of them responded distinctly to the treatments. Cluster 1 and 2 were most impacted by the probiotic treatment, while Cluster 3 responded more to the placebo treatment. Furthermore, Cluster 1 responded throughout the whole treatment, while Cluster 2 and Cluster 3 had, respectively, a late and an early response. Those behaviours reinforce the concept that individuals with diverse bacteriotypes might respond differently to the same treatment. In addition, stratification of individuals according to their bacterial composition may be useful to better understand and predict the responses to specific treatments, such as probiotic interventions (Cheng and Ning, 2019; Christensen *et al.*, 2018).

Maltodextrin has a bifidogenic effect

Focusing on SVs related to the probiotic species, it was observed that some *Bifidobacterium*-related SVs (SV15 and SV121) decreased both in the placebo and probiotic groups, but others, such as SV34-*B. longum* and SV228-*Bifidobacterium* significantly increased in the placebo cohort both in Cluster 1 and Cluster 2. Their presence in the control subjects, who were administered maltodextrin, is also in line with data reported in a previous work (Watson *et al.*, 2013) where authors observed that the majority of the culturable bifidobacterial strains (including 10 strains of *B. longum*) were capable of growing in maltodextrin rich media. Tandon and colleagues (2019) observed the same behaviour of the bifidobacterial population in a randomized, double-blind, placebo-controlled, dose-response relationship study led to investigate the efficacy of fructo-oligosaccharides on human gut microbiota, where maltodextrin was used as control.

From this perspective, this study does not include a true placebo cohort which may have prevented to capture time dependent oscillations in abundances of relevant *taxa*; further, since maltodextrins are broadly used as placebo treatment, their bifidogenic effect needs to be deeply evaluated in future clinical trials involving bifidobacteria. Considering our data, we suggest that participants of this study were subjected to two different treatments rather than one: a 'synbiotic' administration (probiotics and maltodextrin) and a 'prebiotic' assumption (maltodextrin).

Cluster 1 individuals displayed different gut composition following the prebiotic and synbiotic treatments

The gut composition of individuals who received the synbiotics in Cluster 1 are selectively characterised by the presence of *L. rhamnosus* and *C. leptum* at the beginning of the treatments and then of *Mogibacterium* (described in 2000 to include strains isolated from the human periodontal environment; Nakazawa *et al.*, 2000) at the end. *C. leptum* belongs to *Clostridium* Cluster IV which was reduced in patients with depression and anhedonia and it was negatively associated with scores in Quick Inventory of Depressive Symptoms-Self-Rated (QIDS-SR) and the Generalized Anxiety Disorder (GAD)-7 (Mason *et al.*, 2020). The effect of probiotics administration on the abundance of *C. leptum* was also observed by Sato and colleagues (2017) where patients with type-2 diabetes had higher counts of *C. leptum* after 16 weeks of probiotics (*L. casei* Shirota) assumption. The synbiotic treatment specifically reduced the levels of *Faecalibacterium*, which was among the signature *taxa* of this cluster. This taxon is usually lower in MDD patients but there is still a lack of congruence across investigations (Barandouzi *et al.*, 2020; Cheung *et al.*, 2019).

The prebiotic treatment is associated with higher levels of *Anaerotignum* (described in 2017 following the isolation of strains from a methanogenic reactor; Ueki *et al.*, 2017), *S. thermophilus, Turicibacter* and *D. invisus*. Among these species, it is interesting to report a reduced abundance of *Turicibacter* spp. was observed in socially defeated mice and was strongly correlated to pro-inflammatory cytokine changes within the prefrontal cortex (Szyszkowicz *et al.*, 2017). However, this has to be further investigated, as *Turicibacter* levels were also found to be higher in depressed subjects (Kelly *et al.*, 2016). As for *D. invisus*, it is usually lower in MDD patients and in other autoimmune diseases, including Crohn's disease, ulcerative colitis and rheumatoid arthritis (Lee *et al.*, 2019).

Prebiotic administration was also related to reduced levels of *Butyricimonas, Lachnospira*, *Alistipes* and *R. lactatiformans* (isolated in 2016 from human faeces; Shkoporov *et al.*, 2016). The decrease of *Butyricimonas* spp. may have a positive effect on the individuals: *Butyricimonas* members

were found to be at higher levels in the gut microbiota of patients with clinically significant depression compared to control patients (Jiang *et al.*, 2015). As for *Lachnospira*, no consensus data have been obtained so far, as Cheung and colleagues reported that this taxon could be related to MDD as well as to healthy subjects (Barandouzi *et al.*, 2020; Cheung *et al.*, 2019).

A significant association with depression was shown for *Alistipes* both in human cases as well as in mice subjected to stress over an extended time period. Since high levels of this *taxon* in the gut microbiota were also linked to chronic fatigue syndrome and irritable bowel syndrome (IBS), it has been suggested that *Alistipes* may promote depression through inflammatory pathways. In addition, *Alistipes* species are indole-positive and may thus influence tryptophan availability (the precursor of serotonin), disrupting the balance in the intestinal serotonergic system (Naseribafrouei *et al.*, 2014). They are also high metabolisers of proteins and amino acids and, as such, they could trigger the production of toxic compounds such as ammonia, putrescine, and phenol (Cheung *et al.*, 2019). However, these data are not concordant with what observed from Zheng and colleagues who reported that *Alistipes* were overrepresented in healthy control subjects compared to patients diagnosed with MDD (Zheng *et al.*, 2016).

Prebiotic treatment in Cluster 2 has an opposite effect of the synbiotic in Cluster 1

In Cluster 2, the prebiotic supplementation induced a general oscillation of the abundance levels of *Eubacterium*, *Blautia* and *Lachnospiraceae* spp., which characterized the bacteriotype of this group. Kim and colleagues (2020) suggested that the reduction in the relative abundances of *Eubacterium* is related to the increase of the brain-derived neurotrophic factor in the serum, improving brain functions. On the contrary respect to Cluster 1, the comparison between the prebiotic and synbiotic treatments showed that *Alistipes* spp. and *Lachnospira* spp. increased in Cluster 2 individuals.

The synbiotic intervention specifically led to a lower abundance of *Ruminococcaceae* (heterotypic synonym of family *Oscillispiraceae*): at family level, it was observed that these *taxa* were lower in depressed subjects compared to the control group (Jiang *et al.*, 2015) and were correlated with behavioural changes induced by stress in mice (Bangsgaard Bendtsen *et al.*, 2012). Conversely, prebiotics reduced the levels of *Blautia*, *Clostridium*, *Escherichia* and *Agathobaculum* spp.: although no data have been reported yet on the association of *Agathobaculum* (a strictly anaerobic and butyrate-producing strain isolated from the faeces of a healthy 23-year-old Korean female, Ahn *et al.*, 2016), with stress-related disorders and there is a lack of consensus related to the presence of *Escherichia* in MDD patients, this effect could be considered beneficial for this cluster, as both *Blautia* spp. and *Clostridium* are usually found at higher levels in patients with MDD (Cheung *et al.*, 2019; Jiang *et al.*, 2015). Conversely, *Faecalibacterium* and *R. inulinivorans* increased at the end of prebiotic treatment: this species has been shown to have beneficial effects in specific conditions (i.e. atherosclerosis, Liu *et al.*, 2020a) but no particular correlation has been found with mental or stress-related disorders (Cheung *et al.*, 2019).

Treatments in Cluster 3 changed the relative abundance of *Phascholarctobacterium faecium*, *Subdoligranulum* and *Eubacterium* spp.

The assumption of the synbiotic in Cluster 3 individuals increased the relative abundance of *Subdoligranulum* and *P. faecium* and *Eubacterium* spp. (which characterised the bacteriotype at the baseline). It is interesting to note that *P. faecium* and, in general, family *Acidaminococcaceae* are more correlated to patients with active MDD (Jiang *et al.*, 2015) and with both IBS and depression (Jeffery *et al.*, 2012) rather that with healthy subjects. On the contrary, *Subdoligranulum* are depleted in subjects with IBS and depression, so its presence in the synbiotic cohort can be interpreted as a positive effect of this treatment (Liu *et al.*, 2020b). This *taxon* is capable of producing short chain fatty acids (in particular butyrate) that protect the intestinal mucosa and regulate the immune system. More specifically, short-chain fatty acids (SCFA) play an important role in the differentiation of T cells and as histone deacetylase inhibitors, which were found to have immunosuppressive and anti-inflammatory functions and have been explored as potential novel antidepressants (Cheung *et al.*, 2019). *R. hominis*, *Anaerotignum* spp. (similarly to Cluster 1), *Pseudoflavonifractor*, *Eubacterium* (conversely to the synbiotic treatment) and *Sporobacter* (among the signature *taxa* of this Cluster) were significantly abundant following the prebiotic treatment while *I. bartletti*, *Bacteroides* and *B. obeum* decreased. *Sporobacter* and *Pseudoflavonifractor* are among the common *taxa* found in the human gut microbiota; as for *R. hominis*, although no data are available regarding the direct positive or negative connection of this species and stress-related disorders, the reduction in the abundance of butyrate-producing *Lachnospiraceae* members, (including *R. hominis*) which are beneficial for the integrity and function of intestinal barrier, was involved in the formation of stress-induced visceral hypersensitivity for which *R. hominis* was proposed as a candidate potential probiotic (Zhang *et al.*, 2019). As for *I. bartlettii*, it is interesting to report that it was found more frequently in the faecal samples of children with neurodevelopmental disorders compared to the control subjects (Bojovic *et al.*, 2020). Finally, *Bacteroides* spp. exhibited divergent directionality and were found to be associated both with MDD as well as with healthy status, so no conclusions

can be made on the effect of their reduction in this cohort (Barandouzi *et al.*, 2020).

5. Conclusions

Although no consensus observations on the biological significance of particular components of the gut microbiota on mood disorders have been obtained yet, the present study shows that both the 'synbiotic' and the 'prebiotic' intake over a period of 6 weeks significantly changed the composition of the gut microbiota.

A debate is still ongoing whether the probiotic supplementation alters successfully the microbiota composition (Kristensen *et al.*, 2016); in this perspective, Pinto-Sanchez and colleagues (2017) observed that probiotic administration in patients with IBS led to changes in urine metabolic profiles, brain activity and to antidepressant effects, but no detectable effects on the gut microbiota composition were noticed. Nevertheless, it has been demonstrated that probiotic treatments impact on both the gut microbiota gene expression (with potential anti-inflammatory effects) and the gut barrier function (as also shown by the probiotic strains used in the present study – Pane M, personal communication); which can lead to an effect on the cognitive function (Chahwan *et al.*, 2019).

Overall, this study offers evidence that probiotics supplementation has variable impacts depending on the gut microbiota bacteriotypes (i.e. Cluster 1, 2 and 3). In some cases, the shifts were towards microbial populations generally related to a healthy mood status (i.e. higher abundance of *C. leptum* and *Subdoligranulum* in Cluster 1 and 3, respectively) and suggests some mechanisms (i.e. SCFA production related to *Subdoligranulum*) which might rationalise the positive effects of the supplementation on the depressive mood state and sleep quality we observed in our previous work (Marotta *et al.*, 2019). Particularly, variations of microbiota compositions were found to be statistically related to sleep quality improvement and to a descending rate of depression, anger and fatigue in probiotic-treated individuals of Cluster 1.

Overall, these findings should be interpreted with caution: first of all, further studies are necessary on a larger and more homogeneous cohort of individuals, taking fully into account the effects of gender, diet, body mass index, presence of inflammation, bacteriotypes, and other factors that may be important covariates affecting the faecal microbiota.

As for diet, it is well established that it is one of the major modulators of the microbiota, therefore its monitoring is of utmost interest to better understand microbial dynamics and link them to other metabolic and physiological parameters (Bowyer *et al.*, 2018). However, the monitoring of young healthy individuals for 6 weeks (9 including washout) proved to be a very challenging task, with too partial data that could not be used for associations.

Indeed, in this study, we tried to move from an effectiveness perspective (the whole cohort) to an efficacy-focused one (the clusters/bacteriotypes), revealing some complexities on the microbial background related to the effects described by Marotta *et al.* (2019) on almost the same cohort. Shedding light on these variables, especially on a healthy individual's cohort, is expected to allow a better development of psychobiotic treatment strategies. This will contribute to the definition of probiotics as an adjunct therapy or for the prevention of mood-related disorders.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0137.

Table S1. General information about samples and psychological variables.

Figure S1. *Taxa* prevalence exploration and prevalence filtered taxa.

Figure S2. Rarefaction curves as a function of sampling depth.

Figure S3. The top 10 genera and related phyla of membership are depicted in this image; *taxa* are plotted for their relative abundance over the different time points, facetted for the different type of treatment.

Figure S4. Shannon-Wiener α-diversity, over time points, treatment type and faceted by gender.

Figure S5. Statistical analysis steps from β-diversity ordination choice, clustering, and the mixed-effects regression models.

Figure S6. Bidimensional representation of β-diversities.

Figure S7. Bidimensional representation of β-diversity by timepoint and treatment, and by sample.

Figure S8. Linear mixed effects regression model coefficients.

Supplementary Results S1-S3.

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Conflict of interests

Marco Pane and Angela Amoruso are employed by Probiotical Research Srl (Novara, Italy). Microbion is a contract research organization providing support to clinical trials, but no conflict of interest is foreseen within the present study.

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