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ORIGINAL ARTICLE

Impact of a probiotic diet on well-being of healthy senior: THE PROBIOSENIOR PROJECT

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Abstract

Aims: The aim of this work was to assess the effects of a probiotic diet on well-being of healthy seniors living in boarding and private homes in Marche Region, Italy. In particular, we focused on the modulation of high-sensitivity C-reactive protein (HsCRP), intestinal microbiota and short-chain fatty acids (SCFAs).

Methods and Results: Ninety-seven healthy seniors took part in a double-blind, placebo-controlled feeding study (59 fed probiotics, 38 fed placebo) for 6 months. Each volunteer ingested daily one food product or a dietary supplement enriched with Synbio® blend (Synbiotec Srl, Camerino, Italy) or the placebo (control group). Blood and faecal samples were collected before and at the end of the intervention period to perform biochemical and microbiological analyses. The serum HsCRP difference value after 6 months of treatment was significantly higher in the probiotic group than placebo ($p < 0.05$). After the intervention, a significant increase in faecal lactobacilli and a bifidobacteria increase in more participants were observed in the probiotic group. The 16S NGS analysis on the probiotic group showed a decreasing trend of Proteobacteria at the end of the treatment and conversely, an increasing trend of Actinobacteria and Verrucomicrobia phyla, to which the increase of Akkermansiaceae and Bifidobacteriaceae contributes at the family level. Finally, total short-chain fatty acids (SCFAs) and butyric acid were significantly higher in the probiotic group at the end of the treatment respect to the beginning.

Conclusions: Overall, this study emphasizes the beneficial anti-inflammageing effect of a prolonged diet based on functional foods enriched with Synbio® through the modulation of the intestinal microbiota and the consequent increase in the SCFA production.

Significance and Impact of the Study: Synbio® integration in elderly daily diet may be a preventive strategy to support healthy ageing.

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KEYWORDS
butyric acid, functional foods, gut microbiota, healthy ageing, probiotics, short-chain fatty acids, Synbio®

INTRODUCTION

As in many Western countries, in Italy, the elderly population has been increasing markedly. The proportion of elderly in Europe is expected to increase from 17% in 2010 to approximately 30% by the year 2060 (Eurostat, 2011). This demographic shift will most probably increase the number of people with age-related diseases and disabilities; therefore, it is crucial to find strategies that decrease morbidity by slowing down the ageing process and thereby increase the number of elderly in good health. Of all environmental variables, diet and lifestyle are potential factors that can be changed by elderly people themselves. The ageing is associated with a chronic, low-grade inflammatory condition that is a well-established background process in many age-related diseases (Franceschi & Campisi, 2014). Inflammageing in the elder population is considered a risk factor for the development of several pathologies, and many recent studies investigate the use of various inflammatory biomarkers such as C-reactive protein (CRP), interleukin-6 (IL-6) and IL-1, and as predictors of physical and cognitive performance amongst elderly (Pepys & Hirschfield, 2003).

CRP plays important roles in inflammatory and disease processes and host responses to infection, and the normal levels increase with ageing. Its elevated levels in serum have been identified to accompany increased vulnerability for disease and mortality in older patients, such as an increased risk of sarcopenia, cardiovascular pathologies, disability and cognitive decline in individuals over 65 years old (Velissaris et al., 2017). Furthermore, a myriad of clinical changes accompanies ageing. Older adults have a high prevalence of comorbid disease and concomitant exposure to different medications, including antibiotics. The inflammageing, through changes in lifestyle and age-related modifications in intestinal physiology, such as impaired dentition and salivary function, decreased motility with constipation, diverticular disease and dietary changes, profoundly affects also the homeostasis of the gut microbiota (GM) (Zapata & Quagliarello, 2015). The intestinal microbiota, in eubiosis condition, plays a key role in host's well-being, providing nutritional, metabolic and immunological benefits such as the synthesis of folate and Vitamin B12, two critical vitamins in the elderly. Notably, the composition and abundance of individual GM changes until adulthood, but a major shift in its composition, called dysbiosis, can trigger harmful local and systemic inflammation. Recent reports indicate that dysbiosis is increased in ageing and that the gut microbiota of elderly people is enriched in pro-inflammatory commensals at the expense of beneficial microbes. Dysbiosis also triggers a chain of pathological and inflammatory events, such as the alteration of levels of microbiota-affected metabolites, impaired function and integrity of the gastrointestinal tract, and increased gut leakiness (Ragonnaud & Biragyn, 2021). The change of some key GM members, as *Clostridium coccoides* and *Eubacterium rectale* groups and *Clostridium* cluster IV, is associated with the depletion in butyrate and in an overall reduction of short-chain fatty acids (SCFAs) in the gastrointestinal (GI) tract. SCFAs, mostly acetate, butyrate and propionate, are microbiota metabolites representing an important energy source for the host, but of note, they can modulate the immune functions and concur to maintain a functional GI epithelial barrier (Mäkivuokko et al., 2010). Consequently, the reduction of them can involve nutritional and immunological effects, contributing to the health impairment of older people; moreover, the increase in pro-inflammatory Enterobacteriaceae and the concomitant decrease in anti-inflammatory GM components result in the establishment of a pro-inflammatory microbial community, which can support the inflammageing process (Biagi et al., 2012; Rampelli et al., 2013). In this scenario, the microbiome manipulation of elderly adults could be a parallel, innovative strategy to influence the development of ageing-associated comorbidities (Zapata & Quagliarello, 2015). PROBIOSENIOR project wants to develop a platform that enables the use of new functional foods and nutraceuticals containing probiotics, made with innovative technologies. The supplementation with these new foods should be able to improve parameters related to the ageing process, in particular the immune and antioxidant tone, inducing optimal GM changes and so in the gut 'performance'. Different probiotic products are already in the market, but none is specific for the elderly and above all, they do not have an innovative distribution, as proposed by this project. Further innovation factor is the experimental research phase to realize functional foods enriched with appropriate amount of high-quality, patented probiotic bacterial strains to guarantee maximum functionality. In this paper, we reported the effects of probiotic supplemented diet on plasma concentration of high-sensitivity C-reactive protein (hsCRP). HsCRP was selected as a primary target because it is an internationally recognized marker of low-grade inflammation and cardiovascular risk (Pearson et al., 2003). Our secondary

objective was to analyse the modulation of intestinal microbiota composition and SCFA concentration.

MATERIALS AND METHODS

PROBIOSENIOR study design

The study was a randomized, double-blind, placebo-controlled study assessing the effect of daily consumption of Synbio® (Synbiotec Srl, Camerino, Italy), a probiotic mixture 1:1 of *Lacticaseibacillus rhamnosus* IMC 501® and *Lacticaseibacillus paracasei* IMC 502® (Silvi et al., 2014; Verdenelli et al., 2009, 2011) by probiotic-enriched foods or by dietary supplement on healthy seniors' status. Specifically, the supplementation should improve the parameters related to the ageing process, inflammation and the general well-being of subjects.

The study was conducted in five different boarding homes and several private homes of senior, all with the site in Marche Region, Italy. The subjects were randomly assigned to one of two parallel groups, to receive either probiotic-enriched foods and capsules or the respective placebo. For the allocation of the participants, a computer-generated list of random numbers was used. The participants were enrolled by the principal investigators at each site. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and national laws and regulations. The study protocol was approved by the Ethics Committee (CERM, Marche, Italy). The participants gave written informed consent for the enrolment.

Functional products

Six different food products were used as carriers for delivering probiotic bacterial strains (Synbio® blend, lyophilized powder containing 5 billion live cells per daily dose): yoghurt, 'mozzarella' cheese and fruit smoothies (Synbiofood, Civitanova Marche, Italy); 'ricotta' cheese and 'primo sale' cheese (Caseificio Val d'Apsa, Urbino, Italy); chocolate (local chocolate maker). In addition, Synbio® in capsules (containing 5×10^9 CFU/capsule) was also provided (Synbiotec Srl, Camerino, Italy).

All the food products were enriched with the Synbio® mixture during their usual production process, as previously reported (Verdenelli et al., 2009), directly on the production site and after a careful analysis of the best method of inoculum for each specific product. For the placebo control group, the foods without probiotics were produced and provided by the same producers of the foods with supplement. The placebos allocated to the capsules control group were identical capsules containing pure maltodextrin, instead of probiotics. Maltodextrin is completely digested before entering the colon, so it does not affect the intestinal microbiota. The placebo capsules were produced and provided by Synbiotec Srl.

The study was performed with a 4-week run-in period, followed by a 24-week intervention period. Volunteers received weekly six different probiotic or placebo foods, for 6 months, to be consumed one per day as defined by the nutritionist in charge. They had to note any adverse events (local or general) during the study and to consult their physician if necessary.

Participants

Eligible participants were all healthy male and female seniors, aged >65 years with hsCRP >1 mg L^{-1}. Exclusion criteria were assessed through an interview and were based on current physical status and history of health conditions, including chemotherapy treatments, severe respiratory, hepatic and/or renal insufficiency; use of anti-inflammatories in the previous 4 months; malnutrition: BMI <18.5 kg m^{-2} or weight loss >10% in the last 6 months. Individuals having daily habits to consume probiotic or probiotic-based products were either excluded or requested to stop probiotic consumption 8 weeks prior to the current trial. Antibiotic treatment in the previous week was disallowed.

Participants were withdrawn from the study if they had more than 1 day per week or 4 days per month of non-compliance to the assigned diet, used disallowed food/nutraceutical supplementation or experienced concomitant diseases requiring treatment with antibiotics or anti-inflammatory drugs.

Prelude

The past PROBIOSENIOR 18 months, dedicated to experimentation, has heavily suffered the pandemic SARS-CoV-2 effects, compromising both the experimental intervention method and the achievement of users. In detail, the clinical trial started in May 2019, and it was interrupted in February 2020, resulting in partial data loss because of the impossibility to recover the post-treatment biological samples. Due to these criticalities and to guarantee a sufficient number of subjects, 20% of the volunteers shortened the treatment period to 3 months.

Compliance

The caregivers were in charge to check the compliance of the subjects living in the boarding home. The use of the study products was recorded daily, and the records were checked. The subjects living in their own house received the combination of functional foods inside to a smart box designed for monitoring their compliance (Fidoka Srl, San Ginesio, Italy). The box had six places in which the products were positioned and when the subject consumed a functional food, a signal was delivered to an aerial and recorded.

Assessment of outcomes

The primary study outcome was a decrease in plasma concentration of high-sensitivity C-reactive protein (HsCRP). Secondary outcome measures included the monitoring of selected bacterial groups of gut microbiota and SCFA level in faecal samples.

HsCRP level

Blood samples were collected into BD Vacutainer® tubes (Becton Dickinson, NJ, USA). An aliquot of the blood was used for haematological determination. Peripheral blood was taken before (T0) and after (T1) the probiotic/placebo supplementation. The sampling at T1 was performed the day after the end of treatment. Blood samples were delivered to the Fioroni laboratory (San Benedetto del Tronto, AP, Italy) and analysed within 24 h by immunoturbidimetric method.

Monitoring of selected bacterial groups

Faecal samples were collected and delivered to the laboratory and stored at −20°C until use. The sampling was performed before (T0) and after (T1) probiotic/placebo intervention; faecal samples at T1 were collected within 2 days from the end of the supplementation.

For the quantification of the six selected bacterial groups in faecal samples, first bacterial DNA was extracted using a Stool DNA Isolation Kit (Norgen Biotek corp., Thorold, Canada) and then quantified by Real-Time PCR (qPCR) procedure. The bacterial groups of interest were *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides-Prevotella-Porphyromonas* spp., *Staphylococcus* spp., *Clostridium coccoides-Eubacterium rectale* group and Enterobacteriaceae. Specific primers were used and SYBR Green quantitative Real-Time PCR amplification was performed, using an iCycleriQ Real-Time Detection System (Stratagene, La Jolla, California) associated with MXP Software and using the conditions and the standard curves for each bacterial group (Nasuti et al., 2016).

16S NGS and analysis

Faeces collection and DNA extraction

An aliquot of faecal samples was also collected into individual sterile tubes and quickly transferred into a −80°C cryogenic freezer until DNA extraction. The microbial DNA was extracted from faecal samples using a Stool DNA Isolation Kit (Norgen Biotek Corp) in accordance with the manufacturer's protocols.

16S library preparation and next-generation DNA sequencing

The V3-V4 hypervariable regions of 16S rDNA were amplified using universal primers (341F 5′- TCGTCGGCAG CGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG-3′, 805R 5′- GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC -3′) following the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 B).

Libraries were sequenced using the MiSeq Illumina Platform (Illumina Inc. San Diego, CA, USA) with a 2x250 paired-end run. Poor quality reads were filtered with Trimmomatic (Bolger et al., 2014); paired-end reads were merged using FLASH (Magoc & Salzberg, 2011) and processed with VSEARCH (Rognes et al., 2016) to detect potential chimera sequences and to cluster merged amplicons in operational taxonomic units (OTUs), with a minimum pairwise identity threshold of 97%. The NCBI 16S RefSeq database (O'Leary et al., 2016) was employed for taxonomic classification. Evaluation of microbial alpha (Adv, Chao1, Simpson's and Shannon's diversity) and beta (UniFrac distances, Bray–Curtis dissimilarity) diversity measures were performed using an internal pipeline. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways potentially affected by groups of bacteria.

SCFA determination

An aliquot of the same faecal samples, collected at T0 and T1, was used for SCFA analysis. The method consists of extraction of the SCFAs (acetic, propionic, i-butyric, butyric, i-valeric, valeric, i-caproic and caproic

acids) by ethyl ether after acidification of the samples. The SCFAs extracted are directly analysed by GC-FID without derivatization and separated on a polyethylene glycol nitroterephthalic acid modified coated capillary column, following the procedures described by Scortichini et al. (2020).

Sample size

This clinical study aimed to verify if the administration of probiotic functional foods and/or supplements can reduce the levels of HsCRP in the blood, that in the 70% of elderly population are between 1 and 3 $mg\,L^{-1}$ and in the 30% higher than 3 $mg\,L^{-1}$ (Imhof et al., 2003). The calculation of the sample size assumed that the primary objective was to detect a 50% reduction in the levels of HsCRP in subjects who received probiotics, compared to the pretreatment conditions. To detect this difference with a two-tailed test, significance level of 0.05 and a power of 80%, approximately 93 subjects per group had to be recruited. Considering a dropout rate of 10%, the number of subjects to be recruited was at least 100 per group.

Statistical analysis

All the analyses were performed on the per-protocol population. Comparison of response rates between groups was performed using a chi-squared test. For the faecal analysis, one-way analysis of variance (ANOVA) between (treatment) and unpaired *t*-test within (time) was employed. When appropriate, a post hoc analysis was carried out using Tukey's post hoc test. Differences in relative abundances of taxa between time points were evaluated using the Wilcoxon test. Unless otherwise stated, all assays were performed in either triplicate or three independently repeated experiments, and all the values presented here are means ± *SEM*. $p < 0.05$ was considered statistically significant.

RESULTS

Demographic and general characteristics of participants

The experimentation phase was conducted in the three regional areas, with the recruitment of eligible subjects in the residential setting as well as volunteers in their private houses.

In detail, the participants were recruited in boarding homes belonging to different 'Aree Territoriali Sociali (ATS)' of Marche Region, ATS 16 ('P. Burocchi', Penna San Giovanni; 'L'Immacolata', Sant'Angelo in Pontano; 'Ex Opera Pia Bonfranceschi', Loro Piceno - MC), ATS 17 ('ASP Lazzarelli, San Severino Marche; 'A. Chierichetti', Gagliole - MC). Free-living subjects have been recruited in private houses in ATS 18 (Camerino - MC), ATS 16 (San Ginesio - MC) and in nursing homes/long-term hospitalization facilities in Ancona province (Villa Igea - AN). At baseline, PROBIOSENIOR participants were on average 81.0 ± 9.0 years with a BMI of 27.1 ± 4.1 $kg\,m^{-2}$, and 33% of the participants were men (Table 1). The subjects in placebo and probiotic groups did not significantly differ at starting of the study. Nobody experienced side effects related to probiotic treatment. Baseline characteristics were well matched between the two groups. Two hundred and thirty (230) participants were assessed for eligibility (Figure 1), and a total number of 97 participants met the including criteria, coming from boarding homes and private houses.

HsCRP level

For the comparison of the HsCRP level between the placebo and the probiotic groups, the difference occurred from the end (T1) and the start of treatment (T0) has been used (Figure 2). As already mentioned, the number of subjects in the study is not particularly high. However, the HsCRP difference value of the probiotic group was statistically significantly higher than the placebo group ($p < 0.05$), evidencing that the supplementation influenced the low-grade inflammatory status of the subjects. Considering two different periods of probiotic supplementation, the 6 months showed a significant effect ($p < 0.05$) on the protein level of the subjects, respect to those on a shorter supplementation (less than 6 months).

TABLE 1 Characteristics of participants' population in the study

	Entire sample ($n = 97$)	Probiotic ($n = 59$)	Placebo ($n = 38$)
Age, years (Mean ± *SD*)	81.4 ± 9.6	81.3 ± 10.1	81.5 ± 8.9
BMI, kg/m^2 (Mean ± *SD*)	27.1 ± 4.1	26.7 ± 3.5	27.0 ± 4.1
Sex			
Female %	66.7	67.7	66.7
Male %	33.3	32.3	33.3

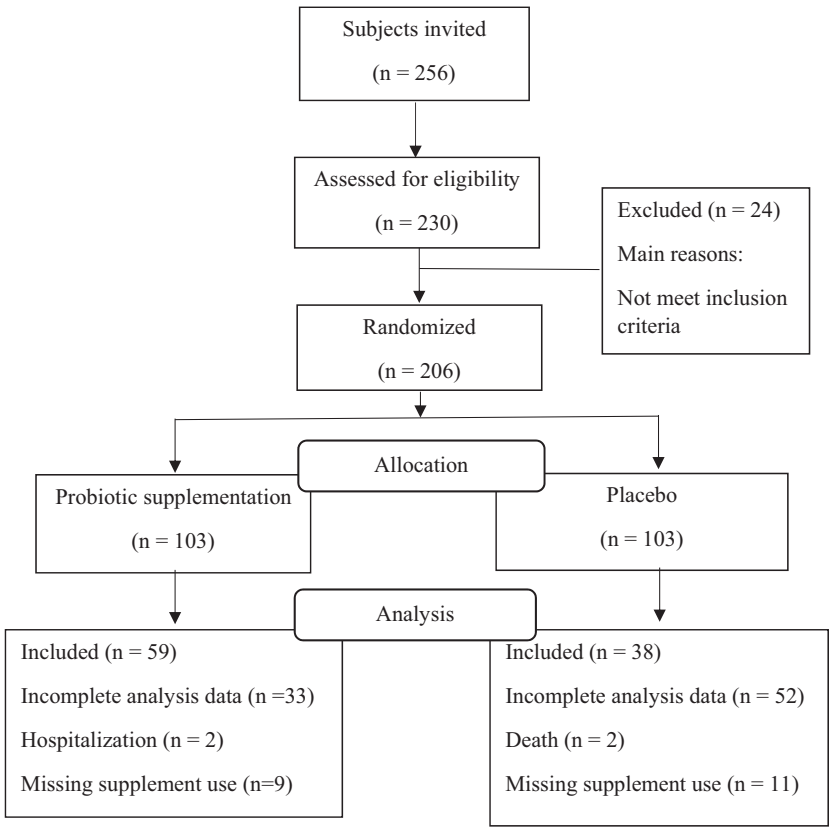

FIGURE 1 Flow chart of participants.

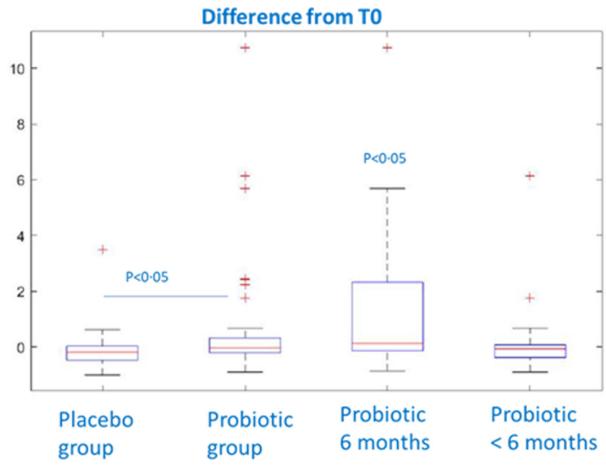

FIGURE 2 Difference values of HsCRP level between T0 and T1 related to the two experimental groups and times of probiotic supplementation.

Monitoring of selected bacterial groups

Figure 3 shows the selected bacterial groups count in the studied population, taking in consideration the different location of the subjects and their gender. The bacterial profile corresponds to the baseline status of the population and it is in line with the expected situation for elderly subjects. We documented a big inter-individual variability in each group, and between different groups or genders. The intestinal microbiota profile has been studied before and after the dietary supplementation to monitor all the changes occurred, considering the mean values of the placebo and probiotic groups. No significant changes were recorded in the placebo group except for a statistically significant decrease in bifidobacteria at T1 (Figure 4). The main finding in the probiotic group was a significant increase in *Lactobacillus* spp. after the administration (Figure 4). Moreover, in the probiotic-supplemented group, the long-term administration positively influenced the effectiveness of the supplementation itself, leading to a significant improvement of *Lactobacillus* spp. count in 100% of subjects who continued for 6 months, respect to the group supplemented for less than 6 months. Overall, no significant changes in the faecal abundances of all other bacterial groups were documented after both interventions. However, the proportion of people with increased/decreased bifidobacterial concentration after intervention looked to be different between the two study arms (chi-square, $p = 0.001$). In particular, *Bifidobacterium* spp. increased in 45/59 (76%) participants in the probiotic group, with a mean 6.3-fold ratio. An opposite trend, with decreasing values, was observed in 24/38 (63%) participants in the placebo group.

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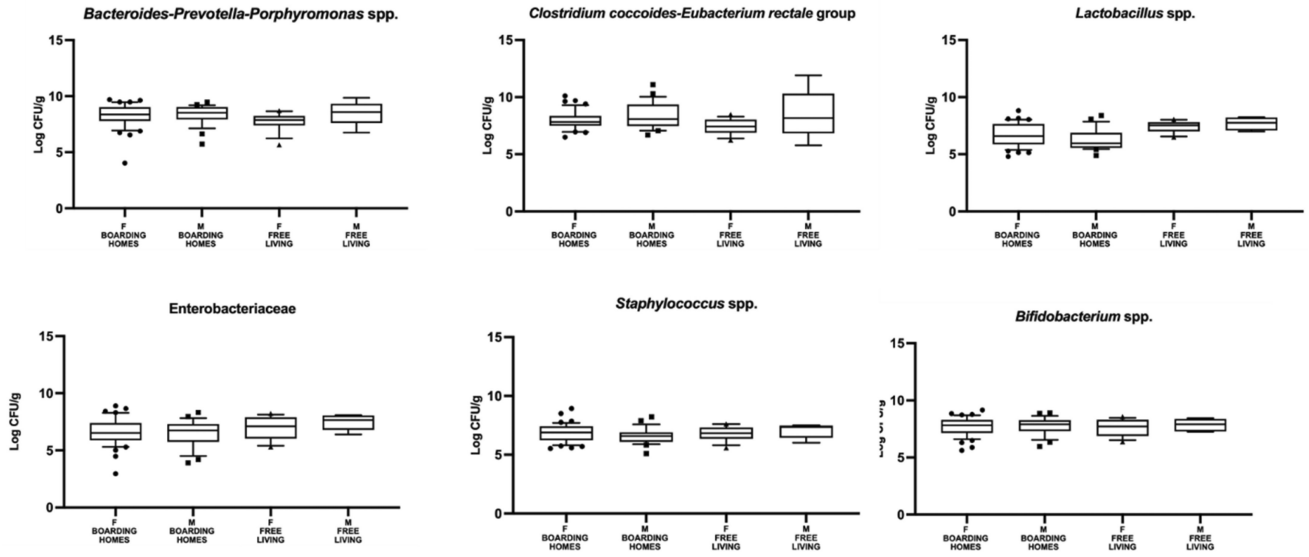

FIGURE 3 Baselines bacterial profiles of the studied population in relation to living locations and gender.

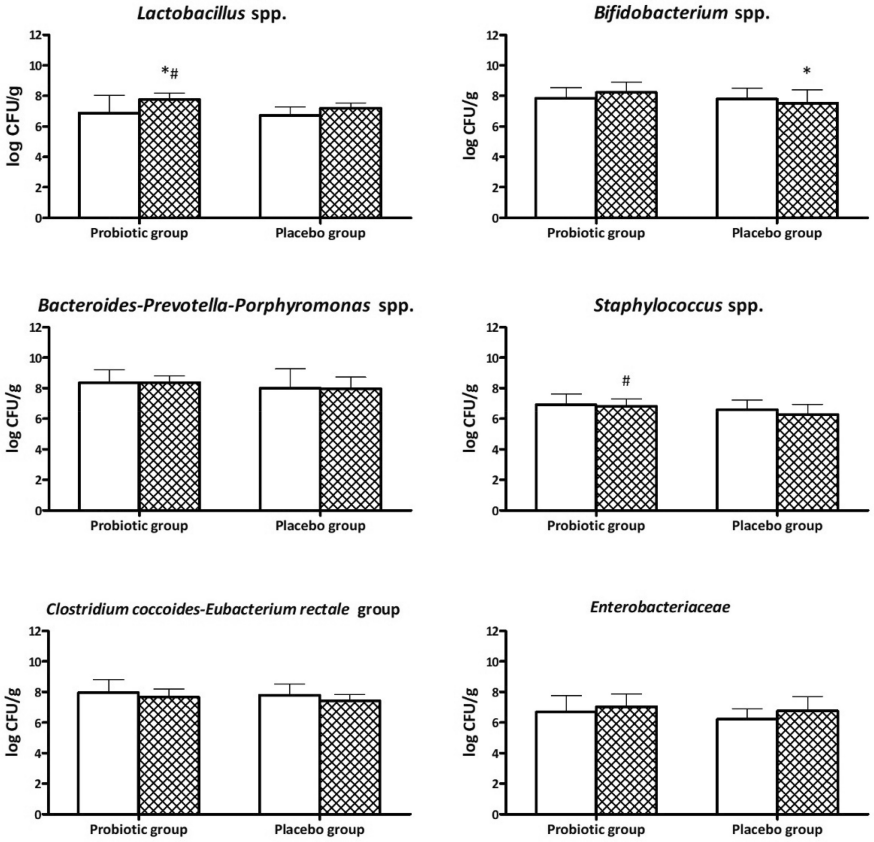

FIGURE 4 Faecal bacteria concentration (log CFU $g^{-1} \pm SD$) of target bacterial groups during the different time points (□T0 and ⊠T1) relative to the two groups of volunteers (probiotic group and placebo group). *significantly different ($p < 0.05$) from T0 and #from placebo group by Tukey's test following one-way ANOVA.

16S NGS and analysis

To characterize the effects of probiotic supplementation on the gut microbiota of participants, V3-V4 regions of the 16S rRNA genes were sequenced using the Illumina MiSeq platform. We analysed the GM composition of all the samples in the probiotic group from two different time points, T0 and T1. The Shannon, Simpson, chao1 and adv indices were used to evaluate the change of α-diversity before and after the probiotics were consumed (Figure 5). No significant difference in terms of species richness was observed ($p > 0.05$). The Bray–Curtis distances used to reveal β-diversity, that is, bacterial structural differences before and after the intervention of probiotics did not show a congruent directional pattern between the two groups. At the phylum level, about 99% of the sequences belonged

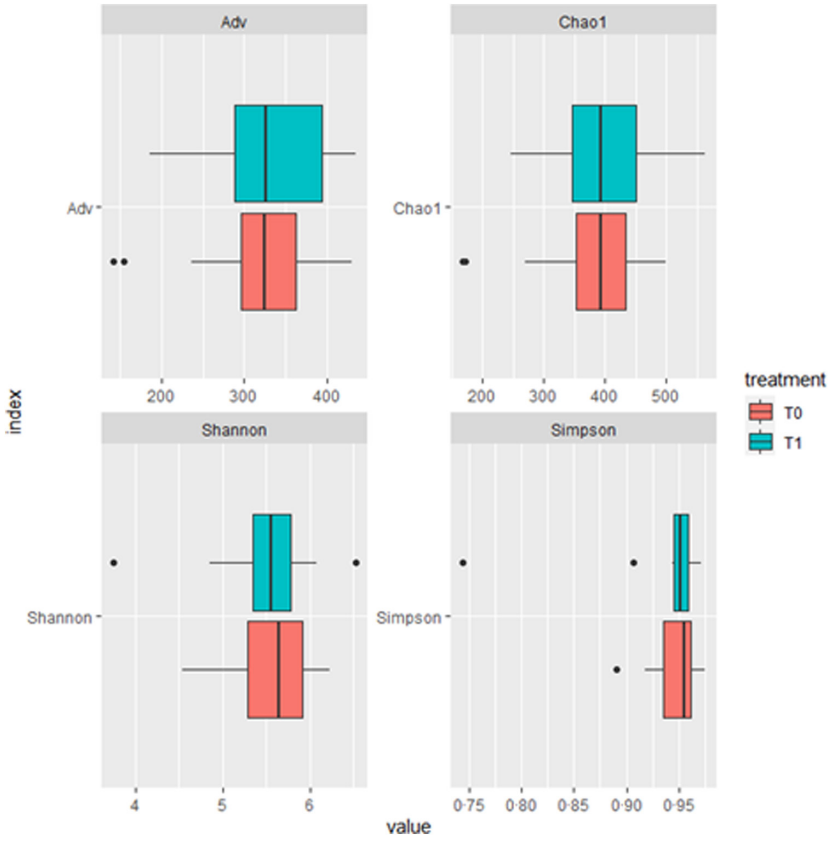

FIGURE 5 Comparison of alpha-diversity indices Adv, Chao1, Shannon and Simpson, in faecal samples collected at T0 and T1 in subjects in the probiotic group.

to the five most populated bacterial phyla in both time points, namely Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia and Actinobacteria (41.95% vs 40.68%, 44.17% vs 45.28%, 8.63% vs 5.72%, 1.84% vs 3.22% and 2.13% vs 3.93%, respectively; Figure 6). At the family and genus level, 25 families and 27 genera were the dominants in both time points (relative abundance of >1%; Figure 6). No significant difference in Firmicutes and Bacteroidetes abundance was identified after probiotic treatment (T1). Anyway, a significant change in the relative abundance of Proteobacteria, Verrucomicrobia and Actinobacteria was documented. Interestingly, Proteobacteria, which has been associated with increased gut inflammation and dysbiosis, was more abundant in seniors at T0, decreasing after the probiotic diet. Conversely, the Actinobacteria and Verrucomicrobia phyla increased after probiotic treatment. Within the Verrucomicrobia phylum, the increase of the Akkermansiaceae and *Akkermansia* spp. at the family and genus level, respectively, is noteworthy. Also, the levels of total bifidobacteria were positively affected by the probiotic treatment as evidenced by significant increase in Actinobacteria at the phylum level, to which contribute an increase in Bifidobacteriaceae and *Bifidobacterium* spp. at the family and genus level, respectively. Next, we explored the metabolic GM activity in the probiotic group through PICRUSt analysis to predict the functional profiling of the microbial communities based on the 16S rRNA gene sequences. Interestingly, after the administration of probiotics, a significant increase in some metabolic pathways was observed (Table 2). In detail, height functional pathways were significantly modulated by the probiotic treatment, especially some amine, aromatic compounds and amino acid degradation pathways.

Characterization of SCFA profile

Figure 7 shows the levels of faecal SCFAs at baseline conditions. We documented a high inter-individual variability in each group, and between different groups or genders.

Figure 8 shows the single and total faecal SCFA levels in placebo and probiotic groups after the supplementation period. Compared with the placebo, the probiotic group had a strong but not significant increase of total SCFA concentration at the end of the intervention. However, analysing the variations within the same group (placebo or probiotic) between time T0 and time T1, a significant difference can be detected in the probiotic group at time T1, for total SCFAs and for butyric acid. Acetic acid was the major SCFA in both groups and a slight, although nonsignificant, increase of acetate could be detected in the probiotic group after treatment. In general, the levels of the resting SCFA remained mainly unchanged without statistically significant differences.

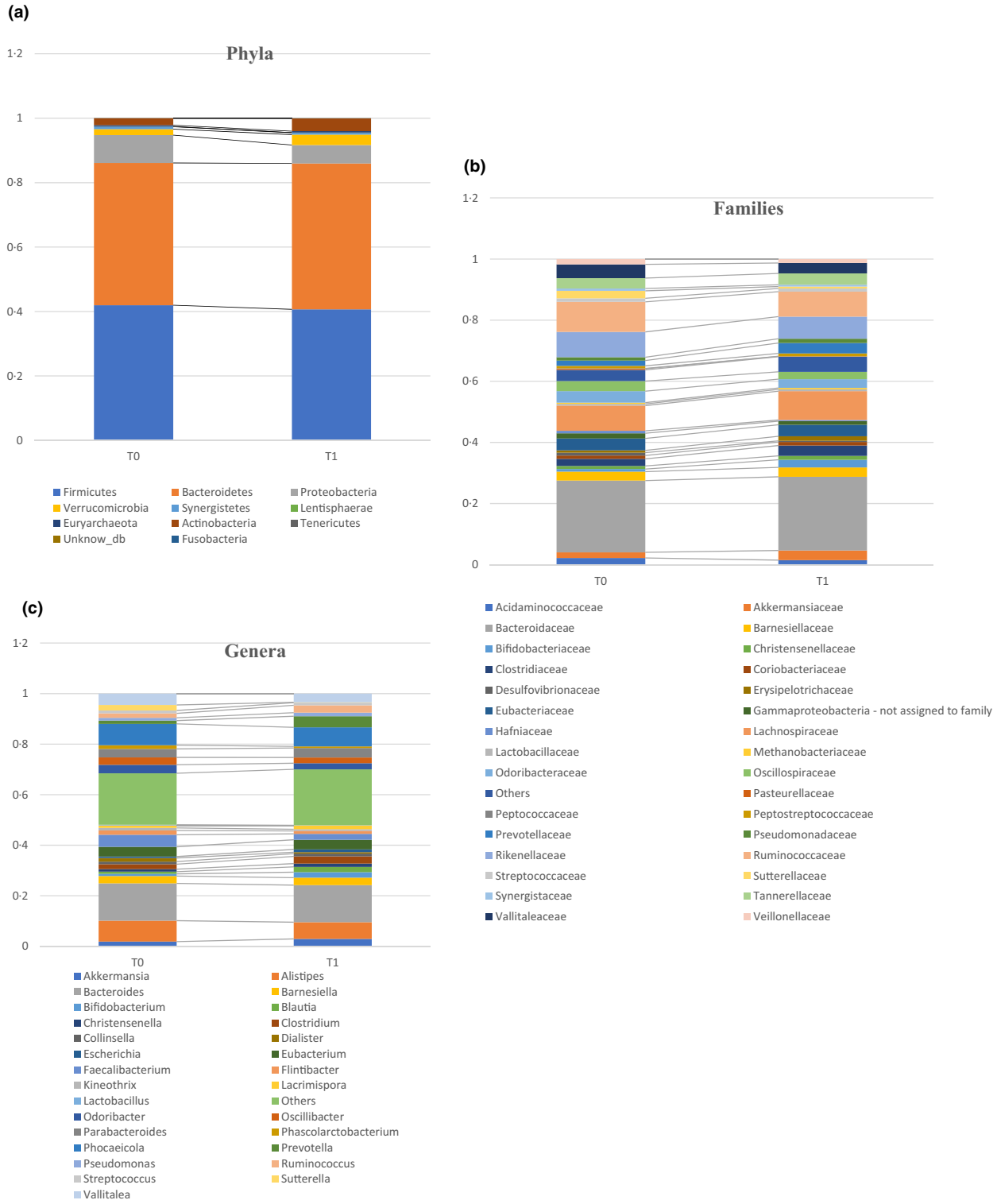

FIGURE 6 Histogram of relative abundance. The *x*-axis represents sampling times (T0 and T1) and the *y*-axis represents relative abundance present. (a) Relative abundance of the phyla; (b) relative abundance of the families; (c) relative abundance of the top 27 genera; other species were combined as 'others.'

DISCUSSION

There are many societal consequences related to ageing population, one of which is the health status of older adults, as it is well known that age increases risk of chronic diseases. Amongst the strategies that can lead to healthy ageing, nutrition is absolutely one of the most effective. It is widely accepted that nutrition plays an important role

TABLE 2 Functional pathways modulated by probiotic treatment

Pathway	Class	T0	T1	*p* value
CRNFORCAT-PWY: creatinine degradation I	Amine degradation	0.00	14.97	<0.05
GALLATE-DEGRADATION-I-PWY	Aromatic compound degradation	0.00	6.75	<0.05
GALLATE-DEGRADATION-II-PWY	Aromatic compound degradation	0.00	6.75	<0.05
METHYL GALLATE-DEGRADATION-PWY	Aromatic compound degradation	0.00	8.41	<0.05
PWY-5005 - biotin biosynthesis	Cofactor Biosynthesis	616.75	303.30	<0.05
PWY-5655 - L-tryptophan degradation	Amino acid degradation	0.00	9.08	<0.05
PWY-5910: superpathway of geranylgeranyl diphosphate biosynthesis I (via mevalonate)	Polyprenyl Biosynthesis	382.63	131.25	<0.05
PWY-6210 - 2-aminophenol degradation	Aromatic compound degradation	0.00	7.17	<0.05
PWY-6505 - L-tryptophan degradation XII	Amino acid degradation	0.00	9.08	<0.05
PWY-7392: taxadiene biosynthesis (engineered)	Secondary metabolites Biosynthesis	3191.12	1772.89	<0.05
PWY-7431 - aromatic biogenic amine degradation	Amine and polyamine degradation	1.76	11.66	<0.05

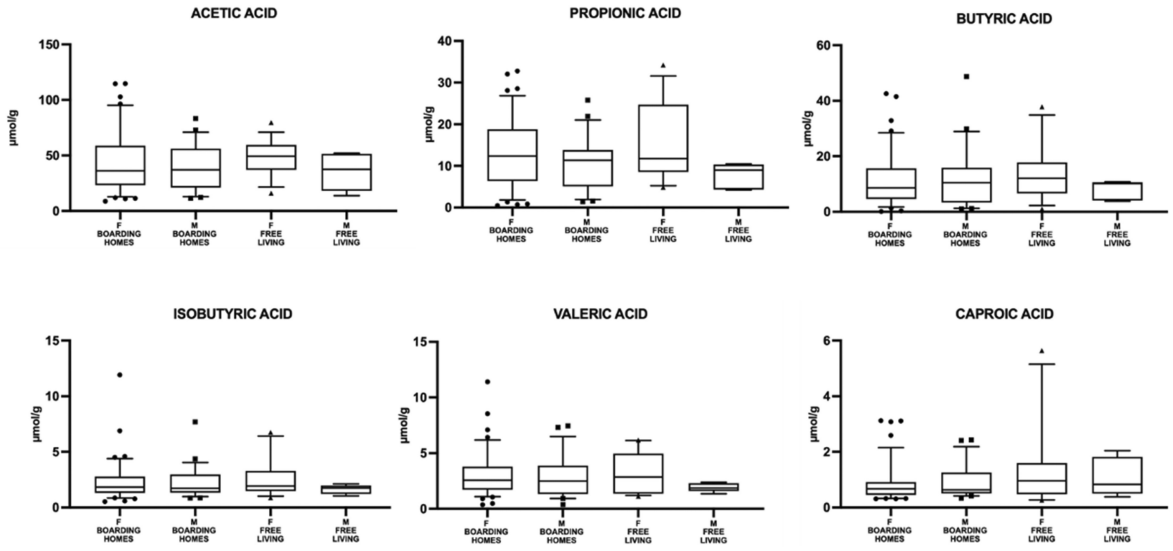

FIGURE 7 Faecal short-chain fatty acids at baseline conditions.

in reducing the risk of chronic diseases such as cancer, heart disease, diabetes and osteoporosis. In this double-blind, placebo-controlled study, aimed to investigate the effects of a probiotics-based diet on the low-grade inflammation in clinically healthy older people, we have obtained some interesting findings that have been demonstrated. Firstly, it confirmed that a significant proportion of healthy elderly people have elevated HsCRP concentrations, as expected for this population and as revealed by epidemiological evidence (Imhof et al., 2003). HsCRP was recognized not only as marker of low-grade inflammation, but also of cardiovascular risk, myocardial infarction and stroke (Pearson et al., 2003). In this study, the difference that occurred from the T1 and T0 was significantly higher after probiotic intervention than the placebo ($p < 0.05$). In addition, a further subgroup analysis, the 6-months probiotic supplementation showed a significant effect ($p < 0.05$) on the protein level respect to the shorter one (less than 6 months). This finding suggests that prolonged intervention is required to counter a chronic situation as in

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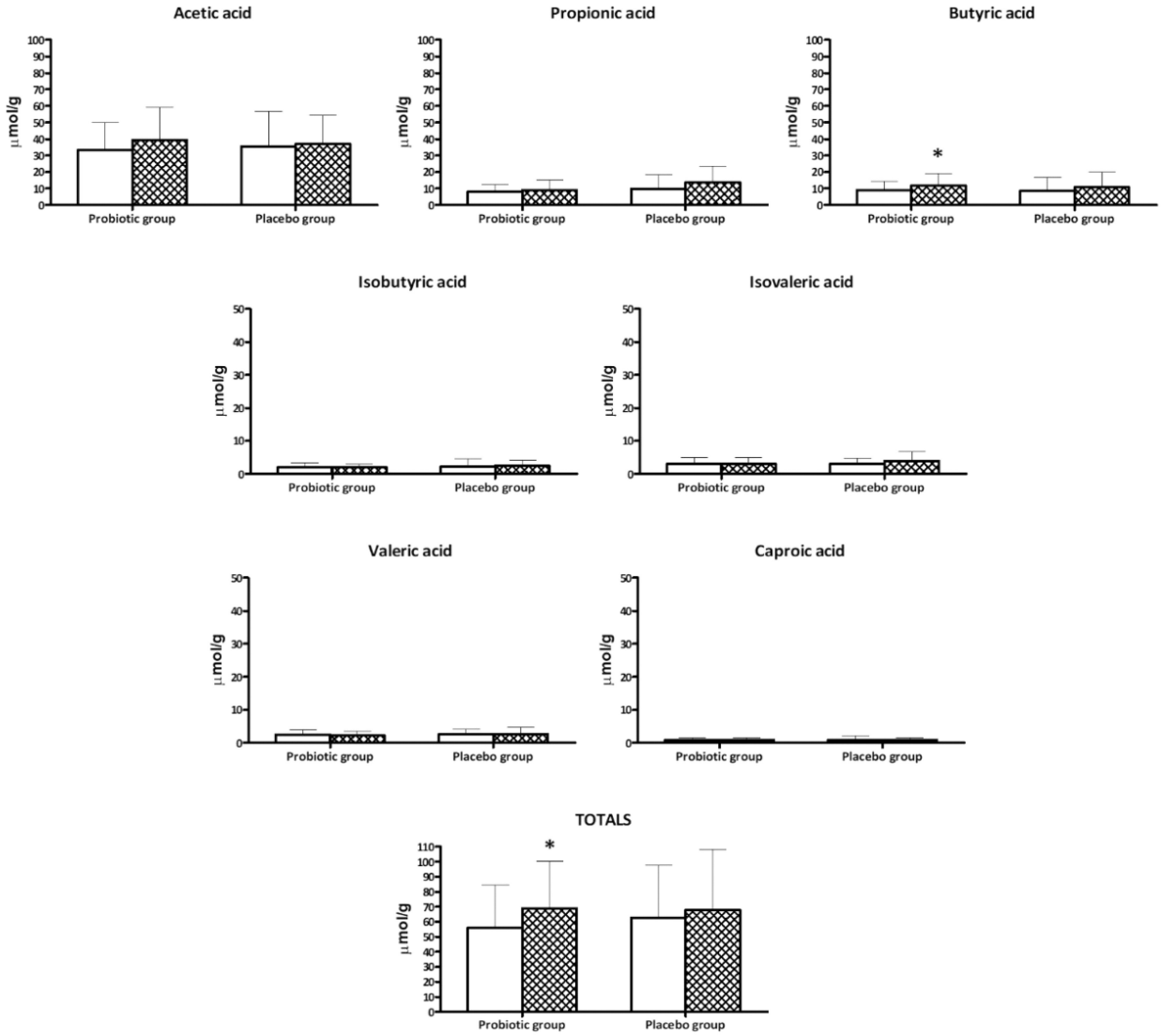

FIGURE 8 Single and total SCFA levels during the different time points (T0 and T1) relative to the two groups of volunteers (probiotic group and placebo group). *significantly different ($p < 0.05$) from T0, by *t*-test.

inflammageing process elderly-associated. The older subjects might need longer treatment to achieve a gut microbiota stabilizing effect. Surely, other parameters strongly linked and related to inflammatory tone are GM composition and SCFA concentration. Both of them change with ageing, due to physiological changes and pathological conditions. The composition of the human gut microbiota is individual-specific at the level of operational taxonomic units (OTUs) and in older people (>65 years) is extremely variable between individuals, groups and genders. The gut bacterial composition of the elderly is characterized by a decrease in *Bacteroides* spp., *Bifidobacterium* spp. and *Lactobacilli* spp. (Odamaki et al., 2016), but in our study, the populations of *Lactobacillus* spp. significantly increased after probiotic treatment. This positive result is probably due to the supplementation used, we supposed. In addition, *Bifidobacterium* spp. increased in the probiotic group after supplementation, though the improvement is not statistically significant. Moreover, previous studies reported an additional increase of facultative anaerobes, including streptococci, staphylococci, enterococci and enterobacteria in the elderly (Mueller et al., 2006; Rajilić-Stojanović et al., 2009). Finally, the prevalence of *Clostridium difficile*, a major intestinal pathogen, dramatically increases in the elderly, probably due to the antibiotic usage. We documented the reduction of cell count of *Clostridium coccoides-Eubacterium rectale* group after both supplementations, with no significant differences for the treatment duration; but the other way around for *Staphylococcus* spp., a significant reduction (respect to baseline data) has been demonstrated in the probiotic group. Of note, the Synbio® blend, used in this study as probiotic, has already demonstrated a microbiota modulation ability in other clinical studies on healthy adults (Silvi

et al., 2014; Verdenelli et al., 2011). It is also interesting to underline how the two bacterial strains making up the blend have been isolated from the intestine of elderly subjects (Silvi et al., 2003). In general, it is hard to define a typical gut microbiota composition of the elderly, since it is highly variable and influenced by various factors such as lifestyle habits or environmental factors (An et al., 2018). However, the overall absence of statistically significant differences, neither in the α- and β-diversity in the samples of our study during the treatment, was not surprising. Indeed, it is well known that the intestinal microbiota tends to be quite stable during time in healthy subjects and it seems to have a documented resilience to perturbations (Dogra et al., 2020). Moreover, inter-individual variations in the gut microbiota may have masked changes due to probiotic intake. Nevertheless, in the present study, a specific effect of the probiotic treatment was observed probably related to the long-term probiotic diet. Results obtained with 16S NGS analysis revealed that the probiotic administration significantly affected some phyla, families and genera abundance. In detail, we observed a significant increase of the Bifidobacteriaceae family and *Bifidobacterium* genus. These data are very important since it is well known that *Bifidobacterium* spp. decreases in ageing and it inversely correlates with inflammageing and consequent ageing-associated morbidity and mortality (Mueller et al., 2006). We also found a significant increase in *Akkermansia* spp., a mucin-degrading microbe that provides energy to other beneficial microbes, including SCFA-producing bacteria. Since centenarians and semi-supercentenarians (individuals with an age of 105 or higher) were found to harbour a higher concentration of *Akkermansia* spp., that is considered a biomarker for healthy ageing (Biagi et al., 2016). It protects the intestinal epithelial integrity, reduces inflammation and induces SCFA production of other commensal microbes. Ageing is strongly associated with a progressively and significant reduction of SCFAs in the GI tract. The SCFAs improve the gut health through a number of local effects, ranging from maintenance of intestinal barrier integrity, mucus production and protection against inflammation to reduction of the risk of colorectal cancer and other diseases (Silva et al., 2020). Considering just the supplementation type, no significant changes occurred in the probiotic group with respect to placebo, after the supplementation. Nevertheless, comparing the level of SCFAs, in the same group at different time points (T0 vs T1), a significant increase has been observed in the probiotic group only. Alterations in the abundance of the gut bacteria mediated by probiotic treatments might change metabolic ability as well. Actually, some metabolic pathways related to the metabolism of amine, aromatic compounds and amino acid degradation were enriched in the probiotic group after the supplementation. This evidence is worthy of further study as the upregulation and downregulation of certain metabolic pathways can be directly linked to specific pathological states. For example, the degradation pathway of tryptophan by bacteria is an important but neglected feature which might be important in microbial regulation of circulating tryptophan availability to the host for kynurenine pathway metabolism in the periphery and central nervous system (CNS). As the precursor molecule to serotonin (5-HT), kynurenine and downstream metabolites of the kynurenine pathway (Palego et al., 2016), changes in the supply and availability of the essential amino acid tryptophan have many implications for the enteric nervous system and CNS functioning and thus brain–gut axis signalling.

Taking together, these results suggest that a balanced microbiota appears to be essential in countering inflammatory events in the elderly and that a probiotic diet intervention could maintain healthy ageing.

In conclusion, PROBIOSENIOR is demonstrated to be an ideal support for healthy ageing and may have a significant impact on the social health system. The 40% of the general population in Western countries is affected by functional disorders such as dyspepsia and irritable bowel syndrome (IBS), which includes constipation, bloating, impaired digestion, digestive pain and intermittent diarrhoea. Combining the knowledge about the influence that diet has on ageing and its potential role to prevent age-related diseases, the way will open for making the microbiota the target of intervention to improve the well-being of the elderly.

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CONFLICT OF INTEREST

None.

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