Higher bacterial DNAemia can affect the impact of a polyphenol-rich dietary pattern on biomarkers of intestinal permeability and cardiovascular risk in older subjects

Running title: Bacterial DNAemia on a PR-diet in older subjects

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Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request in a deidentified form pending review and approval of a formal request to the scientific coordinators of the MaPLE project (patrizia.riso@unimi.it; simone.guglielmetti@unimi.it; candres@ub.edu; paul.kroon@quadram.ac.uk).

ABSTRACT

Purpose: Aging can be characterized by increased systemic low-grade inflammation, altered gut microbiota composition, and increased intestinal permeability (IP). The intake of polyphenol-rich foods is proposed as a promising strategy to positively affect the gut microbiota-immune system-intestinal barrier (IB) axis. In this context, we tested the hypothesis that a PR dietary intervention would affect the presence of bacterial factors in the bloodstream of older adults.

Methods: We collected blood samples within a randomized, controlled, crossover intervention trial in which older volunteers (n=51) received a polyphenol-enriched and a control diet. We quantified the presence of bacterial DNA in blood by qPCR targeting the 16S rRNA gene (16S; bacterial DNAemia). Blood DNA was taxonomically profiled via 16S sequencing.

Results: Higher blood 16S levels were associated with higher BMI and markers of IP, inflammation, and dyslipidemia. PR-intervention did not significantly change bacterial DNAemia in the older population (P=0.103). Nonetheless, the beneficial changes caused by the polyphenol-enriched diet were greatest in participants with higher bacterial DNAemia, specifically in markers related to IP, inflammation, and dyslipidemia. Finally, we found that the bacterial DNA detected in blood mostly belonged to γ-Proteobacteria, whose abundance significantly decreased after the polyphenol-rich diet in subjects with higher bacterial DNAemia at baseline.

Conclusions: This study shows that older subjects with higher bacterial DNAemia experienced a beneficial effect from a polyphenol-rich diet. Bacterial DNAemia may be a further relevant marker for the identification of target populations that could benefit more from a protective dietary treatment.

Registration: This trial was retrospectively registered at www.isrctn.org (ISRCTN10214981) on April 28, 2017.

Key words: MaPLE project; zonulin; *Pseudomonas*; γ-Proteobacteria; interleukin-6.

INTRODUCTION

Aging is characterized by an increase in systemic low-grade inflammation, a process called "inflammaging", which is a highly significant risk factor for morbidity and mortality in the older population [1]. Published reports provide evidence that older people have an altered intestinal permeability (IP), which allows the translocation of microbial factors from the intestine to the bloodstream, plausibly feeding the inflammatory process that promotes inflammaging [2].

Gut microbiota composition, IP and inflammation are intimately linked and interact with each other contributing to the onset of numerous diseases such as obesity [3], rheumatoid arthritis [4], non-alcoholic fatty liver disease [5], cardiovascular diseases [6], inflammatory bowel disease [7] and psychiatric disorders [8]. In particular, the intestinal microbial ecosystem plays an active role in both immune responses and intestinal barrier integrity [9]. Notably, aging is characterized by the deterioration of the gut microbiota community structure, which occurs in association with immune response impairment and intestinal barrier function defects [10]. Interestingly, it was shown in mice that, by reversing age-related gut microbiota changes, it is possible to reduce inflammation, resulting in decreased IP [11].

Numerous studies are providing evidence that the composition of the intestinal microbiota depends on the diet and can be modulated through specific dietary interventions. For example, it was shown that a high fat diet alters the intestinal microbial ecosystem, induces the expression of barrier-disrupting cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and interferon γ (IFNγ)] and results in increased IP [12]. Similarly, a high salt diet was reported to induce modifications in the intestinal microbiota that promote changes in the expression of intestinal immunological genes and alter gut permeability [13]. Conversely, dietary intake of fiber-rich foods such as fruits, vegetables and legumes affects the intestinal microbial ecosystem by promoting the production of short-chain fatty acids (particularly butyrate), which regulate inflammation and strengthens the epithelial tight junctions constituting the intestinal barrier structure [14].

In the context of the gut microbiota-immune system-intestinal barrier axis, the intake of polyphenol-rich foods seems to be particularly relevant. Polyphenols are secondary metabolites of plants, fruits and vegetables commonly consumed in foods such as berries, chocolate, tea and coffee. Polyphenols include well-known antioxidant and anti-inflammatory molecules, which have been demonstrated to preserve intestinal barrier function and positively affect the gut microbiota [15]. Adherence to dietary patterns that include abundant polyphenol-rich foods, such as the Mediterranean diet, has been proposed to counteract the metabolic and physiologic consequences associated with aging [16]. In addition, several polyphenol-rich foods, such as berries, green tea, coffee, cocoa, and extra virgin olive oil, are generally recognized as "anti-senescence" factors [17].

By considering the premises described above, we carried out the MaPLE project (Microbiome Manipulation through Polyphenols for Managing Leakiness in the Elderly) [18], which was based on a randomized, controlled, crossover intervention trial designed to assess the hypothesis that high intake of polyphenol-rich foods in older subjects may reduce IP and improve markers of inflammation, oxidative stress, vascular function and metabolic features of the host. In a previous report, we showed that the 8-week intervention in older volunteers with the MaPLE polyphenol-rich dietary pattern reduced the serum levels of zonulin with stronger effects observed in those subjects with higher zonulin levels, body mass index and insulin resistance at baseline [19].

Since zonulin, which is considered as a marker of IP [20], was demonstrated to be altered by the polyphenol-rich diet compared to the control diet, we hypothesized that the dietary intervention would also have affected the levels/types of bacterial factors in the bloodstream of the older participants participating in the MaPLE trial. To test this hypothesis, we quantified the presence of bacterial DNA in the blood samples collected during the study by using a specific qPCR assay that targets the 16S rRNA gene, and taxonomically profiled the bacterial DNA by 16S rRNA gene sequencing.

MATERIALS AND METHODS

Study participants and trial design

This study is based on the dietary intervention trial carried out within the MaPLE project. The study design and the composition of the diet have been previously described in detail [18,21]. In brief, the intervention trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy), which hosts older subjects in long-term residential care and independent residences. A sample size of 50 was estimated to be required to demonstrate an IP reduction of 30% with 80% power and 0.05 significance and considering a 15% drop-out rate [18]. Subjects were selected in collaboration with physicians and staff at Civitas Vitae in accordance with the exclusion and inclusion criteria previously reported [18]. Specifically, subjects included in the study were ≥ 60 years of age, with an overall good nutritional and cognitive status and increased intestinal permeability evaluated as serum zonulin level. The experimental design is schematically represented in Fig. 1. The trial consisted of an 8-week, randomized, repeated measure crossover intervention study [polyphenol-rich (PR) diet *vs* control (C) diet]. The dietary intervention protocol was designed by substituting low-polyphenol products in the control diet with PR-foods, according to the nutrient composition and total polyphenol content of the daily menu provided to the participants. The PR-foods used in the study were berries and related products, blood orange and juice, pomegranate juice, green tea, *Renetta* apple and purée, and dark chocolate (callets and cocoa powder-based drink). The PR-diet was developed in order to approximately double the total polyphenol intake estimated in the control diet. Specifically, the PR-foods provided on average 724 mg of total polyphenols per day according to quantification by the Folin-Ciocalteu assay [19]. During the PR-dietary intervention, volunteers received 3 portions per day of selected PR-foods. Blood samples were collected at the beginning and at the end of each intervention period. The study protocol was approved by the Ethics Committee of the University of Milan (ref: 6/16/CE_15.02.16_Verbale_All-7). All participants were informed about the study protocol and signed an informed consent before the enrolment. The trial was registered in the ISRCTN public registry (ISRCTN10214981).

Extraction, quantification and microbiomic analysis of blood bacterial DNA

For the analysis of circulating bacterial DNA, 2 ml of blood were collected in EDTA tubes in the morning in fasting condition and stored at -80°C until extraction. A total of 204 blood samples were collected and analyzed. Bacterial DNA quantification and sequencing reactions were performed by Vaiomer SAS (Labège, France) using an optimized blood-specific protocol carefully designed to minimize any risk of contamination [22]. In brief, DNA was extracted with a protocol that combines mechanical cell lysis, enzymatic treatment with proteinase K, and purification with silica membrane spin columns (Macherey-Nagel, Düren, Germany). The quantification of bacterial DNA in the total DNA extracted from blood was carried out by quantitative real-time PCR (qPCR) with pan-bacterial primers EUBF (5'-TCCTACGGGAGGCAGCAGT-3') and EUBR (5'-GGACTACCAGGGTATCTAATCCTGTT-3') [23] targeting the V3-V4 hypervariable regions of the 16S rRNA gene. For the taxonomic profiling, the 16S rRNA gene amplicons were sequenced using the Illumina-MiSeq technology (2 x 300 paired-end MiSeq kit v3, set to encompass 467-bp amplicon). Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB43168. Sequencing reads were clustered in operational taxonomic units (OTUs) using Vaiomer bioinformatic pipeline as described in detail elsewhere [22,24,25]. The potential contribution of contaminant bacterial DNA deriving from reagents, consumables, and the experimenter was assessed by analyzing negative controls represented by molecular grade water in an empty tube, extracted and analyzed at the same time as the blood samples. The analyses of controls indicated that the potential contaminations generated a low overall background signal that only marginally affected the results obtained by qPCR (Supplementary Figure S1A) and 16S rRNA gene profiling (Supplementary Figures S1B, S1C and S1D). Additional information on potential contamination by bacterial DNA occurring during blood microbiomic analysis is available in previous publications [22,25].

Analysis of clinical and functional parameters

The measurement of clinical and functional markers in blood samples was reported previously in details [19]. In brief, fasting serum levels of glucose, insulin, creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), total cholesterol (TC), high- and low-density lipoprotein cholesterol (HDL-C and LDL-C, respectively), and triglycerides (TG) were evaluated through routine biochemical analysis. Zonulin, C-reactive protein (CRP), TNF-α and IL-6 were quantified using ELISA kits (IDK ® Immundiagnostik; R&D Systems - cat# DCRP00, 150 HSTA00E, HS600B - respectively). The concentration of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in serum samples was determined by means of ELISA kit (Booster®, Vinci Biochem S.r.l.). The HOMA and Cockroft-Gault (CG) indexes were calculated according to the formulas previously defined in literature [26].

Statistics

Statistical analyses were performed using R software packages (version 3.1.2). Volunteers that completed the study (n=51) were stratified according to the levels of 16S rRNA gene copies in blood at baseline in tertiles (n = 17 per tertile). Significant differences at baseline between the resulting groups were determined by unpaired Student's t test or Mann–Whitney U test, depending on the normal distribution of data for any single variable under study. The p value adjustment for multiple comparisons was performed by Benjamini-Hochberg correction method. Normality was assessed by the Shapiro–Wilk test. Significant changes during the dietary interventions were assessed by paired Student's t test for variables found to follow a normal distribution; for the other variables, the Wilcoxon signed-rank test was used.

RESULTS

Bacterial DNAemia at baseline

Bacterial DNA was detected by qPCR in the blood of all 51 older adults participating in the study. Stratification of 16S g.c. levels in tertiles (n=17 for each tertile) revealed that subjects with higher blood concentration of 16S g.c. (tertile III) significantly differed from those with lower concentrations (tertile I) for zonulin, BMI, TNF-α, TG, TC/HDL and LDL/HDL ratio, which were higher in tertile III, and HDL-C, which was higher in subjects with lower 16S g.c. levels (tertile I) (Table 1; Fig. 2). In addition, we found that the middle tertile (tertile II) was characterized by significantly higher TG and TC/HDL than the tertile I (Fig. 2).

Overall, these data seem to suggest that, in the older volunteers under study, higher blood concentration of 16S g.c. is associated to higher BMI and markers of IP, inflammation and dyslipidemia.

Effect of the dietary intervention on the markers under study

We previously reported the effect of the PR-diet on IP-related and other clinical markers in the older subjects participating to the intervention study [19]. Here, we failed to find a significant effect of the polyphenol-enriched diet on serum bacterial DNAemia (P=0.103, according to repeated measure ANOVA).

Subsequently, we analyzed whether the effect of the dietary intervention could be different in subgroups of older volunteers, stratified on the basis of 16S g.c. levels in blood at baseline. Whereas no effect was observed following the PR-diet intervention in the group of subjects with the lowest concentration of 16S g.c. (tertile I), those in tertile III showed a significant decrease in zonulin and IL-6, and an increase in uric acid. In addition, we found a trend towards a reduction of the 16S g.c. and TC/HDL ratio. Notably, we did not find any significant change during the control diet for the tertiles I and III (Table 2). In the middle tertile, the only significant result was the decrease of serum GGT after the PR-diet (Table 2).

In summary, these results seem to indicate that subjects with higher bacterial DNAemia calculated as 16S rRNA g.c. per µl of blood could benefit more from the PR-dietary intervention as demonstrated by the reduction of IP and other markers of inflammation and dyslipidemia.

Effect of the dietary interventions on the abundance of blood bacterial taxa

The bacterial DNA isolated from the blood samples collected during the study was taxonomically profiled by 16S rRNA gene sequencing (Supplementary Figure S1BCD). The taxonomic distribution of blood DNA was dominated by the Gram-negative bacteria of the phylum *Proteobacteria*, and particularly by the class *γ-Proteobacteria*, largely represented by the genus *Pseudomonas* (Fig. 3). In addition to *Pseudomonas*, the most prevalent and abundant genera were *Flavobacterium*, *Arthrobacter*, and *Escherichia/Shigella*, respectively belonging to the phyla *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Fig. 3).

The stratification of older subjects in tertiles based on the 16S g.c. levels in blood at baseline revealed that, following the PR-diet intervention, the abundance of several taxa within the *Proteobacteria* phylum decreased exclusively in tertile III, i.e. in subjects with higher basal bacterial DNAemia (Table 3). In particular, in tertile III, the PR-diet was associated with reductions in the most abundant family and genus detected in blood samples, i.e. *Pseudomonadaceae* and *Pseudomonas*, respectively. Conversely, during the control diet, we observed reductions in less abundant taxa, i.e. the class *Chitinophagaceae* in tertile III, and the order *Betaproteobacteriales* and the family *Burkholderiaceae* in tertile II (Table 3).

Overall, these results indicate that the bacterial DNA detected in the blood of the older volunteers mostly belong to taxa within the class *γ-Proteobacteria*, whose abundance significantly decreased after the PR-diet in the group of subjects with higher bacterial DNAemia at baseline.

DISCUSSION

The MaPLE project was designed with the primary aim to test the hypothesis that a polyphenol-rich dietary pattern may preserve and/or improve intestinal barrier function in older people [18]. This hypothesis was confirmed by the results of the intervention trial, which revealed that including a modest number of small portions of PR-food products in the dietary pattern of older subjects could improve intestinal permeability, evidenced by a reduction in serum zonulin (a protein involved in the opening of intercellular tight junctions) [19]. In addition, we recently reported a correlation between serum zonulin levels and bacterial DNAemia in older subjects recruited within the MaPLE trial [25]. This observation supported bacterial DNAemia as a potential candidate biomarker of intestinal permeability. In this context, here we assessed the intestinal permeability of the same volunteers participating to the trial through qPCR quantification and taxonomic profiling of the 16S rRNA gene detected in blood.

Analysis of data at baseline revealed that subjects possessing higher bacterial DNAemia also had increased serum zonulin levels, further supporting the notion previously proposed of a link between zonulin and DNAemia in the older population [25]. In addition, we found that older adults with higher bacterial DNAemia also had significantly higher levels of the inflammatory factor TNF-α and a less healthy lipidemic profile, characterized by higher TG and lower HDL-C levels together with increased TC/HDL and LDL/HDL ratios. Interestingly, serum levels of TNF-α were found often to be associated with lipid dysmetabolism, and both circulating TNF-α and dyslipidemia may act synergistically to promote several pathologic conditions such as rheumatoid arthritis [27], kidney damage [28], and cardiovascular diseases [29]. Moreover, an altered presence of bacterial DNA in blood was observed in patients with liver fibrosis [30], chronic kidney disease [31], type 2 diabetes [32], and myocardial infarction [33], which are also conditions characterized by lipid dysmetabolism and increased TNF-α [34]. Notably, the older subjects with increased bacterial DNAemia were also characterized by significantly higher BMI (a median of 25 in tertile I and II vs 31 in tertile III). Reportedly, obese people are typically characterized by elevated inflammatory

markers and altered lipidemic profile. In addition, circulating zonulin was shown to increase with BMI, resulting to be significantly higher in obese subjects [35,36].

In our previous report, we showed that, within the MaPLE trial, the effects of the PR-diet were strongest in subjects characterized by higher zonulin levels, which had also greater reductions in diastolic blood pressure and serum glucose and IL-6 [19]. Similarly, here we showed that stratification of volunteers according to initial bacterial DNAemia was crucial to unveil the changes in health-relevant parameters specifically modulated by the intervention. In fact, after the PR-diet, we observed a significant reduction in serum levels of both zonulin and IL-6 (decreasing approximately 15% and 21%, respectively) only in older participants with higher DNAemia. In addition, a small but significant increase in uric acid was observed. Notably, none of the investigated markers significantly changed during the intervention with the control diet, suggesting that the observed changes in the treatment arm were a consequence of the polyphenol-enriched diet [18]. Numerous independent studies, including *in vitro*, animal and human studies, have demonstrated the ability of certain polyphenols and polyphenol-rich foods to counteract inflammation and intestinal permeability, including those foods used in the MaPLE intervention, such as dark chocolate (monomeric and oligomeric catechins / flavan-3-ols), green tea (gallated and galloylated flavan-3-ols) and blueberries (i.e. a mixture of anthocyanins, flavan-3-ols, other flavonoids and phenolic acids) [2]. In this respect it is, however, remarkable that increased IP may considerably impact the bioavailability of dietary polyphenols in the older population [37], which could explain at least in part the differential efficacy of the PR-diet between subjects with higher and lower zonulin levels at baseline.

The assay adopted in this study to quantify zonulin was suggested to recognize several members of permeability-regulating proteins belonging to the mannose-associated serine protease family [38], including the pre-haptoglobin 2, *HP2* [39]. Notably, the *HP2* gene possesses a promoter that is under the control of the cytokine IL-6 [40,41]. Therefore, the observed reduction of zonulin levels in older subjects may potentially be, at least in part, a consequence of the effect of the

PR-diet on serum IL-6 levels [11]. Interestingly, Qi et al. found in a small cohort of healthy older adults that zonulin serum concentrations were positively associated with TNF-α and IL-6 levels and negatively correlated with skeletal muscle strength and habitual physical activity, two indices of physical frailty [42]. It is also worth mentioning that a mechanistic link between aging, altered gut microbiota, enhanced intestinal permeability, and increased circulating pro-inflammatory cytokines was finely demonstrated in a murine model [11]. Furthermore, the simultaneous increase of serum zonulin and systemic IL-6 levels was also found in several metabolic disorders such as type 2 diabetes [43], and obesity [44].

We also found an increase of serum uric acid, a degradation product of purine nucleotides, in the older volunteers with higher bacterial DNAemia following the PR-diet. It has been reported that increased uric acid can be associated to kidney dysfunction and to inflammatory conditions and we found higher levels in the group of subjects with higher DNAemia (tertile III) with respect to the tertile I ($P = 0.056$). At the same time, uric acid is also recognized as a relevant antioxidant compound in our body and its duality has been attributed to differences in concentrations and in the surrounding microenvironment [45]. In this regard, it is worth mentioning that the levels of this marker were within the normal range along the entire study, thus the biological relevance of the results we obtained should be further investigated.

The polyphenol rich dietary intervention caused only a trend ($P = 0.099$) towards the reduction of bacterial DNAemia in the older subject with higher 16S g.c. levels. Nonetheless, a significant reduction was found in the circulating 16S g.c. ascribed to the Gram-negative phylum *Proteobacteria*, and in particular to the classes *β*- e *γ-Proteobacteria*, including the *Pseudomonas* genus, which was the most abundant genus found in the blood of older volunteers in the MaPLE study. There are several reports demonstrating that the bacterial DNA detected in the blood of healthy adults and older subjects, and also in several pathological conditions, mainly belong to the phylum *Proteobacteria*, and specifically to the genus *Pseudomonas* [25,32,33,46-49]. The outer membrane in the cell wall of these microorganisms contains the lipopolysaccharides (LPS), also

called endotoxin. LPS is the best-known microbe-associated molecular pattern (MAMP) able to efficiently trigger the production of pro-inflammatory cytokines, such as TNF-α and IL-6, upon its specific CD14-dependent recognition by the toll-like receptor (TLR)-4, which is anchored on the outer surface of immune cells such as neutrophils, macrophages, dendritic cells, mast cells, and B cells [50]. Although speculative, our observations are consistent with the idea that the PR-diet may have reduced the translocation of bacteria (or components from them) possessing the pro-inflammatory molecule LPS into the bloodstream, resulting in a mild decrease of systemic stimulation of the inflammatory response (decreased IL-6 levels) and, therefore, plausibly counteracting inflammaging. However, this hypothesis needs experimental verification, starting from the quantification of circulating LPS, which we have not evaluated here.

Our study has a number of strengths related to the strict control of experimental conditions, including the repeated-measure crossover dietary intervention, the characteristics of the older volunteers and their high adherence to the study protocol. However, there are also several limitations, in particular, the relatively small sample size has reduced the possibility to provide strong evidence for some variables for which inter-individual variability plays a significant role. In addition, the fact that bacterial DNA in blood is a minority in comparison with host DNA, implies that its extraction, quantification and taxonomic profiling is technically very challenging and entails a level of indeterminacy which is not completely known.

Overall, despite the limitations of the present study, the obtained results for older subjects with increased bacterial DNAemia may support the notion that polyphenol intake, IP (i.e. zonulin levels and bacterial DNAemia) and inflammation (i.e. IL-6 and TNF-α) could be mechanistically linked through the following sequence of events: the intake of PR-foods may have exerted anti-inflammatory effects which was evidenced as reductions in serum IL-6; lower levels of circulating IL-6 could justify decreased expression of zonulin, preserving the integrity of the intestinal barrier and consequently preventing the translocation of bacteria (entire cells or fragments) into the bloodstream; the reduction in circulating bacterial components leads to a reduction in systemic

inflammation. This putative cascade of events is mainly supported by data obtained in the subjects with the highest blood bacterial DNAemia, which also had significantly higher BMI, suggesting that the impact of a PR-rich diet may be more evident in more compromised subjects.

In conclusion, this study contributes to validate the primary hypothesis of the MaPLE project that a dietary pattern enriched in polyphenols may reduce IP in older subjects, potentially contributing to the improvement of systemic inflammation. Furthermore, our results show that DNAemia was a significant variable affecting the impact of the PR-diet in the target population under study. We propose bacterial DNAemia as a further relevant marker for the identification of more vulnerable target populations that could benefit more from a protective dietary treatment.

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STATEMENT OF AUTHORSHIP

SG, Guarantor of article. PR and SG designed the trial and in collaboration with AC, CAL and PAK optimized the study protocol including the development of the polyphenol-rich diet. SG drafted the first version of the manuscript in collaboration with GG, CDB, and PR. Laboratory work was carried out by VT, GG, NHL and SB. GG performed bioinformatic analyses in collaboration with SG. GG, SG performed the statistical analysis in collaboration with TM. All the authors critically revised the draft and approved the final version.

CONFLICT OF INTEREST STATMENT

None of the authors declares conflict of interest.

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ETHICS APPROVAL STATEMENT

The study protocol was approved by the Ethics Committee of the University of Milan (ref: 6/16/CE_15.02.16_Verbale_All-7). All participants were informed about the study protocol and signed an informed consent before the enrolment.

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TABLES

Table 1. Baseline characteristics of older volunteers who completed the MaPLE dietary intervention trial (n=51) stratified in tertiles according to the 16S rRNA gene copies (16S g.c.) quantified in the blood samples at baseline. *I*, *II* and *III* indicate the groups of subjects with lower, intermediate, and higher levels of 16S g.c., respectively. Data are reported as mean ± standard deviation; median values are shown between brackets. P values indicate significant differences between groups determined through unpaired Student's t test or Mann-Whitney U test, depending on the normal distribution of the considered variable. P values < 0.05 are shown on a green background; 0.05 ≤ p values < 0.1 are shown on a light-yellow background.

	I (n=17)	***II*** (n=17)	***III*** (n=17)	P value		
				I vs II	***I vs III***	***II vs III***
Age	76.8 ± 10.4; (79)	78.2 ± 10.4; (76)	78.4 ± 10.7; (76)	0.484	0.676	0.790
16S rRNA (g.c./µl)	3604 ± 643; (3762)	4901 ± 432; (4744)	8986 ± 5647; (7246)	**<0.001**	**<0.001**	**<0.001**
Zonulin	38.8 ± 10.4; (38.2)	42.1 ± 15.3; (36.8)	45.6 ± 8.3; (45.7)	0.790	**0.043**	0.136
BMI (kg/m2)	25.3 ± 6.5; (25.1)	26.5 ± 4.7; (24.8)	28.6 ± 5; (30.5)	0.449	**0.023**	0.292
CRP (mg/L)	8.7 ± 10.9; (2.7)	4.7 ± 4.4; (3.3)	7.7 ± 7.1; (6.2)	0.986	0.535	0.177
TNFα (pg/ml)	1.2 ± 0.5; (1.1)	1.3 ± 0.5; (1.2)	2.2 ± 1.7; (1.8)	0.338	**0.020**	0.107
IL6 (pg/ml)	5.7 ± 6.1; (3)	3.2 ± 2.2; (2.1)	4.7 ± 2.7; (3.7)	0.471	0.513	0.090
ICAM-1 (ng/ml)	62 ± 23.9; (55.2)	54 ± 20.4; (49.8)	50.9 ± 16.1; (49.2)	0.272	0.215	0.914
VCAM-1 (ng/ml)	1636 ± 2830; (951)	1166 ± 628; (998)	913 ± 416; (966)	0.488	0.708	0.207
Glucose (mg/dl)	102 ± 21; (95)	98 ± 17; (97)	141 ± 111; (93)	0.639	0.931	0.505
Insuline (U/m)	6.5 ± 3.8; (6)	8.6 ± 5.5; (6.8)	10.1 ± 8.8; (6.2)	0.241	0.270	0.773
HOMA Index	1.7 ± 1.3; (1.4)	2.2 ± 1.6; (1.6)	4.7 ± 9.1; (2.1)	0.460	0.133	0.264
Creatinine (mg/dl)	0.9 ± 0.4; (0.8)	0.8 ± 0.3; (0.8)	0.9 ± 0.3; (1)	0.662	0.444	0.175
Uric Acid (mg/dl)	5.0 ± 1.7; (4.7)	5.5 ± 1.8; (5.6)	6.0 ± 1.7; (5.6)	0.493	0.056	0.407
CG index	79.8 ± 56.7; (69.4)	74.9 ± 33.5; (72.7)	69.8 ± 27.5; (66.1)	0.928	0.823	1.000
TC (mg/dl)	205 ± 49; (194)	203 ± 54; (197)	182 ± 47; (182)	0.785	0.176	0.320
HDL-C (mg/dl)	54.4 ± 13.2; (50)	45.8 ± 15.4; (45)	39.4 ± 12.6; (41)	0.101	**0.002**	0.209
TC/HDL (ratio)	3.8 ± 0.8; (3.8)	4.6 ± 1; (5)	4.9 ± 1.4; (4.6)	**0.049**	**0.016**	0.470
LDL-C (mg/dl)	125 ± 36; (125)	126 ± 40; (124)	111 ± 35; (113)	0.920	0.278	0.367
LDL/HDL (ratio)	2.4 ± 0.7; (2.3)	2.8 ± 0.7; (2.7)	3.0 ± 0.8; (2.9)	0.087	**0.024**	0.542
TG (mg/dl)	98 ± 43; (81)	159 ± 113; (137)	181 ± 94; (142)	**0.038**	**0.002**	0.195
AST (U/L)	19.3 ± 6; (19)	18.2 ± 5.4; (17)	16.1 ± 5.7; (15)	0.504	0.075	0.294
ALT (U/L)	12.9 ± 5.8; (12)	14.1 ± 6.1; (13)	13.1 ± 9.6; (9)	0.587	0.457	0.279
GGT (U/L)	30.7 ± 23.9; (20)	47.8 ± 57.7; (24)	35.8 ± 26.3; (23)	0.639	0.361	0.773

Table 2. Effect of the intervention with the polyphenol-rich (PR) and control (C) diet on the markers under study in the group of older subjects stratified in tertiles (n=17 per tertile) based on the 16S rRNA gene copies (16S g.c.) quantified in the blood samples at baseline. *I*, *II* and *III* indicate the groups of subjects with lower, intermediate, and higher levels of 16S g.c., respectively. P values indicate significant changes before (bef) and after (aft) the interventions determined through paired Student's t test or Wilcoxon signed-rank test, depending on the normal distribution of the considered variable. P values < 0.05 are shown on a green background; 0.05 ≤ p values < 0.1 are shown on a grey background. **, P < 0.01; *, P < 0.05; +, 0.05 ≤ p < 0.1.

	I						*II*						*III*					
	PP-rich diet			Control diet			PP-rich diet			Control diet			PP-rich diet			Control diet		
	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*
16S rRNA (g.c./µl)	3762	3599	*0.404*	3936	3571	*0.329*	4699	4221	*0.818*	4828	4841	*0.963*	**6664**	**5349**	*0.099* +	6585	5991	*0.58*
Zonulin (ng/ml)	37.6	39.3	*0.438*	43.9	45.4	*0.467*	42.1	38.9	*0.226*	40.9	43.7	*0.180*	**46.1**	**38.9**	***0.026*** *	43.8	43.9	*0.73*
BMI (kg/m2)	24.6	24.5	*0.779*	24.3	24.2	*0.674*	25.3	26.1	*0.117*	24.9	25.2	*0.712*	30.5	30.8	*0.075* +	29.4	29.5	*0.97*
CRP (mg/L)	2.3	1.9	*0.394*	1.5	2.2	*0.691*	3.9	3.5	*0.603*	3.3	3.4	*0.518*	4.2	4.6	*0.906*	5.5	4.3	*0.84*
TNFα (pg/ml)	1.1	1.3	*0.218*	1.1	1.2	*0.776*	1.2	1.3	*0.338*	1.2	1.2	*0.938*	1.6	1.6	*0.850*	1.6	1.5	*0.26*
IL6 (pg/ml)	3.8	3.5	*0.925*	2.0	2.4	*0.890*	2.4	2.8	*0.927*	2.3	2.2	*0.431*	**4.3**	**3.4**	***0.048*** *	3.9	3.8	*0.93*
ICAM-1 (ng/ml)	52	61	*0.145*	69	60	*0.404*	50	56	*0.190*	52	56	*0.517*	48	50	*0.966*	44	46	*0.79*
VCAM-1 (ng/ml)	941	804	*1.000*	916	1038	*0.890*	1037	1251	*0.159*	974	1076	*0.611*	1028	743	*0.347*	998	672	*0.64*
Glucose (mg/dl)	95	94	*0.365*	95	92	*0.733*	92	97	*0.534*	100	99	*0.161*	94	94	*0.326*	96	95	*0.27*
Insuline (µU/ml)	6.0	5.9	*0.408*	4.5	4.9	*1.000*	6.2	7.4	*0.981*	7.1	5.0	*0.093* +	6.6	7.5	*0.609*	6.5	7.1	*0.78*
HOMA Index	1.4	1.3	*0.187*	0.9	1.1	*0.943*	1.3	1.7	*0.972*	1.7	1.3	*0.134*	1.9	1.9	*0.463*	2.1	1.9	*0.85*
Creatinine (mg/dl)	0.9	0.9	*0.740*	0.9	0.9	*0.265*	0.9	0.8	*0.248*	0.8	0.8	*0.506*	1.0	1.0	*0.509*	1.0	0.9	*0.55*
Uric Acid (mg/dl)	4.7	4.8	*0.753*	4.8	4.8	*0.118*	5.3	5.4	*0.352*	5.6	5.2	*0.850*	**5.8**	**6.6**	***0.014*** *	6.3	5.8	*0.40*
CG index	69	69	*0.284*	69	68	*0.477*	60	70	*0.243*	72	65	*0.747*	66	64	*0.865*	67	64	*0.74*
TC (mg/dl)	201	195	*0.181*	202	195	*0.274*	199	195	*0.448*	199	195	*0.529*	184	178	*0.472*	174	175	*0.80*
HDL-C (mg/dl)	53.5	53.4	*0.903*	55.3	55.2	*0.949*	47.8	44.9	*0.091* +	45.8	44.9	*0.598*	40.0	41.5	*0.251*	39.8	40.7	*0.55*
TC/HDL ratio	3.8	3.7	*0.301*	3.9	3.7	*0.227*	4.3	4.4	*0.298*	4.7	4.5	*0.698*	4.8	4.6	*0.067* +	4.5	4.4	*0.70*
LDL-C (mg/dl)	123	118	*0.117*	121	115	*0.303*	123	120	*0.480*	123	122	*0.793*	112	108	*0.506*	106	105	*0.48*
LDL/HDL ratio	2.3	2.3	*0.209*	2.3	2.2	*0.252*	2.7	2.8	*0.092* +	2.8	2.8	*0.616*	2.9	2.7	*0.155*	2.8	2.7	*0.37*
TG (mg/dl)	86	76	*1.000*	72	93	*0.438*	112	121	*0.979*	137	107	*0.449*	139	157	*0.433*	139	124	*0.90*
AST (U/l)	19.0	18.0	*0.753*	21.0	22.0	*0.736*	18.0	17.0	*0.567*	17.0	17.0	*0.975*	15.5	14.5	*0.606*	16.5	15.5	*0.90*
ALT (U/l)	12.0	13.0	*1.000*	15.0	13.0	*0.887*	13.0	13.0	*0.117*	11.0	11.0	*0.937*	10.5	10.0	*0.852*	10.00	11.00	*0.29*
GGT (U/l)	20.0	19.0	*0.509*	22.0	20.0	*0.844*	**30.0**	**26.0**	***0.003*** **	24.0	29.0	*0.682*	26.5	30.5	*0.265*	30.0	27.5	*0.61*

Table 3. Effect of the intervention with the polyphenol-rich (PR) and control (C) diet on the abundance of bacterial taxa detected in the blood of older subjects under study. Older volunteers have been stratified in tertiles (n=17 per tertile) based on the 16S rRNA gene copies (16S g.c.) in blood at baseline. *I*, *II* and *III* indicate the groups of subjects with lower, intermediate, and higher levels of 16S g.c., respectively. P values indicate significant changes before (bef) and after (aft) the interventions determined through Wilcoxon signed-rank test. P values < 0.05 are shown on a green background; 0.05 ≤ p values < 0.1 are shown on a grey background. ***, P < 0.001; **, P < 0.01; *, P < 0.05; +, 0.05 ≤ p < 0.1.

	I						*II*						*III*					
	PP rich diet			Control diet			PP rich diet			Control diet			PP rich diet			Control diet		
Taxonomy	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*	bef	aft	*P*
p_Actinobacteria;c_Actinobacteria;o_Micrococcales;f_Micrococcaceae	173	152	*0.890*	181	209	*1.000*	323	344	*0.548*	209	316	*0.459*	526	240	*0.067* +	387	285	*0.246*
p_Bacteroidetes	874	788	*0.712*	885	664	*0.064* +	948	955	*0.782*	1265	915	*0.378*	1449	1190	*0.304*	1765	1189	*0.417*
p_Bacteroidetes;c_[Saprospirae];o_[Saprospirales];f_Chitinophagaceae	101	31	*0.098* +	42	28	*0.207*	25	48	*0.243*	160	53	*0.098* +	113	121	*0.671*	**169**	**85**	***0.024*** *
p_Bacteroidetes;c_Bacteroidia	874	788	*0.678*	883	664	*0.071* +	948	954	*0.782*	1265	915	*0.378*	1449	1188	*0.304*	1765	1189	*0.369*
p_Bacteroidetes;c_Bacteroidia;o_Chitinophagales	199	148	*0.782*	115	70	*0.109*	149	151	*0.927*	213	164	*0.459*	295	230	*0.495*	210	163	*0.196*
p_Bacteroidetes;c_Flavobacteriia;o_Falvobacteriales	564	570	*0.678*	602	412	*0.174*	547	618	*0.284*	696	481	*0.459*	882	796	*0.325*	1069	718	*0.304*
p_Bacteroidetes;c_Flavobacteriia;o_Falvobacteriales;f_Flavobacteriaceae	491	443	*0.782*	555	370	*0.329*	506	581	*0.071* +	630	460	*0.747*	805	669	*0.119*	821	542	*0.212*
p_Bacteroidetes;c_Flavobacteriia;o_Falvobacteriales;f_Flavobacteriaceae;g_*Flavobacterium*	491	443	*0.782*	555	370	*0.329*	506	581	*0.071* +	630	460	*0.747*	805	669	*0.119*	821	542	*0.167*
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae	132	122	*0.611*	125	92	*1.000*	172	130	*0.431*	**261**	**111**	***<0.001*** ***	**375**	**160**	***0.001*** **	232	154	*0.099* +
p_Proteobacteria;c_Gammaproteobacteria	1512	1236	*0.159*	1703	1316	*0.579*	1695	1788	*0.678*	2075	1933	*0.712*	3020	2244	*0.074* +	2474	2193	*0.932*
p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales	187	153	*0.517*	218	162	*0.890*	292	156	*0.243*	**338**	**176**	***0.003*** **	**417**	**224**	***0.009*** **	368	293	*0.246*
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales	959	829	*0.145*	1212	641	*0.459*	1159	1105	*0.678*	902	1163	*0.353*	**1659**	**1263**	***0.038*** *	1231	1358	*0.442*
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae	925	765	*0.284*	680	638	*0.712*	1012	1105	*0.378*	890	1083	*0.459*	**1609**	**1159**	***0.043*** *	1055	1319	*0.417*
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_*Pseudomonas*	925	765	*0.284*	680	638	*0.712*	1012	1105	*0.378*	890	1083	*0.459*	**1609**	**1159**	***0.043*** *	1055	1319	*0.417*

FIGURE LEGENDS

Fig. 1. Study scheme. The number of older subjects allocated to each arm that completed the trial per protocol is indicated on the right. The red arrows indicate blood sample collection. PR, polyphenol-rich.

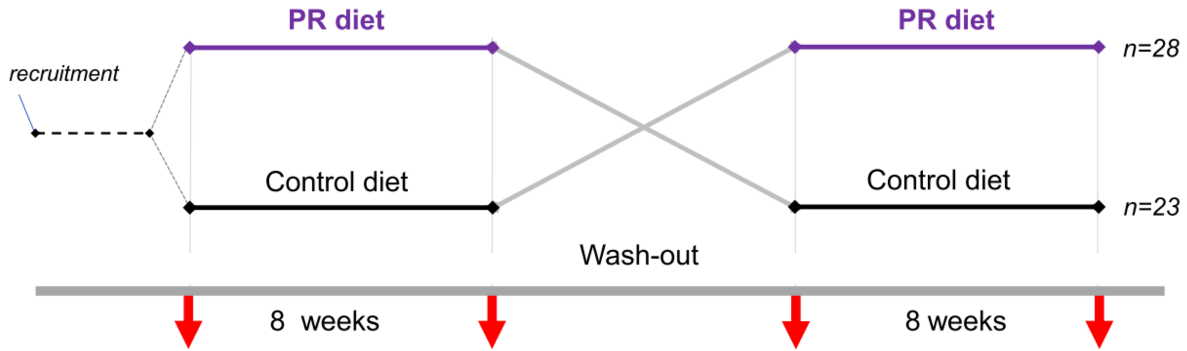
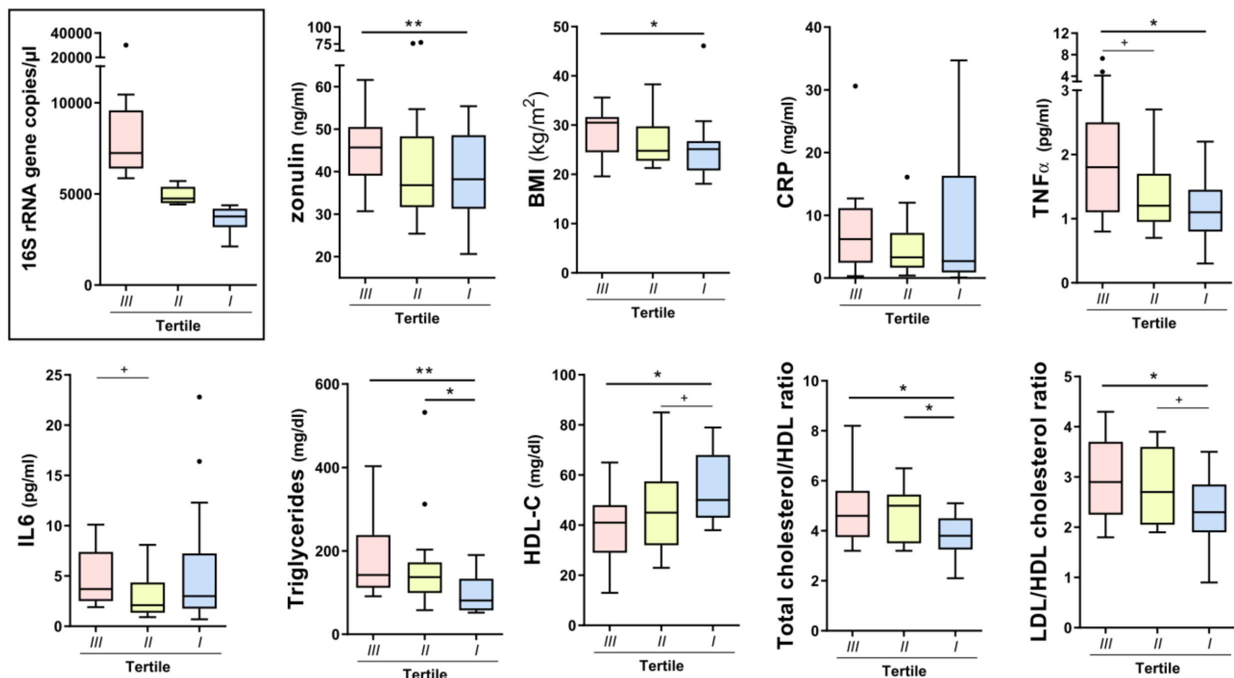
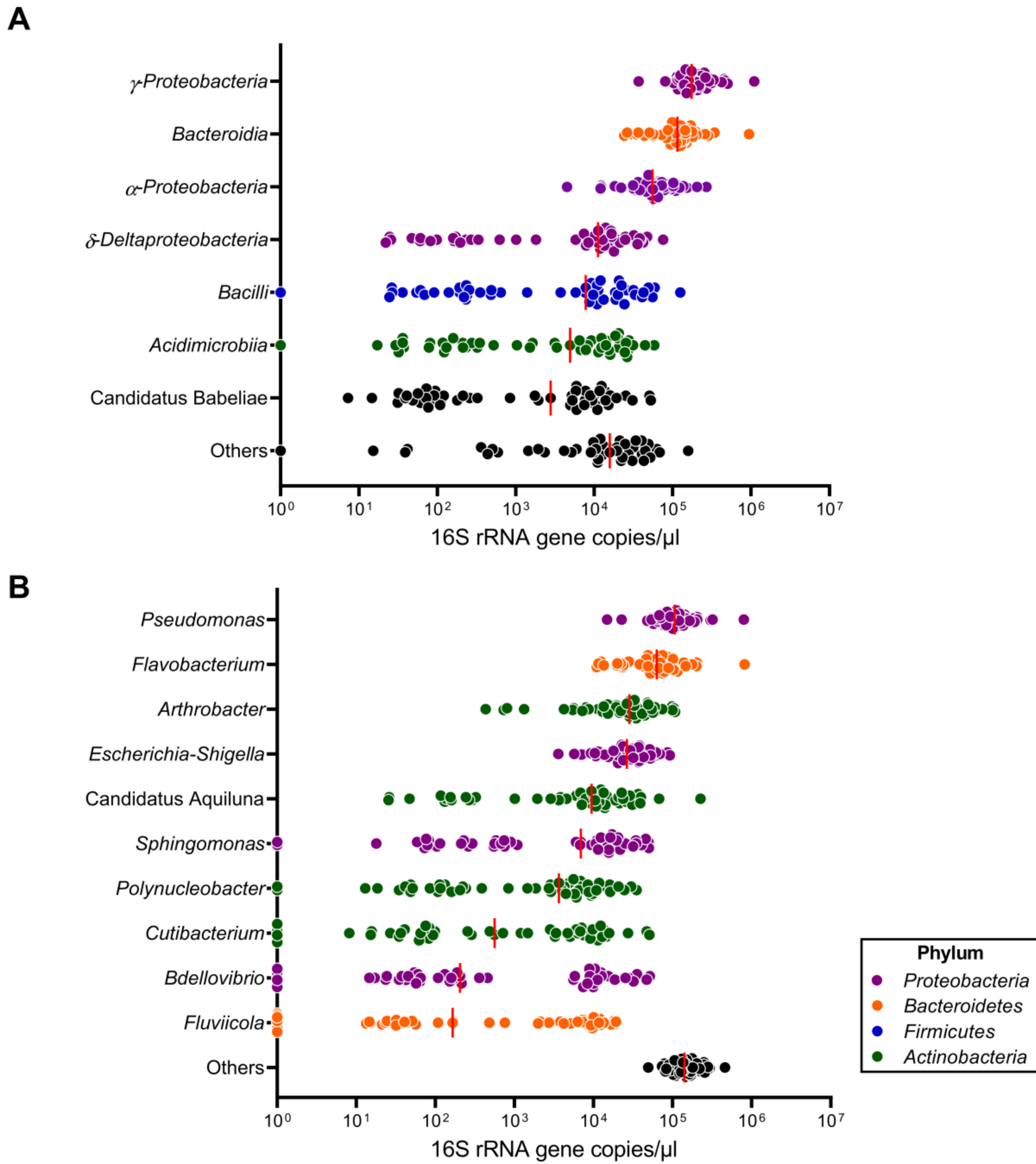


Fig. 2. Tukey's boxplots representing the levels of selected health-related markers in the older volunteers of the MaPLE study stratified according to the levels of bacterial DNAemia. *I*, *II* and *III* indicate the groups of subjects with lower, intermediate, and higher levels of 16S rRNA gene copies in the blood samples at baseline, respectively. Asterisks show significant differences among groups determined through unpaired Student's t test or Mann-Whitney U test, depending on the normal distribution of the considered variable. *, $P < 0.05$; **, $P < 0.01$; $^{+}$, $0.05 \leq p < 0.1$.



28 **Fig. 3**. Taxonomic composition of the bacterial DNA detected in the blood of older subjects at
29 baseline expressed as 16S rRNA gene copies per volume of blood. The most abundant classes (**A**)
30 and genera (**B**) are shown.



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