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Original article

Crosstalk among intestinal barrier, gut microbiota and serum metabolome after a polyphenol-rich diet in older subjects with "leaky gut": The MaPLE trial

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SUMMARY

Background &aim: The MaPLE study was a randomized, controlled, crossover trial involving adults ≥60 y.o. (n = 51) living in a residential care facility during an 8-week polyphenol-rich (PR)-diet. Results from the MaPLE trial showed that the PR-diet reduced the intestinal permeability (IP) in older adults by inducing changes to gut microbiota (GM). The present work aimed at studying the changes in serum metabolome in the MaPLE trial, as a further necessary step to depict the complex crosstalk between dietary polyphenols, GM, and intestinal barrier.

Methods: Serum metabolome was monitored using a semi-targeted UHPLC-MS/MS analysis. Metataxonomic analysis (16S rRNA gene profiling) of GM was performed on faecal samples. Clinical characteristics and serum levels of the IP marker zonulin were linked to GM and metabolomics data in a multi-omics network.

Results: Compared to the control diet, the PR-diet increased serum metabolites related to polyphenols and methylxanthine intake. Theobromine and methylxanthines, derived from cocoa and/or green tea, were positively correlated with butyrate-producing bacteria (the order Clostridiales and the genera *Roseburia*, *Butyricicoccus* and *Faecalibacterium*) and inversely with zonulin. A direct correlation between polyphenol metabolites hydroxyphenylpropionic acid-sulfate, 2-methylpyrogallol-sulfate and catechol-sulfate with *Butyricicoccus* was also observed, while hydroxyphenylpropionic acid-sulfate and 2-methylpyrogallol-sulfate negatively correlated with *Methanobrevibacter*. The multi-omics network indicated that participant's age, baseline zonulin levels, and changes in Porphyromonadaceae abundance were the main factors driving the effects of a PR-diet on zonulin.

Conclusion: Overall, these results reveal the complex relationships among polyphenols consumption, intestinal permeability, and GM composition in older adults, and they may be important when setting personalized dietary interventions for older adults.

Trial registration number: ISRCTN10214981.

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1. Introduction

Increased intestinal permeability (IP), a condition also known as "leaky gut", has been proposed as a potential contributor to inflamm-aging and a wide range of intestinal disorders such as inflammatory bowel disease, coeliac disease, and Crohn's disease, as well as several chronic diseases such as cardio-renal-metabolic diseases [1,2]. Increased IP is characterized by a low-grade systemic inflammation triggered by the diffusion of toxins or bacterial factors to the bloodstream [3].

Age has been reported as an independent risk factor for altered IP, and some studies have shown an increased IP over the age of 50 [4]. Moreover, gut microbiota (GM) is another regulator of IP implicated in the renovation of the intestinal epithelial cells and in maintaining the integrity of tight junctions [5]. Indeed, a detrimental modification of the microbial community structure in the gut (dysbiosis) can lead to a loss of immune tolerance and to the development of a gut inflammatory environment coupled with increased IP [6]. In consequence, dysbiosis and/or altered IP may not only lead to the overproduction and absorption of toxic metabolites with potential deleterious effects on host-health [7–9], but also compromise the bioavailability of nutrients and beneficial food components, such as polyphenols [10].

Among the strategies to prevent "leaky gut" and to decrease IP associated with aging or chronic diseases, changes of lifestyle factors, including diet, should be the most feasible [11]. Higher consumption of fruits, vegetables and other plant-based foods provides dietary fibre and polyphenols which might help to counteract an impairment of IP through aging [12,13]. In addition, GM activity may improve the structure of the tight junctions and modulate the inflammatory environment in the gut through lower molecular weight compounds derived from food components [14,15].

In the MaPLE trial, we found that an 8-week polyphenol-rich (PR) diet comprising 3 daily portions of PR-foods such as cocoa, green tea and berries (1391 mg/day of dietary polyphenols *vs.* 812 mg/day registered in the control diet) led to a significant reduction of the IP marker, zonulin, in older subjects affected by "leaky gut" [16]. Thus, our primary hypothesis is that serum metabolome changes will be associated with the improvement of IP in older adults through GM-dependent and GM-independent pathways. Here, we also investigated the crosstalk between intestinal barrier and the changes of GM composition and serum metabolome linking clinical characteristics to metataxonomics and metabolomics data through a multi-omics network using Mixed Graphical Models.

2. Materials and methods

2.1. Recruitment of the volunteers and assessment of the dietary protocol

The trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy) in residential care and independent residences for older subjects, as previously described [16]. To be included in the trial, the subjects had to be $\geq$ 60 years old and with increased IP, evaluated by means of serum zonulin level. Other inclusion and exclusion criteria were already reported in [16] and the dietary intervention protocol has been previously published [17]. Briefly, the PR dietary pattern was designed by the substitution of some low-polyphenol products in the control diet with other comparable products but high in polyphenols, i.e. PR-products (e.g. foods used for snack or breakfast), while maintaining as much as possible the overall energy and nutrient composition. Specifically, subjects consumed 3 portions/day of selected PR-products including 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), providing a mean of 724 mg/day of total polyphenols as estimated by Folin-Ciocalteu analysis. Mean total polyphenol intake was 1391 mg/day in the PR-diet *vs.* 812 mg/day in the control diet.

The study protocol complied with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of the University of Milan, Italy (ref: 6/16/CE_15.02.16_Verbale_All-7). All subjects and their relatives were informed about the study protocol and they signed an informed consent before the enrolment. The trial was registered under the code: ISRCTN10214981 [17].

2.2. Experimental design

The study consisted of an 8-week, randomized, cross-over intervention trial (PR-diet *vs.* control diet). Volunteers were randomly allocated in one of the two arms of the trial, starting with PR-diet or control diet according to a computerized randomization protocol. Subjects assigned to the PR-diet received the three daily portions of selected PR-foods described before. During the control diet period, subjects followed the regular menu provided by the nursing home, which was previously evaluated for the nutritional and polyphenol composition. After the wash-out period (8 weeks) performed to avoid any carry-over effect, the groups were switched to the other diet.

At four time-points during the trial (visits 1–4), corresponding to the beginning and the end of each intervention period, all participants underwent to physical and general condition examinations (e.g. weight, blood pressure and clinical signs), as already described [16], and blood and faecal samples were collected.

2.3. Dietary assessment

The dietary assessment was performed by weighed food records. Three records per subject were obtained during each intervention period. Energy, macro and micronutrients intakes were estimated with MetaDieta® software (Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) [16]. Total polyphenol estimation was performed using the Phenol Explorer database (phenol-explorer.eu) to provide estimates of polyphenol concentrations in each food. In the cases that no useful values, using our proprietary data or values obtained from the literature, total polyphenol content of the foods was estimated directly using the Folin-Ciocalteau method as described in [17].

2.4. Blood sampling and biomarkers analysis

After an overnight fast, blood samples were drawn in Vacutainer tubes containing silica gel for serum separation. Serum was obtained by tube centrifugation at 1400 g and 4 °C for 15 min, divided in small aliquots into labelled vials and stored at −80 °C until analysis.

Anthropometric, metabolic and functional parameters were measured as previously described [17]. Serum zonulin levels were quantified using a specific ELISA kit as previously reported [17].

2.5. Sample preparation and UHPLC-MS/MS metabolomics analysis of serum

Serum samples were prepared for UHPLC-MS/MS analysis by a simple protein precipitation protocol. After thawing on ice, 100 μL of serum were added of 500 μL ACN containing 1.5% v/v formic acid and 10 mM of ammonium formiate. Samples were mixed and kept at −20 °C for 10 min, then centrifuged at 10,000 rpm and 4 °C for 10 min. After protein precipitation, 500 μL of supernatant were dried on vacuum and the residue was recovered with 100 μL of an 80/20 v/

v mixture of water/ACN, containing 0.5% v/v formic acid, 10 mM of ammonium formiate and 100 ppb of a mixture of 13 internal standards (glutamic acid ^{15}N, phenylalanine ^{15}N, urea d_4, acetyl-L-carnitine d_3, myristoyl-L-carnitine d_9, caffeine $^{13}C_3$, glucose d_2, palmitic acid d_{31}, succinic acid d_4, glycocholic acid ^{13}C, ferulic acid, $^{13}C_3$, epicatechin $^{13}C_3$, and taxifolin, Sigma–Aldrich, Steinheim, Germany). Finally, after centrifugation at 10,000 rpm and 4 °C for 5 min, samples were transferred to a 96-well plate and analysed using the targeted UHPLC-MS/MS method described in [18]. Briefly, an Agilent 1290 Infinity UHPLC system coupled to a Sciex QTRAP 6500 mass spectrometer was used for the analysis. A Phenomenex Luna Omega Polar C18 column (100 mm × 2.1 mm, 1.6 μm) equipped with a fully porous polar C18 security guard cartridge was used as stationary phase. Chromatographic conditions were as follows: column temperature, 40 °C; autosampler temperature, 4 °C; injection volume, 2 μL; flow rate, 0.5 mL/min. In the negative ion mode, the mobile phase consisted in a gradient of 0.1% formic acid and 10 mM ammonium formate in water (A) and pure ACN (B). The gradient program was: 0–8 min, 5–20% B; 8–10 min, 20–100% B; 10–12 min, 100% B; 12–12.1 min, 100-5% B; 12.1–14 min, 5% B. On the other hand, in positive ion mode water and ACN, both containing 0.5% formic acid, were used as mobile phases A and B, respectively. In this case, the gradient profile was: 0–5 min, 5–50% B; 5–8 min, 50–100% B; 8–10 min, 100% B; 10–10.1 min, 100-5% B; 10.1–12 min, 5% B. MS detection was performed by using the scheduled multiple reaction monitoring (sMRM) mode. The mass spectrometer operated in positive and negative ionization modes in separate runs, using the following parameters: ion spray voltage, +4500/-3500 V; source temperature, 600 °C; curtain gas, 30 psi; ion source gas 1 and gas 2, 50 psi each; collision-activated dissociation gas, 3 psi; entrance potential, ±10 V; target scan time, 0.05 s.

Analyst 1.6.2 and Sciex OS software by Sciex were used for data acquisition and data processing, respectively.

The quality control of metabolomics data was performed using the POMA R/Bioconductor package (https://github.com/pcastellanoescuder/POMA) [48]. Data pre-processing included the removal of metabolites with more than 80% missing values in all the study groups [19], the imputation of the remaining missing values using the KNN algorithm, and finally data normalization by means of *log* transformation and Pareto scaling. Afterwards, distances to the group centroid were computed based on Euclidean distances to remove outliers from the data matrix (±1.5 × IQR). Finally, the coefficients of variation for areas, retention times and peak widths of the internal standards added to samples were calculated for analytical reproducibility assessment.

2.6. Metataxonomics of faecal samples

The bacterial community structure of faecal samples was assessed as described [17]. In brief, DNA was isolated from faces resuspended in Lysing Matrix E bead beating tubes (MP Biomedicals, Santa Ana, CA, USA) through the FastDNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. Then, the V3–V4 region of the 16S rRNA gene was amplified with panbacterial primers 16S 341F (5′– TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3′) and 16S 806R (5′–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3′). Finally, amplicons were sequenced using an Illumina MiSeq sequencer (Illumina Inc, San Diego, CA, USA) using a 600 cycle MiSeq v3 reagent kit. Pairing, filtering, taxonomic assignment, and biodiversity analyses of sequencing reads were carried out by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) 2 [20] through the Divisive Amplicon Denoising Algorithm (DADA2) using the Greengenes database (version 13_5). Illumina sequencing generated 4,030,722 filtered paired-end reads (median of 19,328 reads per sample). Bioinformatic analysis was conducted using DADA2; reads were merged, and denoised. Reads were dereplicated and singletons removed. After merging and denoising by DADA2 the final sequences were 1,076,356 (mean = 5,021, SD = 3,306). The sequence length statistics in bp showed: min = 240, max = 457, median = 433, standard deviation = 25. Overall, 7,729 unique amplicon sequence variants (ASVs) were identified [16]. Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB46689.

2.7. Statistical analyses

For each period, the diet effect on clinical and metabolomics data were estimated as the change between the "end of dietary intervention − baseline" measurements. For metabolomics data, after exclusion of the metabolites with more than 80% of missing values, they were imputed using the K-nearest neighbour method, log-transformed and Pareto scaled. Afterwards, dietary intervention effects on normalized metabolite concentrations were compared using a subject-specific random effect linear mixed model using baseline metabolite concentrations (at visit 1 and 3), period, diet, period × diet interaction, age, sex and body mass index (BMI) as fixed factors or covariates. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR).

Correlations between the changes of zonulin levels and plasma metabolites and macro and micronutrients significantly altered by the PR-diet were performed using partial correlation tests accounting for baseline zonulin during the PR-diet intervention. Next, stepwise linear multiple regression was used to assess for significant predictors in a model that included age, sex, period, and metabolites and macro and micronutrients significantly correlated with zonulin changes during PR-diet intervention. Residuals were checked for normality using the Shapiro–Wilk test.

Correlation analyses (Spearman and Kendal test) were carried out between the normalized relative abundance of bacterial taxa and metabolites significantly affected by the PR-diet using median data from the trial time-points.

For the integration of metataxonomics and metabolomics data we used a Mixed Graphical Model (MGM). MGMs are undirected probabilistic graphical models, where each node corresponds to one variable, and the edges between two nodes represent a conditional dependency between them given all other variables in the graphical model [21]. We have used 'mgm' R-package to estimate the network of dependencies [22]. Specifications were set to allow the maximum number of interactions in the network. Variables in the model were changes in zonulin levels during each intervention period, baseline zonulin, age, sex, BMI and randomized allocation sequence, metabolomic set of variables (change in metabolites affected by the PR intervention), and metataxonomic variables (changes in relative abundances of all the analysed gut bacteria at the taxonomic level of family). A subsequent model included also data on macro and micronutrients intake that differed between the PR- and control diets.

All statistical analyses were performed using IBM SPSS Statistics 25 (IBM, USA) and R version 4.0.5 (R foundation, Austria).

3. Results

3.1. Trial outcomes

The baseline clinical parameters of the participants, the intake of macro and micronutrients during the PR- and control diets, changes in GM composition, and the main results of the trial have

already been published [16]. Briefly, fifty-one participants (22 men and 29 women) with a mean age of 78 ± 10 years completed the two, 8-weeks, dietary intervention periods. The intake of energy, total protein, saturated fatty acids, total ω-3 fatty acids, cholesterol, B vitamins (B1, B6, B12), vitamin E, and folates was similar between the PR- and control diet. During the PR-diet participants showed a statistically significant lower intake of animal and plant protein (−6% and −7%, respectively), total lipids (−5%), monounsaturated fatty acids (MUFA, −7%), polyunsaturated fatty acids (PUFA, −20%), total ω-6 fatty acids (−23%), calcium (−16%), and iron (−7%) than during the control diet. Conversely, significantly higher intakes of total carbohydrates (+5%), total dietary fibre (+6%), vitamin C (+15%), and total polyphenols (+71%) were reported during the PR-*vs.* the control diet. The overall bacterial community structure (α- and β-diversity) of faecal microbiota did not change significantly during the PR-diet in comparison to the control one (Supplementary Fig. S2); nonetheless, the following shifts in specific bacterial taxa were observed: i) relative reduction in: *Bacteroides uniformis* (−63%), and *Streptococcus agalactiae* (−83%); and ii) relative increase in: *Alistipes onderdonkii* (+300%), *Anaerobutyricum hallii* (+216%), *Faecalibacterium prausnitzii* (+100%), and bacterial members of the genera *Lactonifactor* (+38%) and *Butyricicoccus* (+59%). Zonulin levels were significantly reduced (−9%, Supplementary Fig. S1) by the PR-diet in comparison to the control diet.

3.2. Variation of serum metabolome induced by the PR-diet intervention

Using a targeted UHPLC-MS/MS approach, the effects of the PR-diet intervention on the serum metabolome of the 51 participants was evaluated. As shown in Fig. 1 and Table 1, ten metabolites were significantly associated to the effects of the PR-diet intervention on serum composition. Among these, catechol sulfate (CAT-S), hippuric acid (HA), 2-methylpyrogallol sulfate (2-MePyr-S), and hydroxyphenylpropionic acid sulfate (HPPA-S) were increased in serum following PR-diet and could be considered as markers of polyphenols intake and subsequent degradation by gut microbiota [23–26], followed by phase II metabolism in the liver. A brief description of these metabolites is reported in the Supplementary Material. Theobromine (TB), also increased in serum after the PR-diet, derived from the consumption of cocoa during the intervention period or from the metabolism of theophylline from green tea [27], as well as 3-methylxanthine (3-MX) and 7-methylxanthine (7-MX) [28].

The serum levels of asparagine, deoxycarnitine and hydroxyhexanoylcarnitine decreased after the PR-diet intervention. Notably, the lowered levels of deoxycarnitine could be associated with the effects of the PR-diet on the IP, considering that a positive correlation between circulating deoxycarnitine and IP has been previously reported [29].

As shown in the heatmap reported in Fig. 2, a positive correlation was observed between the changes in the two methylxanthine metabolites and its parent metabolite, TB. Several other markers of PR-food ingestion were positively correlated with each other, as an indication of the relationship between the ingestion of PR-foods and the production of lower molecular weight derivatives by GM [23]. Most importantly, the two methylxanthine metabolites of TB, 3-MX and 7-MX, were negatively correlated to serum levels of zonulin, expressed as the difference between the levels at the end and at the beginning of the PR-intervention period, respectively (Fig. 2). Correlation analysis using micro and macronutrients data showed an inverse correlation between changes in zonulin levels and the intake of PUFA, iron, and animal protein. Other correlations were observed between dietary data and the changes in the metabolites altered by the PR-diet, specially between calcium intake and hippuric acid and the two methylxanthines (Fig. 2). Multiple regression analysis showed that the increase of 7-MX ($\beta = -0.15$, $p = 0.003$), baseline zonulin levels ($\beta = -0.72$, $p < 0.001$), age ($\beta = 0.008$, $p = 0.024$), and iron intake ($\beta = -0.44$, $p = 0.040$) were significant predictors of the decrease of serum zonulin induced by the PR-diet ($R^2 = 0.52$).

3.3. Correlations between serum metabolome and gut microbiota composition

Significant correlations between plasma metabolites associated to the PR-diet and specific changes of bacterial taxa of the GM were observed (Fig. 3). Among the other metabolites, TB was the one showing more statistically significant correlations. Most importantly, plasma TB was positively correlated with dominant SCFAs-producing bacteria, such as the members of Clostridiales order, *Roseburia*, *Butyricicoccus* and *Faecalibacterium,* and with *Lactonifactor* (all $p < 0.0001$), a bacterial genus involved in the transformation of dietary plant lignin to enterolactone [30]. Statistically significant negative correlations were also observed between TB and potentially pathogenic bacterial genera, such as the *Methanobrevibacter* [31,32], and the members of Proteobacteria phylum (*Desulfovibrio* and Enterobacteriaceae) (all $p < 0.01$). Similarly, methylxanthine metabolites 3-MX and 7-MX showed positive correlations with *Butyricicoccus* and *Lactonifactor,* and negative correlations with the Bacteroidales order (Fig. 3).

Other statistically significant correlations were observed between the polyphenol metabolites HPPA-S, 2-MePyr-S and CAT-S with *Butyricicoccus* (positive correlations), and between HPPA-S and 2-MePyr-S with *Methanobrevibacter* (negative correlations) (Fig. 3).

3.4. Interdependent associations between changes in zonulin, serum metabolome and gut microbiota composition

The changes in zonulin levels during the PR-diet were directly related to age, to changes in serum levels of HA and CAT-S, and to the changes in the relative abundance of the bacterial families Porphyromonadaceae and Coriobacteriaceae in the gut (Fig. 4). In line with previous reports [16], changes in zonulin levels were inversely correlated with baseline zonulin, showing that the effects of the intervention were more evident among participants with an overt alteration in IP. Baseline zonulin was directly related to age, to changes in deoxycarnitine levels and to the relative abundance of Lachnospiraceae family. Conversely, inverse relationships were observed with changes in 7-MX, TB and CAT-S (Fig. 4). Altogether, the multi-omics network shows that changes in zonulin depended on three main factors: a) participant's age, which was also associated to different responses in GM composition (positively associated with changes in members of Lachnospiraceae, Enterobacteriaceae, Porphyromonadaceae and Coriobacteriaceae, while negatively with changes in members of the family Verrucomicrobiaceae), b) baseline zonulin levels, which was one of the main factors affecting serum metabolome changes, and c) changes in Porphyromonadaceae family abundance. Interdependency between these three factors illustrates the complexity of IP regulation, and it shows that changes in serum metabolome were related to changes in zonulin through its association with baseline zonulin and changes in GM composition (Lachnospiraceae, Coriobacteriaceae and Enterobacterioceae). Several of these relationships were specific for the PR-diet period and were not present during the control diet

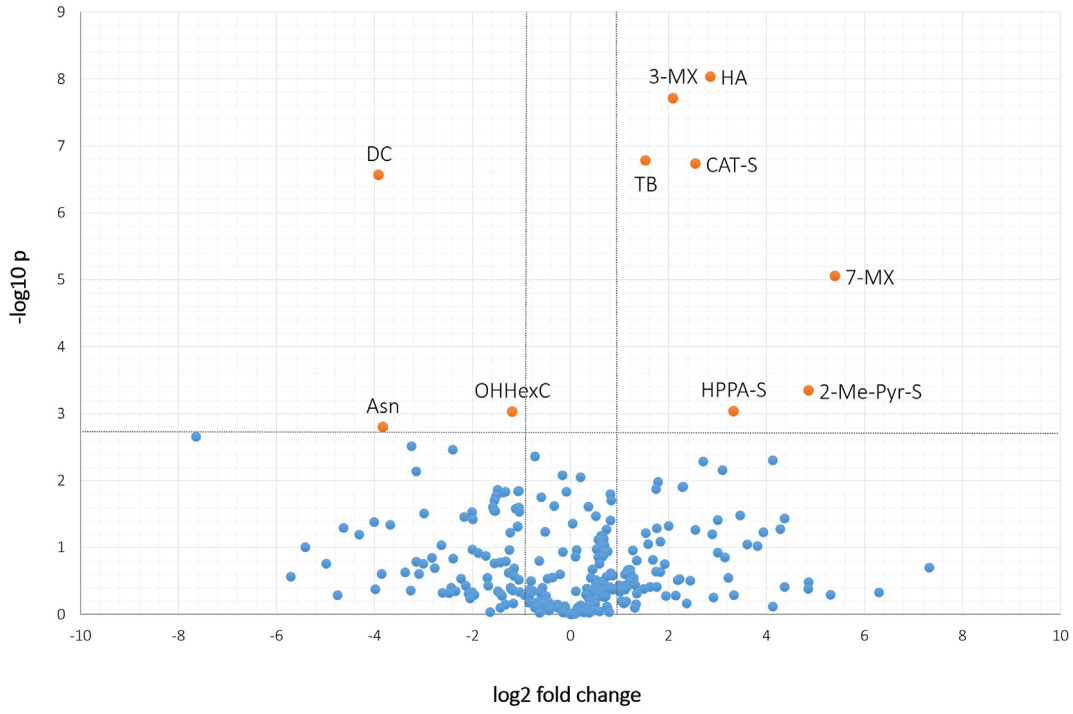

Fig. 1. Volcano plot of log_2 (fold-change) (x-axis) *vs.* $-log_{10}$(FDR-corrected p-value) (y-axis), showing the metabolites significantly associated to the changes of serum metabolome induced by the PR-diet intervention in the whole MaPLE cohort (n = 51). The Fold Change value of each metabolite, reported as log_2(Fold Change), corresponds to the ratio between Δconcentration during the intervention period and Δconcentration during the control period. P-values were calculated by comparison of ΔControl *vs.* ΔPR values using linear mixed models with subject-specific random effects adjusted for age, sex, body mass index, baseline metabolite concentration, period and the period × diet interaction, followed by Benjamini-Hochberg correction for multiple comparisons. HA: hippuric acid; CAT-S: catechol sulfate; 3-MX: 3-methylxanthine; TB: theobromine; DC: deoxycarnitine; 7-MX: 7-methylxanthine; 2-MePyr-S: 2-methylpyrogallol sulfate; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; OHHexC: hydroxyhexanoylcarnitine; Asn: asparagine.

Table 1
Variables significantly (FDR-adjusted p-value <0.05) associated to the changes of serum metabolome induced by the PR-diet intervention in the whole MaPLE cohort (n = 51).

Name	Fold Change[a]	FDR-corrected p value	Origin (Classification)
Hippuric acid	+2.86	<0.0001	Exogenous (microbial polyphenol metabolite)
3-Methylxanthine	+2.40	<0.0001	Exogenous (theophylline metabolite)
Theobromine	+1.97	<0.0001	Exogenous (methylxanthine, from diet)
Catechol sulfate	+2.56	<0.0001	Exogenous (microbial polyphenol metabolite)
Deoxycarnitine	−1.05	<0.0001	Endogenous (carnitine)
7-Methylxanthine	+5.44	0.0004	Exogenous (theophylline metabolite)
2-Methylpyrogallol sulfate	+4.92	0.019	Exogenous (microbial polyphenol metabolite)
Hydroxyhexanoylcarnitine	−1.48	0.032	Endogenous (acylcarnitine)
HPPA-S	+3.47	0.032	Exogenous (microbial polyphenol metabolite)
Asparagine	−0.95	0.048	Endogenous (amino acid)

HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate.
[a] Fold Change corresponds to the ratio between Δ concentration during the PR-diet intervention period and Δconcentration during the control period. It is reported as log_2(Fold Change). Statistical analysis was carried out using linear mixed models with subject-specific random effects adjusted by age, sex, body mass index, baseline metabolite concentration, intervention period and the period × diet interaction, followed by Benjamini-Hochberg correction for multiple comparisons.

(Fig. 4), as for example the direct correlation between baseline zonulin and Lachnospiraceae, or the negative correlation between the changes in zonulin and Clostridiaceae. On the other hand, opposite correlations between nodes during the control and PR-diet periods could be observed, as in the case of the relationship between baseline zonulin and Enterobacteriocaeae, that in control diet was characterized by a direct association. Including the dietary data in the multi-omics network showed inverse associations between total dietary fibre and baseline zonulin, as well as between iron intake and changes in zonulin levels during the PR-dietary intervention (Supplementary Fig. S3). Furthermore, calcium intake showed a positive association with Porphyromonadaceae, only during the PR-diet. The association between total dietary fibre and baseline zonulin was common to both dietary intervention periods (Supplementary Fig. S3).

4. Discussion

The present study shows a determinant role of IP in the metabolomic changes related to the PR-diet intervention and the interdependent associations between changes in zonulin, serum metabolome and gut microbiota composition. These relationships were visualized in a multi-omics network integrating clinical

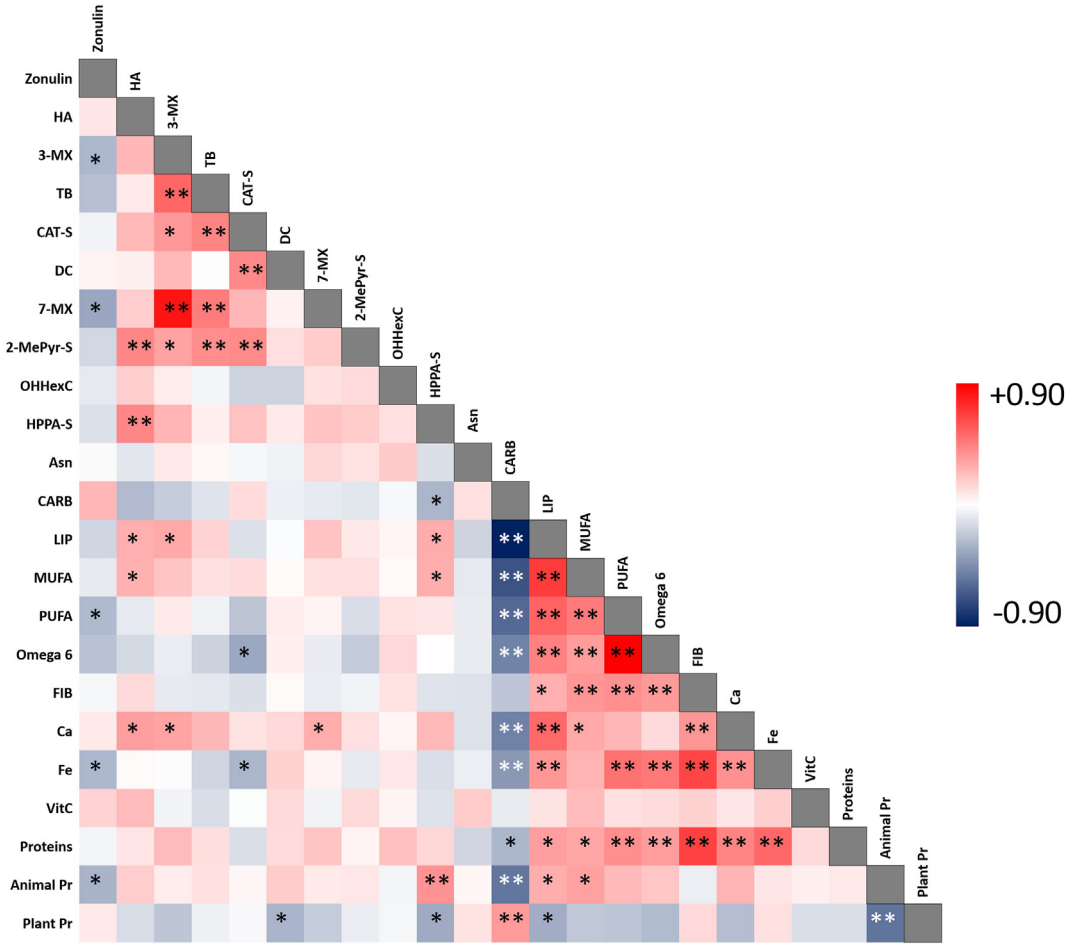

Fig. 2. Heatmap showing the correlations among serum zonulin, serum metabolites significantly altered by the PR-diet intervention, and the amounts of nutrients provided by the MaPLE diet during the trial, specifically: carbohydrates (CARB), lipids (LIP), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA), omega-6 fatty acids, fibers (FIB), calcium (Ca), iron (Fe), vitamin C (VitC) and total, animal and plant proteins (Pr). HA: hippuric acid; 3-MX: 3-methylxanthine; TB: theobromine; CAT-S: catechol sulfate; DC: deoxycarnitine; 7-MX: 7-methylxanthine; 2-MePyr-S: 2-methylpyrogallol sulfate; OHHexC: hydroxyhexanoylcarnitine; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; Asn: asparagine. *: P-value < 0.05; **: P-value < 0.01.

characteristics, baseline zonulin levels, and metabolomic and metataxonomic data.

Metabolomics analysis of serum samples revealed that an 8-week PR-diet significantly altered the levels of 10 metabolites. Seven of these were exogenous, hence derived from the consumption of specific PR-foods or from the metabolism of their constituents by endogenous metabolic pathways or by the intestinal microbiota. Specifically, these were methylxanthine metabolites (TB, 3-MX, and 7-MX) and phenolic compounds derived from the degradation of dietary polyphenols by the GM (HA, CAT-S, HPPA-S and 2-MePyr-S). Among the endogenous metabolites, deoxycarnitine decreased after the PR-diet intervention. In the MGM network, the increase of CAT-S, 7-MX and TB and the decrease of deoxycarnitine levels were correlated with baseline zonulin, and not with its changes, showing that the effects of the intervention on these metabolites depended on higher extent on baseline IP. TB is the most abundant methylxanthine of cocoa, but it could be produced also by the gut microbiota upon the intake of food products containing caffeine, such as coffee or tea [27]. Recent studies show that TB could exert anti-tumour, anti-inflammatory and antioxidant activities, and act as a cardiovascular protector through the inhibition of phosphodiesterases and the blockade of adenosine receptors [33]. In healthy rats, a two-week administration of TB (from cocoa) was shown to induce significant alterations of the GM composition, reducing the proportion of the colitogenic IgA-coated bacteria, and to enhance butyric acid production [34]. The increase of butyrate-producing bacteria belonging to the families Lachnospiraceae and Ruminococcaceae has been already reported in the MaPLE trial, and here, positive correlations were observed between *Butyricicoccus*, *Roseburia*, and *Faecalibacterium* and TB levels. Methylxanthines are metabolites of theophylline and TB mainly formed by endogenous metabolism in the liver, and to lower extent in small intestine and colonic mucosa cells [28]. In the MaPLE trial, they could also be associated to the consumption of green tea and cocoa included in the dietary intervention. 7-MX was negatively correlated with the PR diet-induced variation of serum zonulin in correlation analysis, and with baseline zonulin in the multi-omics network. A possible mechanism behind this relationship could be the inhibition of the enzyme poly (ADP-ribose)polymerase-1 (PARP1) [35,36]. In previous studies on the depletion of this protein in mice, it has been observed that the PARP1 deficiency was associated with a modulation of the colonic microbiota, with increased relative abundance of clostridial clusters IV and XIVa, and a concomitant increase in the frequency of several mucosal regulatory T cells [37]. In another study, it was observed that the inhibition of PARP1, using 3-aminobenzamide, leaded to a decrease of the IP and a local anti-inflammatory effect in an animal model of colitis

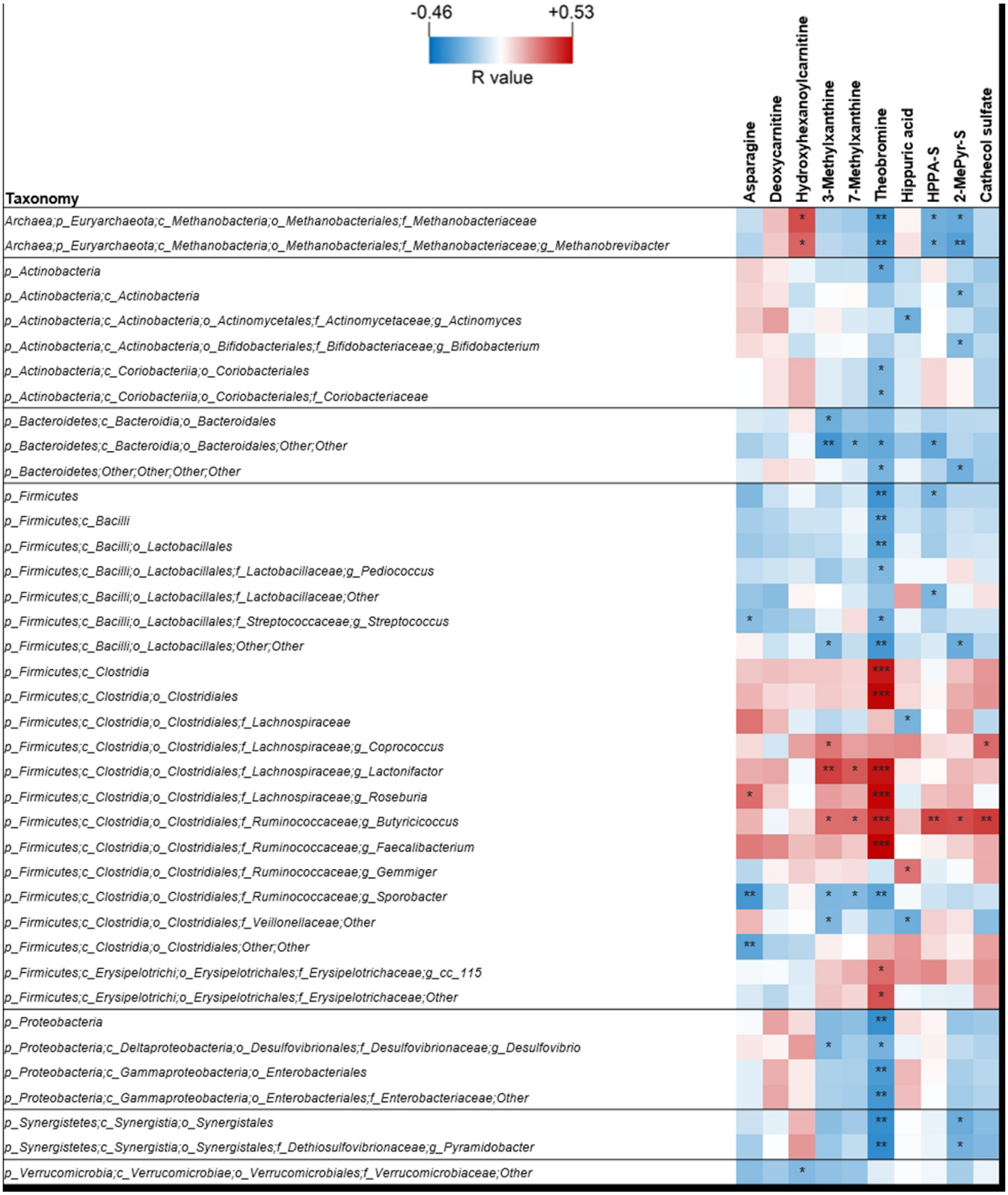

Fig. 3. Heatmap showing correlations between the median relative abundance bacterial taxa in the gut and median concentration of the metabolites that were altered in serum after the polyphenol-rich diet. The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; 2-MePyr-S: 2-methylpyrogallol sulfate.

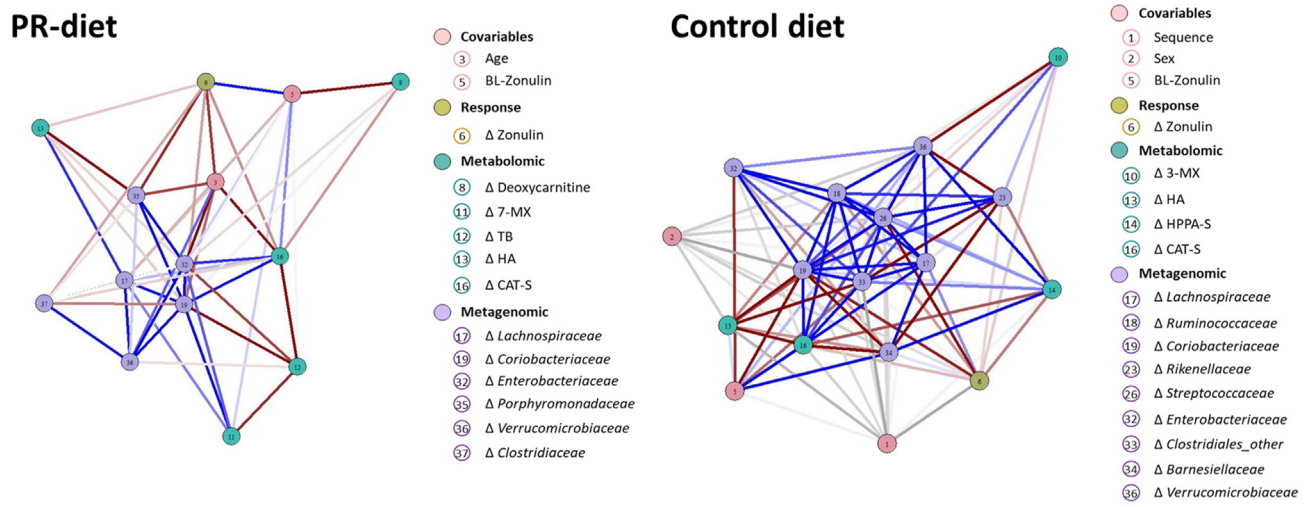

Fig. 4. Neighbourhood of the nodes "Δ Zonulin" and "Baseline Zonulin" during the polyphenol-rich (PR) and control diet. Edge intensity reflects the strength of an association from strong positive (dark red) to strong negative association (dark blue). The node colour indicates the type of data. Variables in the mixed graphical model were changes in zonulin levels (Δ Zonulin), baseline zonulin (BL-Zonulin), age, sex, BMI, and randomized allocation sequence, change in metabolites affected by the PR intervention, and metataxonomic variables (changes in relative abundances of all the analysed gut bacteria at the taxonomic level of family). HA: hippuric acid; CAT-S: catechol sulfate; 3-MX: 3-methylxanthine; 7-MX: 7-methylxanthine; TB: theobromine; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[38]. Considering these preliminary results in literature, it could be hypothesized that 7-MX could have a role modulating the IP through PARP1 inhibition.

In the multi-omics network, while changes in 7-MX levels showed an inverse association with changes in Coriobacteriaceae and Enterobacteriaceae, changes in TB exhibited direct associations. Members of the Coriobacteriaceae family are considered as pathobionts, and *Collinsella* is its dominant taxon [39]. Gnotobiotic approaches have shown that administration of *Collinsella* reduces the expression of tight junction proteins in enterocytes and stimulates gut leakage [40]. On the other hand, Enterobacteriaceae are Gram-negative bacteria that produce the endotoxin lipopolysaccharide, hence they can induce inflammatory reactions in the gut promoting gut barrier disruption and chronic inflammation [41]. Thus, the negative association between 7-MX and these bacterial families could be implicated in the positive effects of cocoa or green tea consumption on GM. However, the inverse associations of TB and of 7-MX with these bacterial members in the multi-omics network are intriguing. One possible explanation could be that GM may modulate the metabolism of TB in colonic cells [42], hence limiting the absorption and posterior metabolism to 7-MX on one side, while promoting the degradation of TB to other metabolites (such as 5-acetylamino-6-amino-3-methyluracil and 3,7-dimethylurate) on the other side [42]. The latter would only be evidenced by determining faecal metabolomics.

Finally, older age, the low levels of zonulin at baseline, and the increase in the relative abundance of Porphyomonadaceae were the main factors limiting the reduction of IP during the PR-diet. The link between ageing and Porphyromonadaceae has been already reported in a cross-sectional study comparing centenarian *vs.* non-centenarian adults [43]. Regarding the relationship between Porphyromonadaceae and health outcomes, it was previously shown that, in a case–control study on patients with liver cirrhosis, poor cognitive performance in older adults was associated with increases of Porphyromonadaceae [44]. However, increased abundance of Porphyromonadaceae was part of a microbe signature in older adults with a better metabolic profile [45]. In animal models, a link between Porphyromonadaceae, gut inflammation and Th17 lymphocytes in the gut has been observed, where mice with colitis exhibited higher Porphyromonodaceae abundance [46]. Therefore, a contribution of Porphyromonadaceae to gut inflammation and other health outcomes can be hypothesized, but needs further experimental confirmation.

Other changes in bacterial taxa during the PR-diet (Lachnospiraceae, Coriobacteriaceae, and Enterobacterioceae) were interrelated in a complex pattern. However, these changes in GM were not directly associated with changes in zonulin. How the PR-diet specifically modulated the GM ecosystem leading to a reduced IP requires further studies. The inclusion of macro and micronutrients data in the analyses allowed the confirmation up to some extent of the positive effect of dietary fibre on intestinal barrier [12,13]. Furthermore, it suggested an association between iron intake and zonulin levels during a PR-dietary pattern, which also merit further studies.

5. Conclusions

Understanding the impact of IP on host responses to dietary interventions is mandatory for personalized dietary counselling. For this purpose, for the first time we present a metabolomics study on the effect of a PR-diet on IP in older subjects with "leaky gut", also evaluating the impact of changes in GM composition on the main outcome of this intervention. Metabolomics affords important data about the molecular effectors that link the consumption of certain foods to their biological activity, and these should be used as pieces of the complex puzzle that represents the different molecular pathways involved. In light of the close interrelations among IP, food constituents and GM composition, such analyses could reveal the complex interplay between GM, the bioavailability of specific dietary constituents, and their effects on the intestinal barrier.

In conclusion, IP was a main factor modulating the serum metabolome changes during a PR dietary intervention in older adults with "leaky gut". The effects of the PR-diet on IP were mainly related with age, baseline IP and relative abundance of Porphyromonadaceae. Possible relationships between cocoa-derived methylxanthines and IP merit further studies.

6. Limitation of the study

It is worth noting that the MaPLE dietary intervention comprised other foods that provide a high amount of biologically active compounds. Thus, other metabolites from these foods could have contributed to generate lower molecular weight derivatives exerting their own activity or playing a synergic role [47]. Therefore, we cannot exclude that other components of the diet or the metabolites deriving from their degradation in the gut could exert a beneficial effect on the IP of the older volunteers.

Authors' contributions

PR and SG designed the trial and in collaboration with AC, CAL and PAK optimised the study protocol including the selection of clinical and biochemical markers and the development of the polyphenol-rich diet. NHL contributed to the elaboration of the dietary polyphenol intake. CDB and SB performed the analysis of zonulin and other clinical and biochemical markers. RGD developed the UHPLC-MS/MS method. GP performed the metabolomics analysis and the statistical elaboration of the data, in collaboration with GG, TM, PCE, AM and EVL. SG and GG performed metataxonomics analysis of faecal samples. AC and BC helped interpreting the clinical outcomes. GP and TM drafted the first version of the manuscript. All the authors critically revised the draft and approved the final version.

Data availability

The data that support the findings of this study are available from the corresponding authors, T. M. and S. G., upon reasonable request.

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Conflict of Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.08.027.

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