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The relevance of urolithins-based metabotyping for assessing the effects of a polyphenol-rich dietary intervention on intestinal permeability: A *post-hoc* analysis of the MaPLE trial

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ABSTRACT

A polyphenol-rich diet reduced intestinal permeability (IP) in older adults. Our aim was to evaluate if participants categorized according to urolithin metabotypes (UMs) exhibited different responses in the MaPLE trial. Fifty-one older adults (mean age: 78 years) completed an 8-week randomized-controlled-crossover trial comparing the effects of a polyphenol-rich *vs.* a control diet on IP, assessed through zonulin levels. Plasma and urinary metabolomics were evaluated with a semi-targeted UHPLC-MS/MS method. Gut microbiota was characterized by 16S rRNA gene profiling. UMs were determined according to urolithin excretion in 24 h urine samples. Multivariate statistics were used to characterize the differences in metabolomic and metataxonomic responses across UMs. Thirty-three participants were classified as urolithin metabotype A (UMA), 13 as urolithin metabotype B (UMB), and 5 as urolithin metabotype 0 (UM0) according to their urinary excretion of urolithins. Clinical, dietary, and biochemical characteristics at baseline were similar between UMs (all $p > 0.05$). After the polyphenol-rich diet, UMB *vs.* UMA participants showed a 2-fold higher improvement of zonulin levels (p for interaction = 0.033). Moreover, UMB *vs.* UMA participants were characterized for alterations in fatty acid metabolism, kynurenine pathway of tryptophan catabolism, and microbial metabolization of phenolic acids. These changes were correlated with the reduction of zonulin levels and modifications of gut microbes (increased Clostridiales, including, *R. lactaris*, and *G. formicilis*). In conclusion, urolithin-based metabotyping identified older adults with a higher improvement of IP after a polyphenol-rich diet. Our results reinforce the concept that UMs may contribute to tailor personalized nutrition interventions.

Abbreviations: 4-HBA-G, 4-hydroxybenzoic acid-glucuronide; ASV, Amplicon sequence variants; LEfSe, Linear discriminant analysis combined with effect size; PLS-DA, Partial least square-discriminant analysis; PR, Polyphenol-rich; URO, Urolithin; UM, Urolithin metabotypes; u3,5-DHPPA, Urinary 3-(3,5 dihydroxyphenyl) propionic acid; u4-MetGA, Urinary 4-O-methyl gallic acid; VIP, Variable Importance in Projection.

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1. Introduction

In the field of personalized nutrition, the study of metabolic phenotypes (metabotypes), that may explain the inter-individual variability in the responses to diet, is an active research field that holds the promise of improving the efficacy of dietary interventions (Adrián Cortés-Martín, Selma, Tomás-Barberán, González-Sarrías, & Espín, 2020). Metabolism by gut microbiota of ellagic acid and ellagitannins, present in foods like berries (strawberries, raspberries, and others), pomegranates, walnuts, some types of teas (X. Yang & Tomás-Barberán, 2019), and oak-aged wines, among others, results in different urolithin metabotypes (UMs) (Adrián Cortés-Martín et al., 2020; Iglesias-Aguirre et al., 2021). UMs are classified according to the presence in urine and(or) feces of distinctive urolithins after the intake of foods rich in ellagic acid or ellagitannins. UMA individuals are those producers of only urolithin A. UMB subjects also produce urolithin A, and distinctively, isourolithin A, and(or) urolithin B. Finally, UM0 individuals cannot produce these urolithins (Adrián Cortés-Martín et al., 2020). In previous studies, overweight-obese individuals with UMB showed increased levels of cardio-metabolic risk factors than those with UMA (González-Sarrías et al., 2017; Selma et al., 2018). In addition, overweight-obese individuals with UMB showed an improvement of cardio-metabolic risk factors in a randomized trial with pomegranate juice, while no changes were observed among UMA subjects (González-Sarrías et al., 2017). On the other hand, a better response to lipid-lowering medical treatment was observed in UMA patients with metabolic syndrome (Selma et al., 2018). However, this could not be confirmed recently in polymedicated patients with metabolic syndrome, since poly-pharmacological treatments disturbed and shaped gut microbiota composition, ultimately affecting UMs distribution (Adrián Cortés-Martín, Iglesias-Aguirre, Meoro, Selma, & Espín, 2021). The present study is in line with the hypothesis that clustering of individuals according to UMs may contribute to understand the relationship between dietary polyphenols and their effects on metabolic health, and to implement personalized nutritional interventions (Adrián Cortés-Martín et al., 2020; Iglesias-Aguirre et al., 2021).

The MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) trial was a dietary intervention showing that a polyphenol-rich (PR) dietary pattern can improve intestinal permeability (IP) markers and blood pressure in older adults (>60 years) (Del Bo' et al., 2021) *via* modifications of gut microbiota composition and plasma metabolome (Peron et al., 2021). Foods containing ellagic acid and ellagitannins among the MaPLE diet were pomegranate juice, green tea, and blueberries. Our hypothesis is that different responses on IP markers and cardio-metabolic risk factors will be observed according to UMs, in relationship with differences in gut microbiota composition and activity. The main aim was to evaluate the relationship between UMs and the response on IP markers and cardio-metabolic risk factors in older adults with "leaky gut" following a PR dietary pattern. In addition, in an exploratory analysis, we characterized the gut microbiota, and serum and urine metabolome changes in the major UMs, UMA and UMB.

2. Materials and methods

2.1. Study protocol

Characteristics of the experimental design, including inclusion and exclusion criteria, have been previously reported (Guglielmetti & Bernardi, 2020). Briefly, the MaPLE trial was an 8-week randomized, controlled, cross-over trial comparing the effects of a PR dietary pattern *vs.* a control diet in a group of 51 older adults (aged $\geq$ 60 years) with "leaky gut". Daily plan of the polyphenol-rich foods provided in the PR diet during the trial are shown in Supplementary Fig. A.1. The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Ethics Cpommittee 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 (Guglielmetti & Bernardi, 2020).

Blood, urine, and faecal samples were collected before and after each 8-week intervention period, with an 8-week washout period in-between.

Patient flowchart and CONSORT checklist and are supplied as supplementary materials in the Appendix B.

2.2. Metabolomics

2.2.1. Urine metabolomics

Details on the urine metabolomics methodology have been previously published (Hidalgo-Liberona et al., 2020). Briefly, a multi-targeted quantitative metabolomics analysis of the urinary metabolome was accomplished by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Urolithins A and B, and isourolithin A as well as their phase-II metabolites were quantified. Details on the on the chemical identification (mass transitions and ions used for quantification and identification), reliability, accuracy, and repeatability of the analyses can be found in a previous publication (González-Domínguez, Jáuregui, Isabel Queipo-Ortuño, & Andres-Lacueva, 2020). More details on urinary urolithins identification, quantification and analytical performance can be found in the Appendix C.

2.2.2. Urolithin metabotypes

UMs were assigned according to the excretion in urine of urolithins during the trial. Individuals with exclusive excretion of urolithin A or its phase-II metabolites were assigned to UMA. Participants excreting urolithin B and(or) isourolithin A in addition to urolithin A (and(or) their respective phase-II metabolites) were assigned to UMB. Participants with undetectable levels of urolithins or isourolithins in urine were assigned to UM0.

2.2.3. Serum metabolomics

Serum metabolomics analysis was carried out following the procedure described in (González-Domínguez, Jáuregui, Mena, et al., 2020). Briefly, serum samples were prepared by protein precipitation with cold acetonitrile containing 1.5% v/v formic acid and 10 mM of ammonium formate. Following centrifugation, supernatants were dried on vacuum and the residue was recovered with 100 μL of an 80/20 v/v mixture of water/acetonitrile, containing 0.5% v/v formic acid, 10 mM of ammonium formate, and a mixture of internal standards. Finally, samples were transferred to a 96-well plate and analysed using the semi-targeted UHPLC-MS/MS (González-Domínguez, Jáuregui, Mena, et al., 2020; González-Domínguez, Urpi-Sarda, et al., 2020). Phase II metabolites of some compounds did not have commercial standards available. Therefore, these phase II metabolites were quantified in a semi-targeted way using the calibration curves of structurally similar metabolites as described previously (González-Domínguez, Urpi-Sarda, et al., 2020).

2.3. Metataxonomics

The bacterial community structure of faecal samples was assessed as described in Guglielmetti et al. (Guglielmetti & Bernardi, 2020). In brief, the V3-V4 region of the 16S rRNA gene was amplified and 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 (Bolyen et al., 2019) through the Divisive Amplicon Denoising Algorithm (DADA2) using the Greengenes database (version 13_5). Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB46689.

2.4. Dietary data

During both intervention periods, weighed food records were used to estimate food, energy, nutrient, and polyphenol intake as previously described (Martini & Bernardi, 2020). Up to six detailed daily diaries (recording the amount of foods provided and the amount actually consumed by weighing the leftovers) were analysed for each subject during the two intervention periods. Data includes total polyphenol content quantified by the Folin-Ciocâlteu method and estimates of total polyphenols. These estimations were performed using an in-house ad-hoc database of food composition on polyphenols, compiled from the USDA (https://fdc.nal.usda.gov/, last access date: August 2020) for databases (for flavonoids, isoflavones and proanthocyanidins) and the Phenol-Explorer (www.phenol-explorer.eu, last access date: August 2020) database (for phenolic compounds lignans, stilbenes and other minor polyphenol classes). To calculate the intake of ellagic acid and ellagitannins + ellagic acid, we used data of Phenol-Explorer on pure pomegranate juice and the mid value of previously reported papers. Content of ellagic acid in blueberries was reported to range between 0.8 and 6.7 mg/100 g FW (Sellappan, Akoh, & Krewer, 2002). In green tea ellagitannins content ranged between 0.5 and 2.5 mg / g of tea in prepared tea infusion (X. Yang & Tomás-Barberán, 2019).

2.5. Outcomes

2.5.1. Cardiometabolic risk factors

Anthropometric, clinical and biochemical parameters were measured as previously described (Guglielmetti & Bernardi, 2020). Cardiometabolic risk factors included in the present analysis were body weight, systolic and diastolic blood pressure, glucose, insulin, HOMA-IR, serum lipids (triglycerides, total cholesterol, and LDL- and HDL-cholesterol) and inflammatory markers (VCAM-1, ICAM-1, CRP, TNF-α, and IL-6). Levels of VCAM-1, ICAM-1 were measured using specific ELISA kits (Booster® from Vinci Biochem S.r.l., Vinci, Italy), as well as CRP, TNF-α and IL-6 (kits DCRP00, HSTA00E, HS600B, respectively, from R&D Systems, Biotechne, Abingdon, UK) (Guglielmetti & Bernardi, 2020).

2.5.2. Intestinal permeability markers

Zonulin levels were measured in serum using the Immunodiagnostik® ELISA kit (Bensheim, Germany), following the procedure already published (Guglielmetti & Bernardi, 2020).

2.6. Statistical analysis

2.6.1. Descriptive statistics

Clinical, biochemical, and dietary characteristics are shown according to UM as mean ± SD or median (Q1-Q3) according to data distribution. Comparisons between UMs were carried out by one-way ANOVA or the Kruskall-Wallis test, for variables with normal or skewed distribution, respectively. To compare the nutrients and polyphenol intake across diets and UMs, linear mixed models using individual-specific random effects, and diet, UMs, and its two-way interaction term as main effects were used.

2.6.2. Analysis of outcomes

Outcome variables were included in statistical analyses as the percentage change during the intervention period as [(Post - Pre)/Pre] *100%. The main outcome was Δzonulin levels and the secondary outcomes were changes in body weight, systolic and diastolic blood pressure, glucose, insulin, HOMA-IR, serum lipids, and inflammatory markers. Linear mixed models including individual-specific random effects, sex, age, BMI, and randomized allocation sequence as covariates, and diet and UM (UMA *vs.* UMB) as main effects, and its two-way interaction term were used to assess the impact of Ums on trial outcomes. When appropriate, pairwise comparisons with Tukey's *post-hoc* test were carried out using the R package ''*multcomp*''.

2.6.3. Exploratory analysis

Multivariate analyses were performed to compare the effect of the PR diet in gut microbiota composition, and plasma and urine metabolomics in UMA and UMB participants. The relative abundances of bacterial taxa were used after data normalization carried out through the negative binomial distribution method (R/Bioconductor DESeq2 package). Differently abundant taxa between UMA and UMB groups of volunteers were identified using linear discriminant analysis (LDA) combined with effect size (LEfSe) algorithm (Segata et al., 2011). A cut-off value of LDA score (log10) above 2.0 was chosen. Diversity indexes at baseline were calculated at the ASV taxonomic level and compared between UMA and UMB. The following α-diversity indexes were calculated: Shannon, Pielou, and Faith phylogenetic diversity index. Unweighted and weighted β-diversity was calculated through UniFrac algorithm and compared using PERMANOVA test with 999 permutations. To compare the changes in gut microbiota induced by the PR diet according to UMs, after filtering amplicon sequence variants (ASVs) present in less than 10% of the population, and imputation of missing values, fold changes were calculated and included in a partial least square-discriminant analysis (PLS-DA) model. Multivariate analysis was performed to compare the effects of the PR diet across UMs using the 'mixOmics' package (Rohart, Gautier, Singh, & Cao, 2017). ASVs with a Variable Importance in Projection (VIP) values $\geq$ 2.0 were selected.

For serum and urine metabolomics analysis, pre-processing was carried out by filtering metabolites with more than 80% missing values and KNN imputation. Next, we calculated the percentage change during each dietary intervention period, similarly to the outcome variables. Changes in 300 serum metabolites and 180 urinary metabolites were included in two PLS-DA models. One without (n = 46) and one with the participants with UM0 (n = 51). Metabolites were selected according to VIP values $\geq$ 2.0. Spearman test was used for correlation analyses and results are shown as a network. R (R software, Vienna, Austria) version 4.1.0 was used for all the statistical analyses.

3. Results

3.1. Clinical and biochemical characteristics at baseline were similar across urolithin metabotypes

The main results of the trial were previously reported (Del Bo' et al., 2021). Among the 51 participants involved in the trial, 33 were classified as UMA, 13 as UMB, and 5 as UM0. Clinical, dietary, and metaboliccharacteristics at baseline were similar across UMs (Table 1 and Supplementary Table A.1). Moreover, no differences were observed in medical treatments between the participants classified according to UMs (Table 1). Urinary urolithin concentrations according to the metabotypes are shown in Supplementary Fig. A.2. Due to the low number of participants with UM0, they were excluded from subsequent analysis.

Participants during the PR diet showed a higher intake of carbohydrates, monounsaturated fatty acids, dietary fiber, and total polyphenols (measured by both USDA and Folin-Ciocâlteu method) than during the control diet. In addition, during the PR diet lower intakes of total lipids and polyunsaturated fatty acids were observed. Out of the different polyphenol subclasses, flavonoid intake, as well as consumption of ellagic acid and ellagitannins + ellagic acid, were significantly higher during the PR diet (Supplementary Table A.2). Between UMA and UMB, there were no significant differences in dietary intake during the trial periods, except for a higher polyphenol intake, measured by the Folin-Ciocâlteu method, in participants with UMB (Supplementary Table A.2). No differences in macronutrient or polyphenol intake were observed across UMs during the trial periods (p for diet × UM interaction > 0.05).

Table 1
Baseline characteristics of the study population according to urolithin metabotypes.

	All (n = 51)	UMA (n = 33)	UMB (n = 13)	UM0 (n = 5)	P
Age (years)	77 (70–87)	76 (67–86)	81 (74–88)	79 (73–91)	0.55
Female sex (n, %)	29 (57%)	16 (49%)	9 (69%)	4 (80%)	0.24
Allocation sequence (n)	28/23	19/14	7/6	2/3	0.76
Weight (kg)	73 ± 14	74 ± 14	70 ± 15	75 ± 13	0.59
BMI Kg/m^2)	26 (23–30)	26 (23–29)	24 (20–31)	30 (24–33)	0.47
Anti-HT (n, %)	12 (23%)	9 (27%)	2 (16%)	1 (20%)	0.68
Anti-DBT (n,%)	11 (22%)	8 (24%)	3 (23%)	0 (0%)	0.46
Lipid-lowering medication (n, %)	34 (67%)	23 (70%)	9 (70%)	2 (40%)	0.47
SBP (mm Hg)	125 (120–130)	125 (120–130)	128 (122–130)	120 (116–131)	0.83
DBP (mm Hg)	75 (70–80)	78 (71–80)	75 (68–79)	78 (72–82)	0.42
Glucose (mg/dL)	95 (86–113)	97 (87–119)	93 (87–105)	84 (80–109)	0.32
Insulin (μU/mL)	6.2 (4.7–9.2)	6.1 (4.6–9.5)	6.4 (4.8–9.0)	6.6 (5.5–12.6)	0.85
HOMA-IR	1.5 (1.1–2.5)	1.5 (1.0–2.6)	1.5 (1.3–2.1)	1.6 (1.1–3.2)	0.96
TG (mg/dL)	117 (89–169)	121 (90–161)	111 (58–182)	127 (107–331)	0.77
TC (mg/dL)	196 ± 50	193 ± 53	192 ± 43	229 ± 44	0.28
HDL-C (mg/dL)	46 ± 15	45 ± 16	49 ± 13	48 ± 16	0.58
LDL-C (mg/dL)	120 ± 37	119 ± 37	116 ± 35	141 ± 41	0.40
sVCAM-1 (μg/mL)	0.97 (0.63–1.33)	0.84 (0.52–1.30)	1.11 (0.97–1.45)	0.97 (0.65–1.45)	0.17
sICAM-1 (ng/mL)	51 (44–65)	50 (40–64)	60 (50–79)	46 (38–48)	0.011
CRP (mg/L)	3.5 (1.6–9.8)	3.5 (1.7–8.8)	2.8 (0.9–10.7)	9.7 (1.9–13.2)	0.87
TNF-α (pg/mL)	1.2 (0.9–1.8)	1.1 (0.9–1.7)	1.5 (1.1–2.4)	1.2 (0.9–1.8)	0.24
IL-6 (pg/mL)	3.1 (1.9–5.4)	3.0 (1.7–4.5)	3.7 (2.2–8.8)	4.0 (2.3–6.2)	0.77
Zonulin (ng/mL)	42 ± 12	42 ± 12	42 ± 9	46 ± 20	0.88

UM, urolithin metabotype; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA, Homeostasis Model Assessment; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; sVCAM-1, soluble vascular cell adhesion molecule; sICAM-1, soluble intercellular cell adhesion molecule; CRP, C-reactive protein; TNF, tumoral necrosis factor; IL, interleukin. Values are presented as mean ± S.D. or median (Q1-Q3), according to data normal or skewed distribution, respectively.

3.2. Zonulin levels were significantly reduced in participants with the urolithin metabotype B

Zonulin levels were significantly reduced after the PR diet in participants with UMB, but not in those with UMA (UMB Cohen's effect size$_{PR vs Control\ diet}$ = −1.26, UMA Cohen's effect size$_{PR vs Control\ diet}$ = −0.13, Fig. 1). On the other hand, there were no statistically significant interactions between diet and UMs for the secondary outcomes, except for HDL-Cholesterol (Table 2 and Fig. 1). During the PR diet, HDL-Cholesterol slightly increased in participants with UMB. However, in pairwise comparisons no statistically significant difference was observed between the PR and control diet (Fig. 1). Results were similar when total polyphenol intake (assessed by both USDA or Folin-Ciocâlteu method) and pharmacological treatments were included as covariates (data not shown).

3.3. Gut microbiota composition differed between urolithin metabotypes and showed specific modifications in response to the PR diet

The comparison of the overall bacterial community structure (α- and β-diversity indexes calculated at the ASV taxonomic level) of faecal microbiota between participants with UMA and UMB at baseline is shown in Supplementary Table A.3. At the taxonomic level of ASV, gut bacteria evenness was significantly higher in participants with UMB. Similarly, bacterial community richness, assessed through the Shannon index, was higher in participants with UMB with a borderline statistically significance (p = 0.095). In particular, UMB participants showed higher abundances of members of the family Enterobacteriaceae, of the

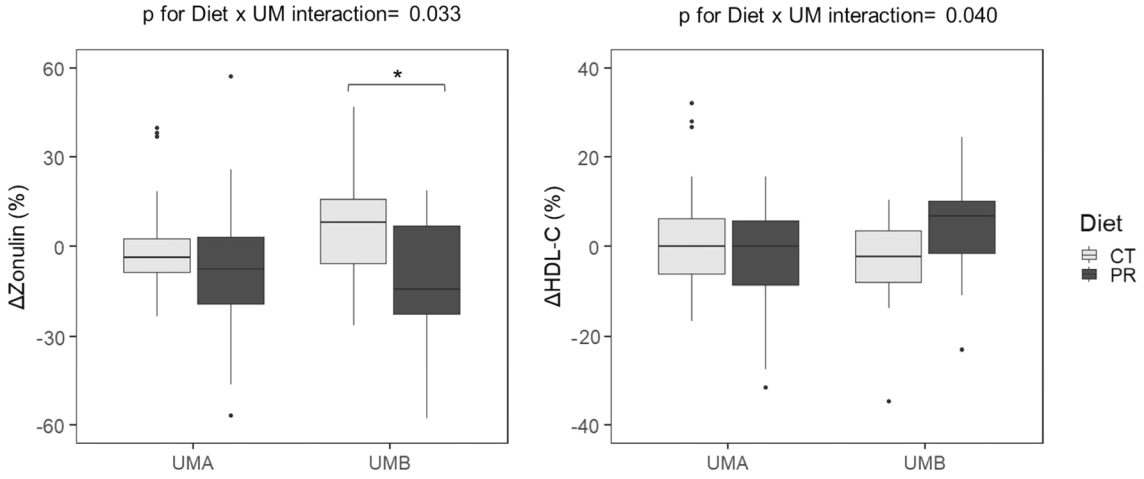

Fig. 1. Interaction between diet and urolithin metabotypes for zonulin and HDL-C levels. UM, urolithin metabotype; PR, polyphenol-rich; CT, control. 33 participants were UMA, and 13 UMB. Linear mixed models including individual-specific random effects, sex, age, BMI, and randomized allocation sequence as covariates, and diet (PR *vs.* control) and urolithin metabotype (UMA *vs.* UMB) as main effects, with its two-way interaction. *p < 0.01 in pairwise comparisons adjusted by the Benjamini-Hochberg procedure.

Table 2
Influence of urolithin metabotypes on changes in cardio metabolic risk factors in older adults following a polyphenol-rich diet.

	All (n = 51)				**UMA (n = 33)**				**UMB (n = 13)**				**P for Diet**	**P for UM**	**P for Diet × UM**
Treatment	**PR**		**CT**		**PR**		**CT**		**PR**		**CT**				
Time	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**			
Weight (kg)	74 ± 14	74 ± 14	73 ± 14	73 ± 14	74 ± 14	74 ± 14	73 ± 14	73 ± 14	70 ± 16	71 ± 16	71 ± 15	71 ± 16	0.06	0.06	0.61
SBP (mm Hg)	126 (120–130)	123 (120–130)	125 (120–130)	125 (120–130)	126 (121–132)	130 (120–136)	125 (120–135)	130 (120–133)	128 (120–130)	123 (120–130)	128 (121–130)	120 (120–128)	0.19	0.17	0.39
DBP (mm Hg)	77 (70–80)	78 (65–80)	75 (70–80)	80 (70–80)	78 (70–85)	80 (70–80)	75 (70–80)	80 (70–80)	75 (68–79)	70 (65–80)	74 (69–80)	78 (70–80)	0.21	0.63	0.24
Glucose (mg/dL)	94 (85–113)	94 (86–110)	97 (85–112)	94 (87–109)	94 (87–129)	94 (87–118)	99 (85–118)	99 (87–116)	95 (87–109)	98 (90–105)	97 (89–110)	91 (88–108)	0.42	0.52	0.31
Insulin (μU/mL)	6.2 (4.5–9.3)	6.6 (4.3–9.4)	6.4 (4.4–9.4)	6.2 (4.1–9.3)	6.6 (4.5–10.0)	7.4 (3.7–9.5)	6.3 (4.2–9.5)	5.9 (3.7–9.5)	6.1 (4.8–9.0)	6.1 (5.5–8.7)	6.6 (5.3–10.0)	5.9 (3.8–7.8)	0.85	0.77	0.65
HOMA-IR	1.8 (1.0–2.4)	1.7 (1.1–2.3)	1.5 (0.9–2.9)	1.7 (0.9–2.3)	2.0 (0.9–2.9)	1.7 (0.9–2.5)	1.4 (0.9–3.1)	1.7 (0.8–2.6)	1.6 (1.3–2.0)	1.4 (1.2–2.4)	1.7 (1.1–2.9)	1.4 (0.8–1.8)	0.45	0.61	0.42
TG (mg/dL)	118 (89–151)	122 (79–171)	123 (72–169)	108 (88–137)	121 (88–150)	121 (76–163)	132 (78–172)	106 (88–135)	111 (69–182)	108 (74–188)	103 (57–187)	111 (88–182)	0.63	0.06	0.19
TC (mg/dL)	195 ± 51	189 ± 50	192 ± 49	188 ± 51	192 ± 53	183 ± 47	190 ± 54	187 ± 55	193 ± 48	194 ± 58	187 ± 44	184 ± 39	0.40	0.96	0.55
HDL-C (mg/dL)	47 ± 15	47 ± 15	47 ± 15	47 ± 15	47 ± 15	45 ± 15	46 ± 16	47 ± 16	48 ± 12	50 ± 12	50 ± 14	48 ± 13	0.18	0.19	**0.040**
LDL-C (mg/dl)	119 ± 37	115 ± 34	116 ± 35	114 ± 37	118 ± 36	113 ± 31	117 ± 37	114 ± 38	116 ± 37	116 ± 40	111 ± 34	109 ± 32	0.59	0.89	0.73
sVCAM-1 (μg/mL)	0.97 (0.54–1.23)	0.87 (0.52–1.45)	0.96 (0.63–1.43)	1.03 (0.63–1.45)	0.88 (0.46–1.20)-	0.71 (0.43–1.26)	0.80 (0.51–1.37)	0.75 (0.49–1.46)-	1.04 (0.94–1.37)	1.00 (0.71–1.67)	1.37 (0.95–1.56)	1.04 (0.67–1.83)	0.46	0.85	0.75
sICAM-1 (ng/mL)	50 (43–64)	55 (43–68)	52 (42–71)	53 (41–63)	48 (40–61)	52 (35–61)	50 (42–66)	49 (37–61)	63 (49–85)	67 (56–92)	68 (46–82)	62 (47–93)	0.39	0.66	0.64
CRP (mg/L)	4.0 (1.6–9.2)	3.5 (1.7–7.4)	3.2 (1.3–6.2)	3.1 (1.4–7.4)	3.9 (1.7–9.7)	2.9 (1.8–5.8)	3.3 (1.5–6.2)	3.1 (2.1–6.0)	4.0 (0.7–10.7)	2.2 (0.6–12.2)	1.5 (0.5–6.5)	2.2 (0.8–8.8)	0.42	0.26	0.50
TNF-α (pg/mL)	1.2 (0.9–1.7)	1.3 (1.0–1.7)	1.2 (0.9–1.6)	1.2 (0.9–1.6)	1.2 (0.9–1.6)	1.2 (0.9–1.6)	1.1 (0.9–1.5)	1.2 (0.9–1.5)	1.4 (1.2–2.1)	1.6 (1.5–2.0)	1.5 (1.2–2.3)	1.6 (1.3–2.1)	0.94	0.40	0.88
IL-6 (pg/mL)	3.2 (2.0–6.0)	3.1 (1.6–5.3)	3.1 (1.8–5.4)	2.6 (1.7–5.6)	3.0 (1.8–5.0)	3.1 (1.2–4.8)	2.6 (1.7–4.5)	2.2 (1.4–5.4)	4.4 (2.2–8.2)	3.4 (1.6–8.2)	3.6 (1.7–8.4)	2.9 (1.7–8.6)	0.16	0.13	0.29
Zonulin (ng/ml)	42 ± 10	39 ± 9	43 ± 11	44 ± 13	40 ± 9	39 ± 9	42 ± 9	42 ± 9	44 ± 8	38 ± 9	40 ± 7	45 ± 9	0.55	0.19	**0.033**

UM, urolithin metabotype; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; sVCAM-1, soluble vascular cell adhesion molecule; sICAM-1, soluble intercellular cell adhesion molecule; CRP, C-reactive protein; TNF, tumoral necrosis factor; IL, interleukin. Values are presented as mean ± S.D. or median (Q1-Q3), according to data normal or skewed distribution, respectively. P-values from linear mixed models including individual-specific random effects, sex, age, BMI, and randomized allocation sequence as covariates, and diet (PR *vs.* control) and urolithin metabotype (UMA *vs.* UMB) as main effects, with its two-way interaction.

genera *Succinispira* and *Desulfovibrio*, and of *Gemminger formicilis* (3 ASVs, Supplementary Fig. A.3). No statistically significant differences were detected in the abundance of members of the class Coriobacteriia ($p > 0.05$), which are phylogenetically close to known urolithin producers of the genera *Gordonibacter* and *Ellagibacter* (Beltrán, Romo-Vaquero, Espín, Tomás-Barberán, & Selma, 2018; Selma, Tomás-Barberán, Beltrán, García-Villalba, & Espín, 2014).

During the PR diet, differential changes were observed in uncharacterized spp. of the Clostridiales order (one ASV), in bacteria of the Lactobacillaceae and Lachnospiraceae familiy (one ASV), *Prevotella* genus, and in four ASVs from *Ruminococcus lactaris* and *Gemmiger formicilis* between participants with UMA *vs.* UMB (Table 3 and Supplementary Fig. A.4).

3.4. Urolithin metabotypes were associated with specific changes in serum and urine metabolites during the PR diet.

There was an increase in the urinary levels of urolithin A and its phase II metabolites in the MaPLE trial which was independent of UMs (p for interaction > 0.05, Supplementary Table A.4). Metabolomics analysis of serum showed several changes specific to each UM in response to the PR diet (Fig. 2). The top discriminant metabolites (13 metabolites with a VIP $\geq$ 2.0) were mostly serum metabolites related with fatty acids metabolism [up-regulated in UMB *vs.* UMA: margaric (C17:0), linolenic (C18:3), 3-hydroxybutiryc, palmitic (C16:0), palmitoleic (C16:1), and oleic (C18:1) acids, as well as tiglyl-carnitine and trimethyl-lysine (Fig. 2)]. In addition, the tryptophan-derived metabolites 3-hydroxykynurenine and xanthurenic acid were down-regulated as a consequence of PR diet in UMB compared to UMA participants.

Table 3
Gut bacterial taxa characterizing the response to the polyphenol-rich diet of participants according to their urolithin metabotype.

ASV nr.	Taxonomy	VIP	UMA (log_2FC)	UMB (log_2FC)	*p*
Dada_13	*p_Firmicutes;c_Clostridia; o_Clostridiales;f_;g_;s_*	2.016	0.56	2.78	**0.07**
	p_Firmicutes;c_Clostridia; o_Clostridiales; f_Lachnospiraceae	2.163	−0.22	0.09	**0.039**
Dada_171	*p_Firmicutes;c_Clostridia; o_Clostridiales; f_Lachnospiraceae*	2.174	−0.04	1.02	0.11
Dada_8	*p_Firmicutes;c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Ruminococcuss;s_lactaris*	2.860	0.07	1.58	**0.008**
Dada_33	*p_Firmicutes;c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Ruminococcus;s_lactaris*	2.314	0.16	1.35	**0.010**
Dada_12	*p_Firmicutes;c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Gemmigers;s_formicilis*	2.423	−0.04	1.22	0.16
Dada_31	*p_Firmicutes;c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Gemmigers;s_formicilis*	2.158	0.57	2.57	0.06
	p_Firmicutes; c_Bacillio_Lactobacillales; f_Lactobacillaceae; Other	2.394	−0.20	2.27	**0.018**
	p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotella	2.347	1.31	4.51	0.12

FC, fold change for diet PR/CT. VIP, variable importance in projection from PLS-DA analysis. The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. ASV, amplicon sequence variant. UM, urolithin metabotype.

Last, three polyphenol metabolites were up-regulated in UMB participants: serum 4-hydroxybenzoic acid-glucuronide (4-HBA-G), and urinary 3-(3,5 dihydroxyphenyl) propionic acid (u3,5-DHPPA) and 4-O-methyl gallic acid (u4-MetGA) (Fig. 2). The inclusion of participants with UM0 in the PLS-DA also achieved separation of the three groups (Supplementary Fig. A.5) and discriminant metabolites (14 metabolites with VIP $\geq$ 2.0; Supplementary Fig. A.5) were mostly the same described above (Supplementary Fig. A.6).

3.5. Specific changes of gut microbiota and polyphenol metabolites correlated with the improvement of zonulin.

Fig. 3 shows the correlations between UM-discriminant gut bacterial taxa, metabolites, and zonulin levels during the PR diet in the whole study population (n = 51). The group of metabolites related to fatty acid metabolism were correlated one with each other, and with *R. lactaris* changes. Two metabolites, 3-hydroxybutyric acid and palmitoleic acid, were inversely correlated with xanthurenic acid, a tryptophan metabolite produced from 3-hydroxykynurenine. Regarding polyphenol metabolites, u4-MetGA and 4-HBA-G were inversely correlated with quinolinic acid and 3-hydroxykynurenine, respectively. In addition, changes in *G. formicilis* were positively correlated with changes in u4-MetGA. On the other hand, u3,5 DHPPA and changes in uncharacterized spp. of the Clostridiales order were inversely correlated with zonulin. There were no statistically significant correlations between urolithin A (or its phase II metabolites) with the discriminant gut bacterial taxa, metabolites, or zonulin levels (all $p > 0.05$).

4. Discussion

The present study shows that the effect of a PR diet on the IP marker, zonulin, was different according to urolithin metabotypes. Indeed, while the median reduction of zonulin in participants with UMB was around 15%, in those with UMA was 6%. Most importantly, there were no differences at baseline in clinical, dietary, and biochemical variables between UMs, and they only differed in the urinary excretion of urolithins. Participants with UMB showed a higher total polyphenol intake than participants with UMA all along the trial. Nonetheless, results remained similar when total polyphenol intake was included in the models, attesting for an effect related with UMs intrinsic characteristics. Compared to the previously published results of the trial (Del Bo' et al., 2021), this post-hoc analysis shows that urolithin-based metabotyping could serve as a basis for personalized nutrition approaches. Dietary interventions are commonly affected by large inter-individual variation, lowering its overall efficacy (Morand et al., 2020). The present results highlight the fact that dietary interventions would show a higher efficacy in targeted populations, which can be identified by means of metabolomic analyses.

The differential modulation of three main metabolic pathways may explain the variability in the response across UMs, namely fatty acids metabolism, the kynurenine pathway of tryptophan catabolism, and the degradation of polyphenols by gut microbiota.

4.1. Fatty acid metabolism

It was shown that urolithins may promote mitochondrial metabolism of fatty acids, inducing browning of white adipose tissue and enhancing skeletal muscle lipid metabolism in animal and human studies (Andreux, Blanco-Bose, Ryu, Burdet, Ibberson, Aebischer, & Rinsch, 2019; Xia et al., 2020). Thus, the up regulation of different fatty acids in serum may reflect an enhancement of fatty acid oxidation in participants with UMB. Indeed, 3-hydroxybutirate and tiglyl-carnitine, which are related to utilization of ketone bodies (Almanza-Aguilera et al., 2018; Lin et al., 2021), were also increased during the PR diet in participants with UMB compared to those with UMA. In a caloric restriction study, 3-hydroxybutirate plasma concentration was associated with increased fatty acid

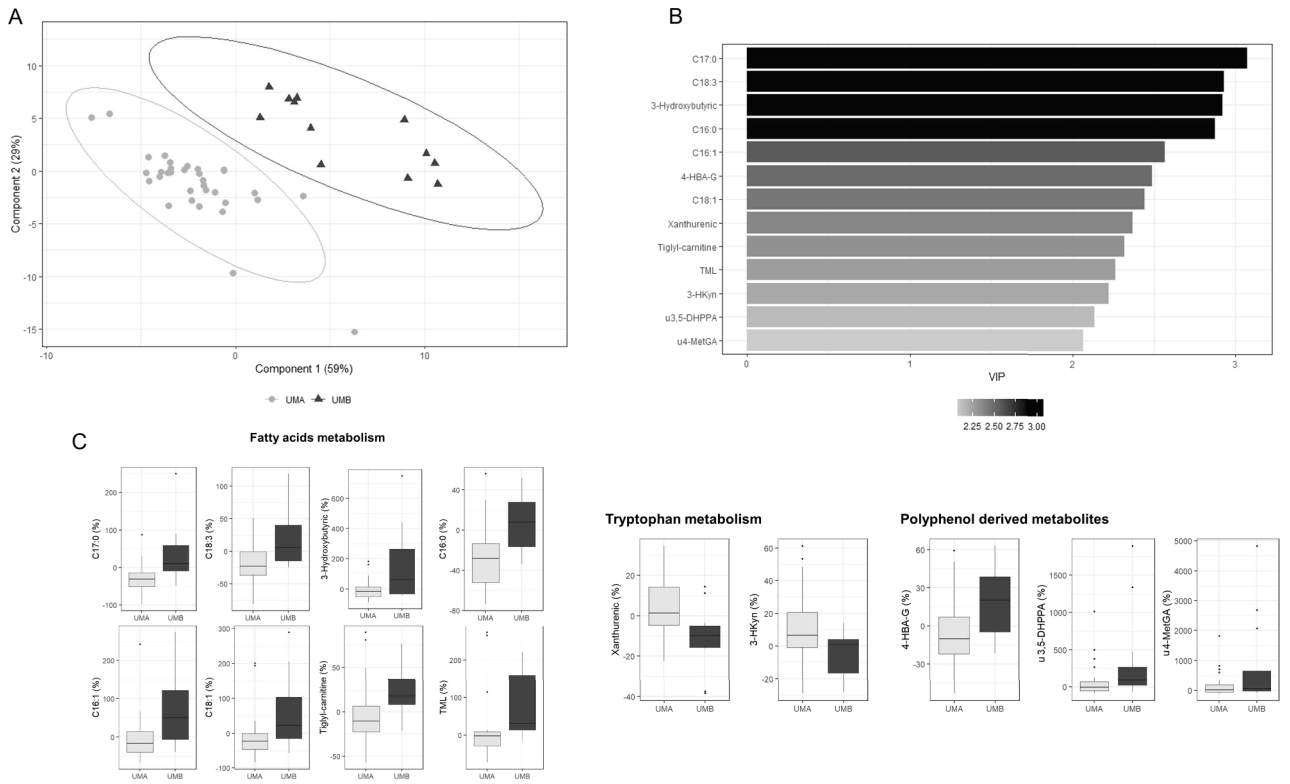

Fig. 2. PLS-DA analysis of serum metabolomics to differentiate the effects of the PR diet in participants according to the main urolithin-metabotypes (UM). Score plot of the first two principal components (Panel A), metabolites with a VIP > 2.0 (Panel B), and boxplots of the percentage change in metabolites concentration with a VIP ≥ 2.0 according to the urolithin metabotype during the PR diet (Panel C). C17:0, margaric acid; C18:3, linolenic acid; C16:0 palmitic acid; C16:1, palmitoleic acid; 4-HBA-G, 4-hydroxybenzoic acid-glucuronide; C18:1, oleic; TML, trimethyl-lysine; 3-HKyn, 3-hydroxykinurenine; u3,5-DHPPA, urinary 3-(3,5 dihydroxyphenyl) propionic acid; u4-MetGA, urinary 4-O-methyl gallic acid.

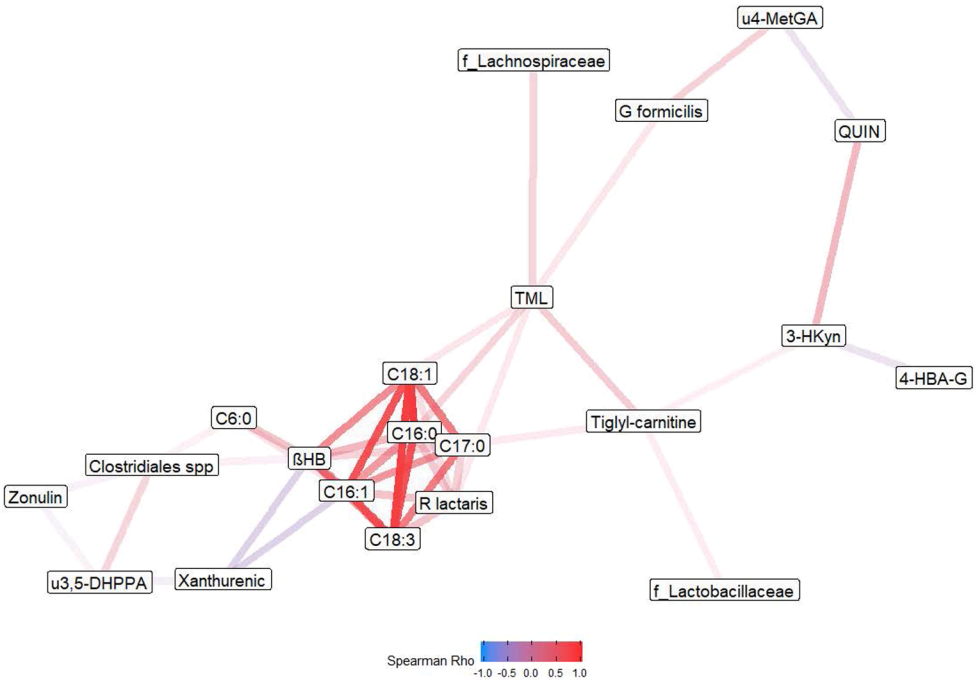

Fig. 3. Network showing the significant correlations between the urolithin-metabotypes' top discriminant metabolites and zonulin levels during the PR diet (n = 51). C17:0, margaric acid; C18:3, linolenic acid; C16:0 palmitic acid; C16:1, palmitoleic acid; C18:1, oleic acid; 3-HB, 3-hydroxybutyric acid; 3-HKyn, 3-hydroxykinurenine; QUIN, quinolinic acid; 4-HBA-G, 4-hydroxybenzoic acid-glucuronide; u3,5-DHPPA, urinary 3-(3,5 dihydroxyphenyl) propionic acid; TML, trimethyl-lysine; u4-MetGA, urinary 4-O-methyl gallic acid; C6:0, hexanoic acid. Correlations of data from the ASVs of *G. formicilis* and *R. lactaris* were similar and for simplification only one is shown in the graphic.

oxidation and weight loss (Almanza-Aguilera et al., 2018). In addition, the higher tiglyl-carnitine, an intermediate by-product of isoleucine catabolism, in UMB subjects may be alternatively associated with: i) increased ketogenesis in the liver and possible accumulation due to saturation of a common mitochondrial enzyme that intersect both metabolic pathways, acetoacetyl-CoA thiolase, (Fukao, Sasai, Aoyama, Otsuka, Ago, Matsumoto, & Abdelkreem, 2018), and/or ii) increased contribution of muscle isoleucine to the liver for ketogenesis (Holeček,

2018). Overall, in response to a PR diet fatty acid oxidation and utilization of ketone bodies appears to be upregulated in participants with UMB compared to those with UMA. Considering the novel beneficial effects of ketone bodies, and particularly, 3-hydroxybutirate, in animal models of longevity (Møller, 2020), this UM-dependent effect of a PR diet in older adults deserves further research.

Margaric acid (C17:0) was the single metabolite with the highest VIP in the PLS-DA analyses. Plasma levels of this odd-chain fatty acid have been associated with reduced incidence of type 2 diabetes (B. J. Jenkins et al., 2017) and is thought to mediate the relationship between dairy intake and its health benefits. However, the impact of dietary fat on the plasma levels of margaric acid have been questioned (B. Jenkins, Aoun, Feillet-Coudray, Coudray, Ronis, & Koulman, 2018), and the possible involvement of an endogenous metabolic pathway behind its positive health effects is under study (B. Jenkins, De Schryver, Van Veldhoven, & Koulman, 2017).

4.2. Kynurenine pathway of tryptophan catabolism

Two metabolites of this metabolic pathway were down-regulated in participants with UMB during the PR diet, 3-hydroxykynurenine and xanthurenic acid. In the analysis including participants with UM0, quinolinic acid, another downstream metabolite of the same metabolic pathway, replaced xanthurenic acid. The modulation of the Kynurenine pathway of tryptophan metabolism by polyphenols and urolithins has been addressed in recent studies (Westfall, Caracci, Zhao, & Wu, 2021; J. Yang et al., 2020). In mice, oral administration of ellagic acid and urolithin A led to a decrease in serum kynurenine levels and to an increase in indole propionic acid, a microbial metabolite with anti-inflammatory effects (Westfall et al., 2021; J. Yang et al., 2020). This decrease in serum kynurenine could be related with the down-regulation of key liver enzymes of the kynurenine pathway, tryptophan-2,3-dioxygenase and kynurenine-3-monooxygenase, as observed in mice models of chronic stress treated with a combination of polyphenols (Westfall et al., 2021). Moreover, in a recent double-blind, placebo-controlled trial, the consumption of pomegranate juice was associated with a higher production of indole propionic acid (J. Yang et al., 2020). Therefore, dietary polyphenols, in general, and urolithins, in particular, may modulate tryptophan metabolism. However, the molecular basis leading to a higher accumulation of some tryptophan metabolites in participants with UMA is not known. It could be that the improvement of IP in participants with UMB reduced the amount of dietary tryptophan metabolized through the endogenous kynurenine pathway.

4.3. Degradation of polyphenols by gut microbiota into phenolic acids

The changes in 4-HBA-G, u3,5-DHPPA, and u4-MetGA could be related to differences in gut bacterial community structure between UMs. Indeed, changes in uncharacterized *Clotridiales* spp. were correlated with changes in u3,5-DHPPA, as well as changes in *G. formicilis* (another member of the Clostridiales order) were positively correlated with changes in u4-MetGA levels. Previous studies showed that gut microbiota taxonomic diversity was higher in participants with UMB compared with other UMs, consistent with a more extensive metabolism of urolithins produced from the same dietary substrate, ellagic acid (Romo-Vaquero et al., 2019). In our study, β-diversity was not different when comparing UMA *vs*. UMB, but participants with UMB showed a gut microbiota with a more even distribution, that responded differently to the PR diet. We observed statistically significant correlations between different members of the Clostridiales order and polyphenol metabolites, which were particularly affected by the PR diet among participants with UMB. A discussion about the origin and effects of these polyphenol metabolites is provided in the Supplementary Material (Appendix A).

The Mediterranean diet, another healthy, plant-based dietary pattern was also shown to increase the urinary excretion of urolithins (Meslier et al., 2020). Meslier et al. showed that a Mediterranean diet intervention led to higher urolithin excretion and this was associated with an improvement in cardiometabolic risk factors (Meslier et al., 2020). Although we have observed an increase in the excretion of urinary urolithin A in the MaPLE trial (Hidalgo-Liberona et al., 2020), there were no correlations between urolithins and cardiometabolic risk factors. Moreover, in this analysis we observed that the increase of urolithin A and its phase II metabolites in urine was independent of UMs; ruling out the possibility that urolithin production/absorption rates were behind the different responses of the PR diet on intestinal permeability. In this regard, further analyses of the effects of different dietary interventions stratified by UMs are needed.

4.4. Limitations

This is a pilot trial in which the proportion of participants within each UM was not balanced and our results need further confirmation. In fact, age is an important factor determining UMs, as the percentage of subjects with UMB tend to increase with age (A. Cortés-Martín et al., 2018). However, in our study, UMB was present in only 25% of the study population, against a nearly 40% estimated in an analysis of 839 subjects with ages from 5 to 90 years (A. Cortés-Martín et al., 2018). Thus, randomized controlled trials designed to evaluate the impact of UMs during a polyphenol-rich diet in older adults are warranted. Our results cannot be extrapolated to different dietary interventions, or to diets including different foods apart from the ones used in the MaPLE project. Different polyphenol-rich foods could have elicited different responses and be more favorable instead to participants with UMA. In addition, we did not conduct analysis by sex because there is no evidence in literature for an association between UMs and sex, as well as we did not observe a statistically significant difference in sex distribution across UMs. Furthermore, the proportion of participants with UM0 could have been overestimated because we did not measure urolithins in feces. Last, more precise methods measuring the abundance of specific gut bacteria related with urolithin production could have provided more insights in their role on the differential effects observed.

5. Conclusions

Our results support previous findings and reinforce the concept that UMs may contribute to tailor personalized nutrition interventions. The present study confirmed this hypothesis and associated UMs with a differential reduction of intestinal permeability in older adults in response to a polyphenol-rich diet.

6. Data transparency statement

Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB46689. Deidentified metabolomics data and metadata may be available upon justified request to Dr. Andrés-Lacueva (candres@ub.edu).

7. Clinical trial registration

The trial was registered in https://www.isrctn.com/ under the code: ISRCTN10214981.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111632.

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