Deciphering the human gut microbiome of urolithin metabotypes: association with enterotypes and potential cardiometabolic health implications.

María Romo-Vaquero[a], *Adrián Cortés-Martín*[a], *Viviana Loria-Kohen*[b], *Ana Ramírez-de-Molina*[b], *Izaskun García-Mantrana*[c], *María Carmen Collado*[c], *Juan Carlos Espín*[a], *and María Victoria Selma*[a*]

[a]Laboratory of Food & Health
Research Group on Quality, Safety and Bioactivity of Plant Foods,
CEBAS-CSIC, Murcia, Spain.
[b]GENYAL Platform on Nutrition and Health, IMDEA Food Institute, CEI UAM + CSIC, Madrid, Spain.
[c]Group of Lactic Bacteria and Probiotics, Department of Biotechnology, IATA-CSIC, Valencia, Spain.

***Correspondence:** Maria Victoria Selma, Ph.D., *Laboratory of Food & Health*, Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Campus de Espinardo Nº25, 30100 Murcia, Spain. **E-mail:** mvselma@cebas.csic.es; **Fax:** +34-968-396213.

List of Abbreviations: ApoA-1, apolipoprotein A-1; **ApoB**, apolipoprotein B; **BMI**, body mass index; **CVD**, cardiovascular disease; **EA**, ellagic acid; **HDLc**, high-density lipoprotein cholesterol; **HOMA-IR**, insulin resistance index; **IDLc**, intermediate-density lipoprotein cholesterol; **IsoUro-A**, isourolithin A; **LDLc**, low-density lipoprotein cholesterol; **non-HDLc**, non-high-density lipoprotein cholesterol; **oxLDLc**, oxidised low-density lipoprotein cholesterol; **PCA,** principal component analysis; **PE**, pomegranate extract; **RATIOHDLc**, Large HDLc/Small HDLc; **RATIOLDLc**, Large LDLc/Small LDLc; **Tchol**, total cholesterol; **Uro-A**, urolithin A; **Uro-B**, urolithin B; **UM**, urolithin metabotype; **UM-A**, urolithin metabotype A; **UM-B**, urolithin metabotype B; **UM-0**, urolithin metabotype 0 (urolithin non-producers); **UPLC-ESI-qToF-MS**, Ultra Performance Liquid Chromatography–Electro Spray Ionization–Quadrupole Time of Flight–Mass Spectrometry; **VLDLc**, very low-density lipoprotein cholesterol.

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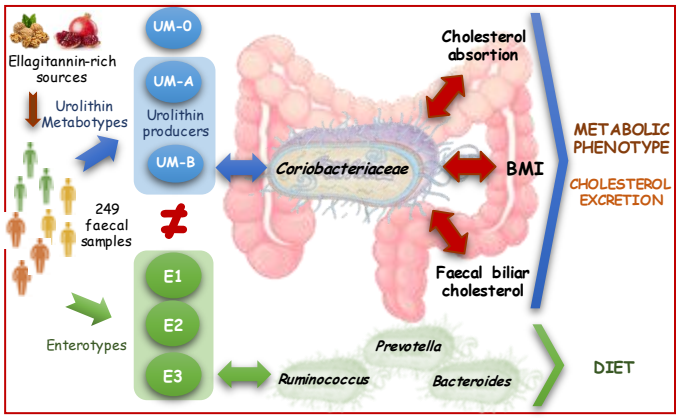
Abstract

Scope: The gut microbiota ellagitannin-metabolizing phenotypes (i.e. urolithin metabotypes, UMs) have been proposed as potential cardiovascular disease (CVD) risk biomarkers because the host blood lipid profile was reported to be associated with specific UMs. However, the link for this association remains unknown so far.

Methods & Results: We analysed the gut microbiome of 249 healthy individuals by 16S rDNA sequencing analysis. Individuals were also stratified by UMs (UM-A, UM-B, and UM-0) and enterotypes (*Bacteroides*, *Prevotella*, and *Ruminococcus*). Associations of UMs discriminating bacteria with CVD risk markers were investigated. Distribution and gut microbiota composition of UMs and enterotypes were not coincident. Almost half of discriminating genera between UM-A and UM-B belonged to the *Coriobacteriaceae* family. UM-B individuals presented higher blood cholesterol levels and higher alpha-diversity, including *Coriobacteriaceae* family than those of UM-A. *Coriobacteriaceae*, whose abundance was the highest in UM-B, was positively correlated with total-cholesterol, LDL-cholesterol, and BMI.

Conclusions: Our results suggest that the family *Coriobacteriaceae* could be a link between individuals' UMs and their blood cholesterol levels. Further research is needed to explore the mechanisms of host metabolic phenotype, including cholesterol excretion products, to modulate this bacterial family.



The gut microbiota ellagitannin-metabolizing phenotypes (i.e. urolithin metabotypes, UMs) have been proposed as potential cardiovascular disease risk biomarkers because the host blood lipid profile was reported to be associated with specific UMs. However, the link for this association remains unknown so far. The results of this study suggest that the family *Coriobacteriaceae* could be a link between individuals' UMs and their blood cholesterol levels.

1. Introduction

Dietary polyphenols are poorly absorbed, and they reach the colon where they are exposed to the gut microbes. The colonic microbiota transforms the unabsorbed polyphenols into better-absorbed metabolites. Some of these metabolites are considered biologically active compounds whereas others are inactive.[1,2] However, the latest investigations have shown large inter-individual differences in the production of bioactive microbial metabolites from dietary polyphenols because of the specific gut microbiome of each individual. Therefore, the potential biological activity derived from polyphenols intake is conditioned by the gut microbiome. Also, inter-individual differences in polyphenol metabolism could indirectly reflect the status of the individuals' gut microbiome (biomarkers of gut microbiota).[3] Three enterotypes of the human gut microbiome were identified in 272 human faecal samples from individuals of four countries, which were subsequently validated using the Human Microbiome Project (HMP) dataset.[4,5] The drivers in these enterotypes were *Bacteroides*, *Prevotella*, and *Ruminococcus*. Individual features such as body mass index (BMI), gender, or age cannot explain the observed enterotypes, but it has been suggested that they can be functional markers and they could allow classification of human groups that respond differently to drugs or dietary compounds intake.[4] In contrast, several studies have not favoured the enterotype concept.[6–8] Those studies focused on the pattern of faecal microbiome distribution in the human population and concluded that the stool microbiota was not a discrete distribution (three or two enterotypes) but rather a smooth gradient. In any case, configuration and functional stratification in the gut microbiome profile (i.e. enterotypes, polyphenol-metabolizing phenotypes) are gaining great attention for elucidating the interindividual variability observed in the health effects of plant food bioactives.[9–11]

Human intervention studies with ellagitannin-rich foods (pomegranate, strawberries, raspberries, oak-aged wines, walnuts, etc.) clearly illustrate the inter-individual variability in polyphenol metabolism. Three different urolithin metabotypes (UMs) depending on the type of the urolithins formed have been described in the population.[10] Thus, urolithin metabotype A (UM-A) is distinguished by the production of urolithin A (Uro-A), in metabotype B (UM-B) individuals produce isourolithin A (IsoUro-A) and urolithin B (Uro-B) besides Uro-A, and those with metabotype 0 (UM-

0) do not produce these final urolithins (only the precursor so-called M5).[10] This inter-individual variability in urolithin production has been related to some dissimilarity in the intestinal microbiota. Indeed, *Gordonibacter* species can transform ellagic acid (EA) into different urolithins in pure culture [12,13] and are positively correlated with Uro-A and UM-A in faeces and urine, whereas the occurrence of IsoUro-A, Uro-B and UM-B are inversely correlated with faecal concentration of *Gordonibacter* spp.[14] *Ellagibacter isourolithinifaciens*, another human gut bacteria of the family *Coriobacteriaceae-Eggerthellaceae*, shows IsoUro-A production capacity in vitro but in vivo correlations have not been investigated.[15,16] Thus, the gut microbiome pattern associated with each urolithin metabotype (UM) has not been explored in depth so far. Elucidation of the microbiome associated with UMs becomes more and more relevant because of the increasing evidence that links UMs with health status. Indeed, recent studies showed that healthy overweight-obese individuals belonging to UM-B presented increased cardiovascular disease (CVD) risk, whereas UM-A seemed to be a protective metabotype against CVD risk factors.[17,18]

Urolithins have shown a range of biological effects in vitro and animal studies including anti-inflammatory, neuroprotective, cardioprotective, 'prebiotic-like', antidiabetic, antiobesity, antioxidant, and chemopreventive activity, and enhancement of the muscular performance.[19] A few human trials have shown the biological effects of ellagitannin-rich food intake with controversial results among them. Indeed, a recent human study with an ellagitannin-rich pomegranate extract showed the inter-individual variability of effects improving CVD risk biomarkers, and it was associated with differences in UMs.[18] In that study, individuals belonging to UM-B improved their blood lipid profile better than those of UM-A upon consumption of an ellagitannin-rich pomegranate extract. Authors suggested that this could be due to either the inability of UM-B individuals to produce relevant levels of Uro-A before the intervention with pomegranate and(or) to indirect effects through modulatory interactions with the gut microbiota that affected its composition and(or) functionality.[18] These results highlight the importance of deepening the knowledge about the gut microbiome of the three UMs as a tool to understand better the different response of individuals to

ellagitannin intervention trials as well as to elucidate the association of UMs with health status through the faecal microbiome composition.

In the present study, we analysed the gut microbiome of faecal samples from 249 healthy individuals by 16S rDNA in order to explain the previously suggested association between CVD risk (BMI, glucose, insulin, and blood lipid profile) and the presence of specific UMs.[17,18] After stratification by UMs, we identified differences among UMs in diversity, richness, and microbial composition. Association of UMs with enterotypes in the faecal microbiome as well as its potential correlation with health status has also been discussed.

2. Experimental Section

2.1. Chemicals and study products

Urolithins and derived phase-II conjugated metabolites were obtained as described previously.[20] Purity was higher than 95% in all tested compounds. Capsules of pomegranate extract were kindly provided by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain).[18] The unpeeled walnuts used in the study were kindly supplied by Borges S.A. (Reus, Tarragona, Spain).

2.2. Study design and participants

Individuals from three trials (n_{total} = 249) were included in the Spanish National Project AGL2015-64124-R ('*PolyMicroBio*') The trials were conducted in line with the Helsinki Declaration and ethical principles for medical research involving human subjects (Seoul, Korea, 2008). The objective was to characterise the individuals' faecal microbiota at baseline and to stratify the individuals according to their different capacity to metabolise ellagic acid derivatives into urolithins (UMs). For UMs stratification, and using a protocol previously described,[21] volunteers consumed an ellagitannin-rich food for three days as detailed below.

2.3. Dosage information

Briefly, in the first trial, normoweight-overweight healthy individuals recruited at CEBAS-CSIC (Murcia, Spain) and IATA-CSIC (Valencia, Spain) (*n* = 50, 27 women and 23 men; mean BMI = 23.7 ± 3.2 kg/m^2) consumed daily 30 g of unpeeled walnuts for three days[17] (**Table 1**, Fig. S1A, Supporting Information). In the second trial (NCT02061098), non-medicated overweight-obese healthy individuals (*n* = 49, 17 women and 32 men; mean BMI = 30.4 ± 3.4 kg/m^2), with mild hyperlipidaemia, ingested 1 daily capsule of pomegranate extract (450 mg/day) for three days[18] (Table 1, Figure S1B, Supporting Information). In the third intervention study, healthy normoweight-overweight-obese volunteers recruited at IMDEA-Alimentación (Madrid, Spain) (*n* = 150; 51 men and 99 women; mean BMI = 27.5 ± 4.7 kg/m^2) ingested three daily capsules of pomegranate extract (1,350 mg/day)[21] (Figure S1C, Supporting Information).

2.4. Sampling procedure and determinations

Urine and faecal samples were collected before and after the three days of the dietary intervention with ellagitannin sources in the three trials. Basal serobiochemical variables were previously described elsewhere.[17,18] Urolithins were determined by UPLC-ESI-qToF-MS as previously described in the samples of urine and faeces obtained after the three-day intervention.[20] The individuals were stratified according to their different capacity to metabolise EA derivatives into urolithins, i.e. UMs (UM-A, UM-B, and UM-0) as previously described.[10]

Gut microbiota analysis was carried out in the faecal samples at baseline. Protocols for bacterial DNA isolation, 16S rDNA gene amplification, metagenomic sequencing library preparation Illumina (Illumina Inc., San Diego, USA) as well as sequence processing and taxonomic classification of gut microbiota were carried out in the same lab (in a centralised manner) as described previously.[22] Paired-end sequencing with a read length of 2 × 300 bp was carried out using a MiSeq Reagent kit v3

(MS-102-3001) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Quality assessment was performed by the use of a prinseq-lite program[23] and applying the following parameters: min_length: 50; trim_qual_right: 30; trim_qual_type: mean; trim_qual_window: 20. R1 and R2 from Illumina sequencing were joined using fastq-join from ea-tools suite. The data obtained in the fastaq format were processed using the Galaxy tool.[24] Chimeric sequences and sequences that could not be aligned were also removed from the data set, and filtered out with UCHIME.[25] The clustered sequences were utilised to construct Operational Taxonomic Units (OTUs) tables with 97% identity, and representative sequences were classified into the respective taxonomical level from phylum to genus using the RDP classifier.[26]

Analyses with RDPipeline (http://pyro.cme.msu.edu/) involved 16S rRNA gene sequence alignment (Aligner), 16S rRNA gene sequence clustering (Complete Linkage Clustering) and alpha-diversity indexes (Shannon Index and Chao1 estimator) at the genus level. Alpha-diversity indexes (Chao1 and Shannon), based on a randomly selected 21181 reads per sample, were used to estimate the samples' richness and diversity at the genus, family, and phylum levels. Shannon and Chao 1 indexes at phylum and family levels were calculated by PAST version 2.17c (http://folk.uio/ohammer/past). The LDA Effect Size (LEfSe) algorithm with the online interface Galaxy (http://huttenhower.sph.harvard.edu/galaxy/root) were used to identify taxa with differentiating abundance among UMs. UM-A and UM-B were assigned as the respective comparison. LEfSe identified features that were statistically different among the different groups and performed non-parametric factorial Kruskal-Wallis sum-rank tests and Linear Discriminant Analysis (LDA) to determine whether these features were consistent concerning the expected behaviour of the different UMs. Annotated heatmap based on relative abundance clustering of the top 70 genera was plotted using gplots and pheatmap functions in R software.

2.5. Statistical analysis

Statistical analysis was carried out using the SPSS Software, version 23.0 (SPSS Inc., Chicago, IL, USA). The individuals of the three trials were included for the bacterial community analysis in faeces. The Shapiro-Wilk test indicated that data of relative abundance of microbial genera, families and phyla did not follow a normal distribution. The Wilcoxon Signed Rank Test was performed to detect significant differences in bacterial data by UMs. When more than two groups were compared, analyses of variance (ANOVA), and Bonferroni t-test or the Kruskal-Wallis followed by Dunn's test were used for normally and non-normally distributed data, respectively. The individuals were clustered in groups by UMs and also by enterotypes using principal component analysis (PCA) and hierarchical clustering analysis (HCA) via R commander.[27] Enterotypes were identified by variations in the levels of one of genera *Bacteroides*, *Prevotella* and *Ruminococcus* whereas UMs were identified by urolithin profile as previously described.[21] Two principal components were plotted using ggbiplot function in R software. Plots of data were performed using Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). We applied a multinomial logit model to evaluate a possible association between UMs and enterotypes.[21] Spearman's rank correlation was applied to explore the link between lipid profile and the bacterial genera. Heatmap of data was performed using the free tool Metaboanalyst.[28] Statistical significance was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Marginal significance was also indicated when $0.1 > {}^{\#}p > 0.05$.

3. Results

3.1. Identification of the faecal microbiome enterotypes and association with UMs

Individuals from the three trials ($n = 249$) were clustered by enterotypes and UMs (Table 1). Each enterotype proportion widely changed among the three trials ($p < 0.001$). Enterotype distribution in trial 1 was significantly different from that in trials 2 and 3 ($p < 0.001$), whereas trials 2 and 3 showed a similar enterotype distribution ($p = 0.148$). When individuals of the three trials were considered together, enterotype 2 (*Prevotella*-type) was the most abundant one (63%) followed by enterotype 3 (*Ruminococcus*-type) (27%) and enterotype 1 (*Bacteroides*-type) (10%) (Table 1). UM percentages were consistent among the three trials independently of mean BMI values of each trial ($p = 0.656$).

UM-A was the most abundant metabotype (55% mean value), followed by UM-B (35%) and finally by UM-0 (10%). In each enterotype, a similar distribution of UMs was observed ($p = 0.642$). Thus, the UM-0, UM-A and UM-B percentages in all the enterotypes were 11 ± 4%, 54 ± 3% and 34 ± 3%, respectively.

PCA and clustering analysis using these three enterotype discriminating genera (*Bacteroides*, *Prevotella* and *Ruminococcus*) showed that samples formed three distinct clusters corresponding to the enterotypes (**Figure 1A**). However, the relative abundance of these three bacterial genera did not discriminate among UMs (**Figure 1B**). The *Prevotella* to *Bacteroides* ratio was higher in individuals classified as enterotype 2 with respect to the enterotype 1 group (**Figure 1C**). In contrast, the *Prevotella* to *Bacteroides* ratio did not differ among UMs, and only a tendency of higher level in UM-B was observed (**Figure 1D**). PCA plot of genera from the *Coriobacteriaceae* family illustrated that PC1 and PC2 were not able to discriminate among the three enterotypes (**Figure 1E**). Only the enterotype 1 (*Bacteroides*-type) was different to the others (enterotypes 2 and 3) in PC1 ($p < 0.05$) and PC2 ($p < 0.01$) according to the statistically lower abundance of *Gordonibacter*, *Ellagibacter*, *Senegalimassilia*, and *Collinsella* in enterotype 1 vs the others (Figure 1E). Conversely, PCA of genera from the *Coriobacteriaceae* family discriminated among UMs in the first and second principal component (PC1 and PC2) (**Figure 1F**). PC1 clearly differentiated UM-B from the other two UMs (UM-A and UM-0) ($p < 0.001$) while PC2 distinguished UM-A with respect to UM-B ($p < 0.001$) and UM-0 ($p = 0.029$) (Figure 1F).

3.2. Richness and diversity of microbiota

The alpha-diversity indexes are summarized in **Table 2** and rarefaction curves in Fig. S2 (Supporting Information). At the phylum level, diversity and richness were higher in enterotype 1 vs enterotypes 2 and 3. At the family level, diversity and richness were higher in enterotype 1 vs enterotype 3 (Table 2). However, diversity and richness were not different between the enterotypes at genus levels. In contrast to enterotypes, diversity and richness differed among UMs at genus level where UM-0

individuals showed a lower diversity and richness than UM-B and UM-A individuals ($p < 0.01$) (Table 2). At phylum and family levels, richness was significantly higher in UM-B than in UM-A and UM-0.

3.3. Composition of the microbial community at the phylum, family and genus levels in UMs

At the phylum level, the three UMs mainly consisted of Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Verrucomicrobia and Euryarchaeota (**Figure 2A**). Among them, only Euryarchaeota abundance was higher in UM-B than in UM-A and UM-0 individuals ($p < 0.006$). Furthermore, phylum Synergistetes whose abundance is lower than 0.05% was also significantly higher in UM-B than in UM-A and UM-0 individuals ($p < 0.05$) (Figure 2A). The Firmicutes to Bacteroidetes ratio was similar among UMs whereas enterotypes 2 and 3 showed higher levels of this ratio than enterotype 1 ($p < 0.01$) (data not shown). At the family level, more differences among UMs were observed. The families *Methanobacteriaceae* from Euryarchaeota phylum, *Synergistaceae* from Synergistetes phylum as well as *Coriobacteriaceae, Clostridiaceae, Enterobacteriaceae* and *Clostridiales incertae sedis* XI were more abundant in UM-B than in UM-A and UM-0 ($p < 0.05$). In contrast, *Lachnospiraceae* and *Eubacteriacea* abundance predominated in UM-A and UM-0 with respect to UM-B ($p < 0.05$).

The 70 most abundant genera in each UM, covering 77.3±2.1% relative abundance, are shown in **Figure 2B**. *Blautia* and *Faecalibacterium* were the most abundant genera without differences among UMs (Figure 2B). Figure 2A shows bacterial genera that differed between UM-A and UM-B covering less than 15% of the total relative abundance. *Lachnospiraceae incertae sedis*, *Clostridium* XVIII, *Prevotella* and three genera from *Coriobacteriaceae* family (*Gordonibacter, Eggerthella,* and *Adlercreutzia*) predominated in UM-A vs UM-B. In contrast, *Clostridium sensu stricto*, *Holdemanella, Parvimonas, Anaerobacter, Intestimonas, Paraprevotella*, one genus of *Synergistaceae* family (*Cloacibacillus*), two genera of *Methanobacteriaceae* family (*Methanobrevibacter and Methanosphaera*), five classified genera of *Coriobacteriaceae* family

(*Collinsella, Olsenella, Senegalimassilia, Slackia, Ellagibacter*) as well as an unclassified *Coriobacteriaceae* predominated in UM-B vs UM-A. Although *Enterobacteriaceae* family predominated in UM-B vs UM-A, *Escherichia coli_Shigella* predominance in UM-B vs UM-A was marginally significant ($p = 0.078$).

Taxonomic representation of statistically and biologically consistent differences between UM-A and UM-B are shown in **Figure 3**. LDA (**Figure 3A**) and the cladogram (**Figure 3B**) generated from LEfSe analysis confirmed the differences between UM-A and UM-B at genus and family levels as well as the differences at the class and order levels. At the class and subclass levels, increased abundance of *Gammaproteobacteria*, *Methanobacteria* and *Synergistia*, as well as *Coriobacteridae*, were observed in UM-B vs UM-A. At the order level, *Coriobacteriales* and *Aeromonadales* were also higher in UM-B than UM-A (Figures 3A and 3B). UM-0 was not significantly different from either UM-A or UM-B in *Parvimonas, Holdemanella, and Slackia.* However, UM-0 differed from UM-B but not from UM-A, for the rest of the bacterial genera that discriminated between UM-A and UM-B. Only *Gordonibacter* predominated in UM-A vs either UM-B or UM-0 (Figure 2A). According to the lower diversity and richness at genus level in UM-0 individuals vs UM-A and UM-B (Table 2), lower levels of other genera, whose abundance was less than 1% such as *Phascolarctobacterium* ($p = 0.012$), *Bilophila* ($p = 0.001$), *Alistiper* ($p = 0.009$), and *Butyricimonas* ($p = 0.002$), characterised UM-0 vs UM-A and UM-B. Consequently, the heat map of relative abundance at the genus level firstly differed two groups, i.e. non-producers (UM-0) and producers (UM-A and UM-B), and secondly subdivided producers into two more similar groups (UM-A and UM-B) (Figure 2B).

3.4. Associations of UMs discriminating genera with urolithin production, BMI and CVD markers

In order to elucidate associations of UMs discriminating genera with urolithin production, CVD risk markers, including BMI, glucose, insulin, insulin resistance index (HOMA_IR), and blood lipid profile, forty-nine non-medicated healthy overweight-obese volunteers with mild hyperlipidaemia

(trial 2) were analysed (**Figure 4**). Some bacteria genera from the *Coriobacteriaceae* family showed positive and negative associations among them (Figure 4A). Remarkably, *Slackia* was negatively correlated with the three *Coriobacteriaceae* genera more abundant in UM-A (*Gordonibacter, Eggerthella*, and *Adlercreutzia*). Furthermore, *Gordonibacter* was positively correlated with *Eggerthella* and negatively correlated with two more abundant genera in UM-B (*Slackia* and *Senegalimassilia*) (Figure 4A). Interestingly, *Slackia* was positively correlated with *Parvimonas* from *Peptoniphilaceae* family whereas *Olsenella* and *Senegalimassilia* were positively correlated with *Methanobrevibacter* which is a methanogenic archaea from the *Methanobacteriaceae* family (Figure 4A).

IsoUro-A and Uro-B production (characteristic of UM-B) was positively correlated with some genera from *Coriobacteriaceae* family (*Olsenella, Senegalismassilia* and *Slackia*) (Figure 4B). IsoUro-A and Uro-B production was negatively correlated to other genera from the *Coriobacteriaceae* family whose abundance was increased in UM-A (*Gordonibacter* and *Eggerthella*) (Figure 4B). In contrast, Uro-A (the main urolithin in UM-A) was only positively correlated with *Gordonibacter* (Figure 4B). Tchol, LDLc, ApoB and non-HDLc were positively correlated with one of the *Coriobacteriaceae* genera (*Slackia*), whose abundance was increased in UM-B (Figure 4B). BMI negatively correlated with *Intestinimonas* and *Prevotella* but not with *Coriobacteriaceae* genera in overweight-obese volunteers (trial 2, n=49, BMI range=27.1-43.0) (Figure 4B). However, when data from the three trials were considered (n_{total}=249, BMI range=18.0-43.3), BMI correlated positively with the *Coriobacteriaceae* family ($p < 0.001$) and with one of the *Coriobacteriaceae* genera (*Collinsella*) ($p < 0.001$) (data not shown). At the family level, the *Coriobacteriaceae* family, whose abundance was increased in UM-B vs UM-A and UM-0, was also positively correlated with the blood levels of some CVD markers (Tchol, LDLc and ApoA-1) ($p < 0.05$) (Fig. S3, Supporting Information). The relative abundance of unknown genera of *Coriobacteriaceae* family (unclassified *Coriobacteriaceae* genera) showed a marginal significant positive correlation with Tchol ($p = 0.097$), LDLc ($p = 0.06$), ApoA ($p = 0.08$), oxLDLc ($p = 0.09$), and HDLc ($p = 0.06$) (Fig.S3). On the other hand, *Eubacteriaceae*, which was increased in UM-A vs UM-B, was positively correlated with ApoA

(p = 0.04) and marginally correlated with HDLc (p = 0.097), glucose (p = 0.09), and insulin (p = 0.09). Besides, *Erysipelotrichaceae*, another family previously associated with host lipid metabolism[29] was not significantly correlated with any CVD biomarkers (Fig.S3). In contrast to UMs, where associations with blood lipid profile were found,[17,18] CVD biomarkers studied in the present study did not differ among enterotypes.

4. Discussion

The number of studies dealing with enterotypes and their biological significance is still limited. A long-term diet enriched in carbohydrates has been linked to the *Prevotella* enterotype, while protein and animal fat have been linked to the *Bacteroides* enterotype.[30] The results of the present study conducted in 249 healthy volunteers showed that UMs and enterotypes distribution was not coincident. In a previous study, enterotypes have been merely inferred to the *Prevotella* to *Bacteroides* ratio.[31] In the present study, *Bacteroides* and *Prevotella* enterotypes differed in the *Prevotella* to *Bacteroides* ratio. However, in the case of UMs, the difference between UM-A and UM-B in the *Prevotella* to *Bacteroides* ratio was only marginal (p = 0.075). From the three enterotypes drivers (*Bacteroides*, *Prevotella*, and *Ruminococcus*), only *Prevotella* abundance was higher in UM-A than UM-B. Almost half of the bacterial genera (8/18) that differ between UM-A and UM-B belonged to the *Coriobacteriaceae* family from the Actinobacteria phylum. Several bacterial species from the *Coriobacteriaceae* family have been reported to be involved in polyphenolic metabolism.[2] Furthermore, it is known that the consumption of several polyphenol-rich foods modulates the gut microbiota composition.[1] Indeed, in a recent study, urolithin non-producers (UM-0) became producers (UM-A or UM-B) following ellagitannin-rich food consumption.[18] Therefore, the ability to produce urolithins (UM-A and UM-B) vs non-producers (UM-0) could be partially related to the consumption of polyphenol-rich diets.

UM-B individuals have a higher diversity of intestinal microbiota with respect to UM-A, but especially regarding UM-0 individuals (urolithin non-producers). Thus, alpha-diversity could explain the capacity of UM-B to produce a higher number of urolithin metabolites than UM-A and this in turn more than UM-0 individuals. The diversity of gut microbiota increases with age (birth to

adulthood),[8] and it is also closely associated with host health.[32] Accordingly, the relation between UMs distribution and ageing has been demonstrated.[21] Thus, the increase of diversity is accompanied by UM-A decrease concomitant with the increase of UM-B. Some studies have suggested the correlation of UMs with gut dysbiosis, obesity and host health status,[10,33] although the relation between BMI and UM-B has not yet been unequivocally proven, probably because obesity has a multifactorial aetiology. In the present study, we found a strong positive correlation of BMI with *Coriobacteriaceae* whose abundance was increased in UM-B. In contrast to *Coriobacteriaceae*, the Firmicutes to Bacteroidetes ratio previously associated with obesity and gut dysbiosis[34,35] did not differ among UMs. Therefore, our results suggest that UMs and their discriminating bacteria could not be considered an obesity predisposition biomarker in healthy people.

The potential health implications of the *Coribacteriaceae* family, whose abundance was found to be higher in UM-B vs UM-A and UM-0, are still poorly understood. *Coriobacteriaceae* are prevalent and dominant members of the human gut microbiota and can metabolise bile acids, steroid hormones, lipids and xenobiotics such as polyphenols.[36,37] Several studies associated *Coriobacteriaceae* with an increased blood cholesterol and lipid metabolism, as well as obesity in animal studies[36–39], whereas higher faecal cholesterol excretion (lower cholesterol absorption by dietary addition of plant sterol esters) has been associated with a decreased relative abundance of *Coriobacteriaceae* in the gut of hamsters.[29] Indeed, *Coriobacteriaceae* abundance was linked to hypercholesterolemia regardless of diet. *Coriobacteriaceae* was positively correlated with cholesterol absorption, liver free cholesterol, plasma non-HDLc, total cholesterol, liver weight and white adipose tissue mass. In contrast, *Coriobacteriaceae* showed a negative association with whole-body cholesterol synthesis and faecal biliary cholesterol excretion in hamsters.[29] Accordingly, in our present human study, we found a positive correlation of the *Coriobacteriaceae* family and also one of its genera (*Slackia*) with blood cholesterol levels whose abundance was higher in UM-B vs UM-A and UM-0. The Tchol excreted by the host via bile appears to inhibit specific bacterial taxa, especially *Coriobacteriaceae*.[29] UM-A, where the levels of *Coriobacteriaceae* and blood cholesterol was lower, differed from UM-B in higher levels of *Gordonibacter*, one specific *Coriobacteriaceae* previously correlated with Uro-A

production and UM-A.[14,17,18] The *Eubacteriaceae* family was also increased in UM-A and UM-0 vs UM-B. Accordingly, in hamsters consuming plant sterol esters, the *Eubacteriaceae* family was increased while blood Tchol and *Coriobacteriaceae* were reduced and a negative correlation of *Eubacteriaceae* with blood cholesterol was found. Authors hypothesised that *Eubacteriaceae* increase was due to the ability of this organism to use the additional faecal cholesterol that became available as a growth substrate in the gut.[29] Thus, cholesterol excreted in the gut could modulate both the abundance and composition of the gut microbiota, especially the *Eubacteriaceae* and *Coriobacteriaceae* families. Therefore, the *Coriobacteriaceae* family could be the link between UMs and host blood levels of cholesterol. Further research is needed to explore the mechanisms of host metabolic phenotype (cholesterol excretion products) to modulate this bacterial family.

In a more recent study, *Coriobacteriaceae* has been reported to affect bile acid metabolism in mice.[37] Thus, *Coriobacteriaceae* increases the expression of ileal bile acid absorption transporter ASBT, ileal *Fxr* (a key modulator of bile acid metabolism) and the absorption of bile acids that can directly or indirectly modulate hepatic bile acid metabolism including de novo synthesis via FXR target gene *Shp*.[37] Bile acid synthesis in the liver generates bile flow which is important for biliary excretion of free cholesterol, endogenous metabolites, and xenobiotics. Therefore, there is a two-way interaction between *Coriobacteriaceae* and host cholesterol because gut cholesterol can modulate *Coriobacteriaceae* abundance and composition, but at the same time *Coriobacteriaceae* can affect cholesterol metabolism and reduce its faecal excretion through increasing bile acid absorption. Unlike *Coriobacteriaceae,* some polyphenol-rich foods such as pomegranate promote the efflux of cholesterol through raising bile acids in the excretion, a similar effect to that of some lipid-lowering drugs such as simvastatin.[40] Pomegranate has been reported to increase the faecal bile acid concentration mainly by influencing two LXR/PPARABCA1 genetic pathways.[40] Further studies should be done to elucidate the role of the UMs discriminating microbiota, mainly *Coriobacteriaceae*, in the effect of polyphenols as potential reducers of CVD risk factors.

In addition to bacteria from the *Coriobacteriaceae* and *Eubacteriaceae* families, other genera differed UM-A from UM-B. Remarkably, pro-inflammatory microorganisms including *Parvimonas*,

Methanobrevibacter, *Methanosphaera*, and Gammaproteobacteria were increased in UM-B vs UM-A. The latter could suggest a higher pro-inflammatory status in UM-B individuals vs UM-A. In a recently published study with these 49 overweight-obese individuals (trial 2) whose endotoxemia marker lipopolysaccharide-binding protein (LBP) and blood cholesterol were reduced by pomegranate extract consumption, *Parvimonas* and *Methanobrevibacter* were also reduced. Furthermore, plasma LBP reduction was associated with *Parvimonas* decrease.[22] However, associations between the decrease of LBP values and individuals' UMs was not found[22] while cholesterol reduction was higher in UM-B volunteers.[18] Therefore, although some of these pro-inflammatory bacteria are increased in UM-B, we cannot confirm that UMs, and particularly UM-B vs UM-A, could be a suitable biomarker for subclinical metabolic endotoxemia probably because other bacteria and host properties, not associated with UMs, are also determining the pro-inflammatory status. Hydrogenotrophic methanogens (*Methanobrevibacter* and *Methanosphaera*), as well as *Parvimonas*, have also been related to obesity.[41] Despite their low abundance, they seem to be involved in specialised functions on the host although further research is needed.

Considering the differences between UM-A and UM-B in alpha-diversity, *Coriobacteriaceae* composition, host blood levels of cholesterol, as well as the previously reported two-way interaction between *Coriobacteriaceae* and cholesterol excretion products,[29,36,37] we hypothesise that cholesterol could play a primary role in the modulation of *Coriobacteriaceae* family. In this regard, the gut microbiota composition associated with UMs might be a consequence rather than a cause of the host phenotype. Besides, UMs and particularly UM-B vs UM-A and UM-0 could serve as an indirect biomarker of the cholesterol excretion products. Therefore, the results obtained in this study support the evidence that interactions between the gut microbiota and host metabolism are bidirectional. Inter-individual differences on the effects upon lipid-lowering therapies should be taken into account, and UMs could be used as a suitable indirect marker of gut composition and host phenotype for individual stratification.

From a clinical viewpoint, the gut microbiota can be targeted for the prevention or treatment of metabolic diseases. Modulation of the gut microbiome for the treatment and prevention of diseases is

being assayed, but scarce information is still available. However, recent advances in the gut microbiome analysis highlight both the potential and promise of targeting intestinal microbes and its functionality for therapeutic gain.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Figure S1. Study design that shows the flow of participants through the trials. (**A**) Walnut intervention in normoweight-overweight volunteers, (**B**) pomegranate intervention in overweight-obese volunteers, and (**C**) pomegranate intervention in normoweight-overweight-obese volunteers.

Figure S2. Bacterial diversity. Figure S2 shows the rarefaction curves of the three UMs, relating the sequencing effort with an estimate of the number of bacterial species, as inferred by the number of OTUs. An OTU is a cluster of 16SrRNA sequences that were >95% identical, a conservative estimate for the boundary between species, established at 97% for full-length 16S rRNA sequences.

Figure S3. Spearman's correlations heatmap. (**A**) Correlation analyses within UMs discriminating families from trial 2 (n=49). (**B**) Correlation analyses of UMs discriminating families with urolithins and clinical parameters in trial 2 (n=49). ★: Spearman's correlation values with $p < 0.05$. ▲: Spearman's correlation values when $0.1 > {}^{\#}p > 0.05$. ${}^{\delta}$*Erysipelotrichaceae*, another family previously associated with host lipid metabolism[29] has also been included, although its abundance was not different among UMs.

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M.V.S. and J.C.E. designed the study; M.V.S., J.C.E., M.C.C., A.R.M. and V.L.K. recruited the volunteers and conducted the trial; MCC and IGM provided samples and sequences for microbiota analysis; M.R.V. processed all the samples; M.R.V. performed sequence processing and taxonomic classification of gut microbiota; M.V.S., M.R.V. and A.C.M. performed statistical analyses; M.V.S. wrote the manuscript; J.C.E. critically reviewed the manuscript; All authors have read and approved the final manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

Keywords

Urolithins / metabotype / cardiometabolic / polyphenol / *Coriobacteriaceae* / gut microbiota

Table 1. Demographic characteristics and serobiochemical variables of participants in the three dietary intervention studies.

	All individuals (*n* = 249)	**Trial 1 (*n* = 50)**	**Trial 2 (*n* = 49)[a]**	**Trial 3 (*n* = 150)**
	Walnuts or Pomegranate extract	Unpeeled walnuts (30 g/day)	Pomegranate extract (450 mg/day)	Pomegranate extract (1,350 mg/day)
Mean age (y) and range	42.4 ± 11.5, (19–72)	36.9 ± 9.0, (19–55)	46.2 ± 6.3, (40–65)	43.3 ± 12.8, (19–72)
Mean BMI (kg/m^2) and range	27.2 ± 4.7, (18.0–43.3)	23.7 ± 3.2, (18.4–34.3)	30.4 ± 3.4, (27.1–43.0)	27.5 ± 4.7, (18.0–39.9)
Gender (female/male)	143/106	27/23	17/32	99/51
Enterotype 1 (%)	10	44	2	2
Enterotype 2 (%)	63	42	59	71
Enterotype 3 (%)	27	14	39	27
UM-0 (%)	10	8	14	10
UM-A (%)	55	58	53	53
UM-B (%)	35	34	33	37
Tchol (mg/dL)	NA	NA	209 ± 38[a]	NA
Triglycerides (mg/dL)	NA	NA	124 ± 66[a]	NA
LDLc (mg/dL)	NA	NA	152 ± 31[a]	NA
HDLc (mg/dL)	NA	NA	53 ± 11[a]	NA

[a]Data previously published.[18] NA, not available

Table 2. Alpha-diversity of each urolithin metabotype at phylum, family and genus levels.

	Diversity (Shannon index)	Richness (Chao Index)		Diversity (Shannon index)	Richness (Chao Index)
Phylum			**Phylum**		
UM-A	0.6 ± 0.2a	7.7 ± 1.7b	Enterotype 1	0.8 ± 0.2a	9.4 ± 2.8a
UM-B	0.6 ± 0.2a	8.5 ± 1.9a	Enterotype 2	0.6 ± 0.2b	7.7 ± 1.7b
UM-0	0.5 ± 0.2a	7.2 ± 1.9b	Enterotype 3	0.5 ± 0.2b	7.8 ± 1.6b
Family			**Family**		
UM-A	1.6 ± 0.2b	36.0 ± 8.1b	Enterotype 1	1.7 ± 0.2a	39.9 ± 11.6a
UM-B	1.7 ± 0.3a	39.2 ± 8.5a	Enterotype 2	1.7 ± 0.3ab	37.0 ± 8.2ab
UM-0	1.5 ± 0.2b	32.6 ± 8.0b	Enterotype 3	1.6 ± 0.3b	35.2± 7.5b
Genus			**Genus**		
UM-A	4.3 ± 0.3a	1,870.8 ± 603.9a	Enterotype 1	4.4 ± 0.3a	1,765.8 ± 675.7a
UM-B	4.4 ± 0.3a	1,946.5 ± 572.9a	Enterotype 2	4.3 ± 0.4a	1,831.8 ± 568.2a
UM-0	4.0 ± 0.5b	1,514.4 ± 572.1b	Enterotype 3	4.3 ± 0.3a	1,963.6 ± 641.8a

Values are expressed as mean ± SD. Different letters mean statistical significance in the diversity and richness indexes between different UMs ($p < 0.05$).

Figure Captions

Figure 1. (**A, E**) PCA and clustering analysis showing differences among enterotypes and (**B, F**) among UMs. Box plots of (**C, D**) *Prevotella_Bacteroides* ratio.

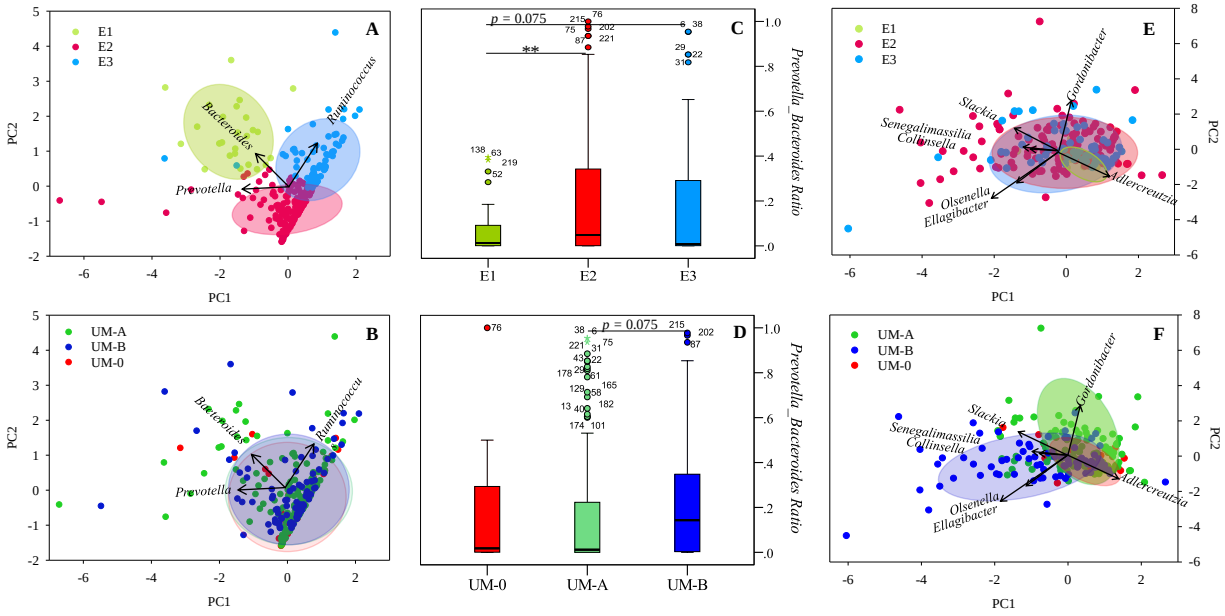

Figure 1

Figure 2. Microbial taxonomic composition in faecal samples of individuals grouped by UMs. (**A**) The bars show the mean proportion at phylum, family and genus level. In the family and genus diagrams only those with significant differences between UM-A and UM-B groups are shown. #Higher abundance in UM-A than UM-B; *Higher abundance in UM-B than UM-A. (**B**) The heatmap shows the relative abundance of the most abundant 70 genera.

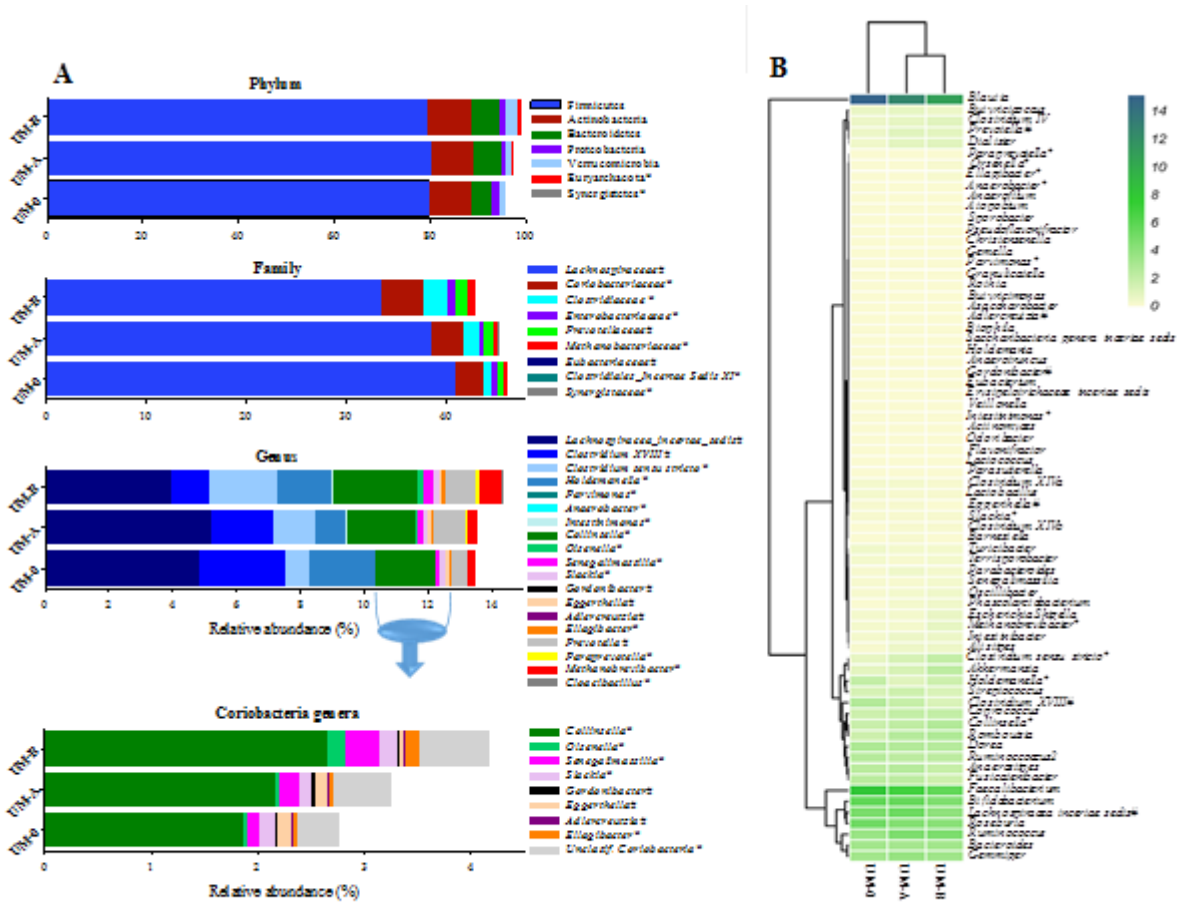

Figure 2

Figure 3. LEfSe analysis where (**A**) LDA score plot and (**B**) cladogram plot were generated showing different abundances in bacterial communities between UM-A and UM-B. The microbial taxa shown in the Figure have a LDA score higher than 2. Red nodes represent taxa significantly ($p < 0.05$) overabundant in UM-A; green nodes represent taxa significantly ($p < 0.05$) overabundant in UM-B; nodes remaining yellow indicate taxa that were not significantly differentially represented ($p > 0.05$). Each circle's diameter is proportional to the taxon's abundance.

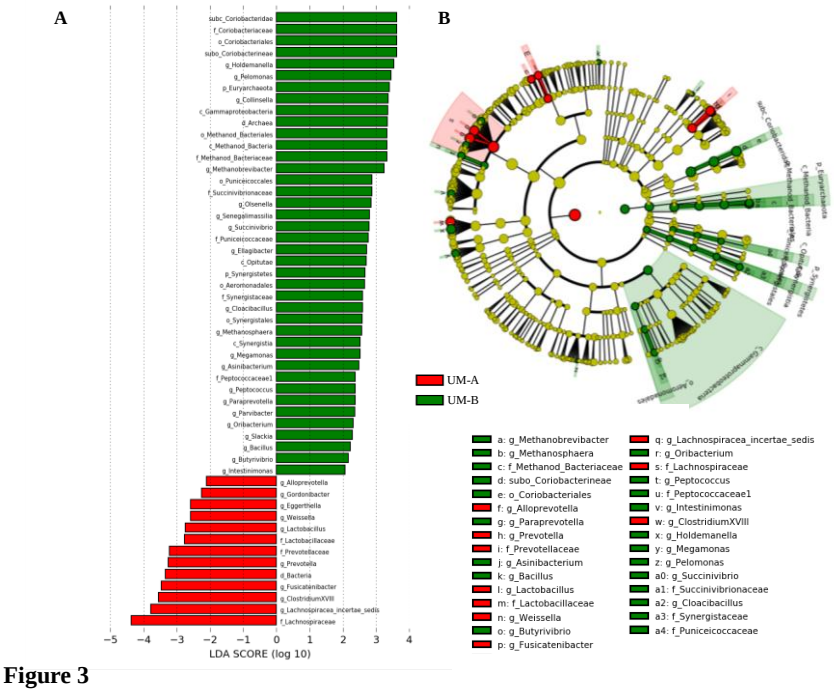

Figure 3

Figure 4. Spearman's correlations heatmap. (**A**) Correlation analyses within UMs discriminating genera from trial 2 (n=49) (**B**) Correlation analyses of UMs discriminating genera with urolithins and clinical parameters in trial 2 (n=49). ★**:** Spearman's correlation values with $p < 0.05$.

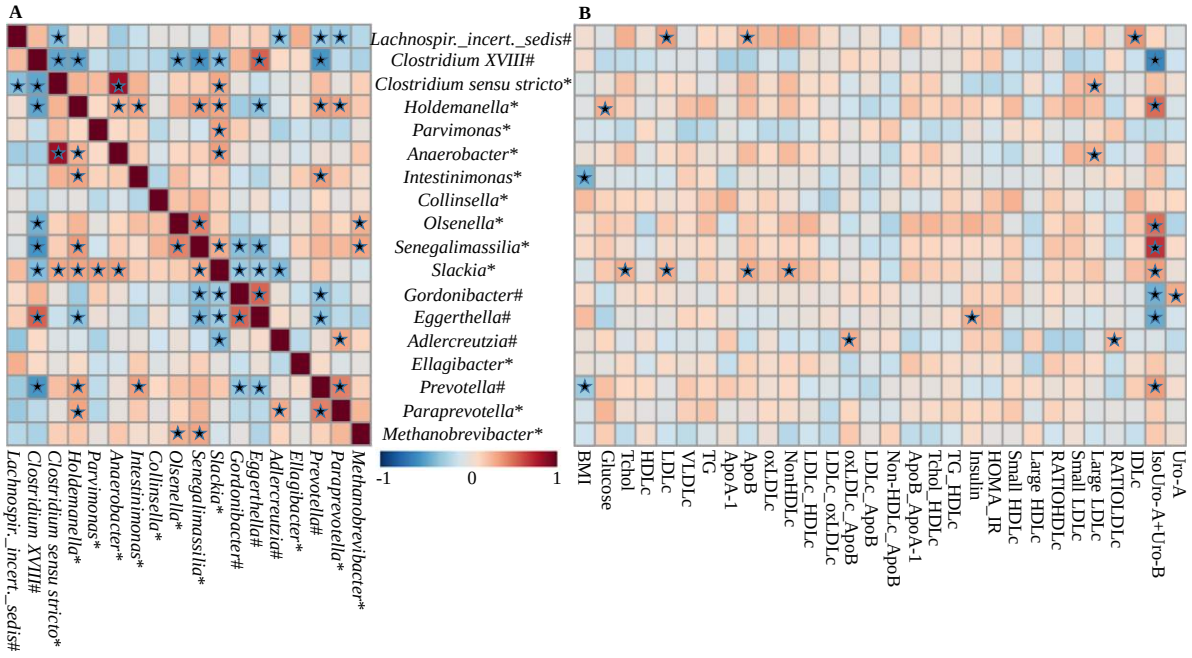

Figure 4