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Yibo Xian

Rong Fan

Jing Shao

Ashley Mulcahy Toney

Soonkyu Chung

See next page for additional authors

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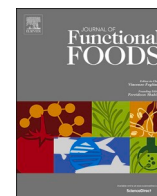


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Authors

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Polyphenolic fractions isolated from red raspberry whole fruit, pulp, and seed differentially alter the gut microbiota of mice with diet-induced obesity

Yibo Xian^{a,b}, Rong Fan^c, Jing Shao^{a,b}, Ashley Mulcahy Toney^c, Soonkyu Chung^{c,d}, Amanda E. Ramer-Tait^{a,b,*}

^a Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

^b Nebraska Food for Health Center, University of Nebraska-Lincoln, Lincoln, NE, USA

^c Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA

^d Department of Nutrition, University of Massachusetts Amherst, Amherst, MA, USA

ARTICLE INFO

Keywords:

Polyphenol extracts
Red raspberry
Seed
Pulp
Gut microbiota
Urolithins

ABSTRACT

Polyphenol extracts from red raspberry (RR) whole fruit or pulp, but not seed, attenuate high-fat (HF) diet-induced obesity in mice. Because host metabolism is linked to the microbiota, we investigated the effects of polyphenols from RR fruit, pulp, and seed on the microbiome. RR polyphenols significantly decreased the abundance of specific taxa that were increased during HF feeding relative to a low-fat diet, including *Ruminococcus* and an unclassified genus from Clostridiales. Compared to a HF diet, pulp and seed polyphenols increased *Roseburia* abundance and decreased levels of an unclassified genus from *Mogibacteriaceae*. RR seed polyphenols uniquely increased *Bifidobacterium* compared to a HF diet. The most notable taxon driving differential abundance among all diets was an unclassified genus from *Coriobacteriaceae*. Importantly, host metabolic markers improved by pulp polyphenols were strongly correlated with select microbiome features, indicating that specific gut bacteria may be involved in RR polyphenol catabolism and/or mediating health benefits.

1. Introduction

Over 650 million adults worldwide were considered obese in 2016 (World Health Organization, 2018). Obese individuals often suffer from metabolic abnormalities such as elevated blood pressure, glucose, and triglycerides that increase their likelihood of developing cardiovascular disease, type 2 diabetes, and certain types of cancer (Haslam & James, 2005). Dietary interventions and physical activity are common approaches to achieve weight loss and glycemic control. Red raspberries (RR), a rich source of ellagitannins (ETs), have been studied extensively for their ability to prevent or treat diet-induced obesity and subsequently improve metabolic function (Calvano et al., 2019; Puupponen-

Pimia et al., 2013). These health benefits are thought to be primarily mediated by polyphenolic compounds, such as ETs and/or anthocyanins (Garcia-Nino & Zazueta, 2015; Overall et al., 2017). ETs are resistant to degradation in the stomach but are hydrolyzed to ellagic acid (EA) and absorbed in the small intestine (Lipinska, Klewicka, & Sojka, 2014). A substantial amount of unabsorbed ETs and free EA also reach the colon, where they are metabolized by the gut microbiota to yield a family of microbial metabolites called urolithins (Uros) (Espin, Larrosa, Garcia-Conesa, & Tomas-Barberan, 2013; Tomas-Barberan, Garcia-Villalba, Gonzalez-Sarrias, Selma, & Espin, 2014).

A growing body of evidence suggests that Uros convey the metabolic benefits of ET- and EA-containing foods (Espin et al., 2013; Kang,

Abbreviations: *A. muciniphila*, *Akkermansia muciniphila*; ASVs, amplicon sequence variants; ATM, adipose tissue macrophages; EA, ellagic acid; ETs, ellagitannins; *G. pamelaeae*, *Gordonibacter pamelaeae*; *G. urolithinfaciens*, *Gordonibacter urolithinfaciens*; GAE, gallic acid equivalent; LDA, linear discriminant analyses; LEfSe, linear discriminant analysis effect size; LF, low-fat; HCA, hierarchical clustering analyses; HF, high-fat; HFPulp, red raspberry pulp polyphenols; HFseed, red raspberry seed polyphenols; HFwhole, whole red raspberry polyphenols; MDP, marker data profiling; PCoA, principal coordinate analyses; qPCR, quantitative PCR; RR, red raspberries; rRNA, ribosomal RNA; Uros, urolithins; WAT, white adipose tissue.

* Corresponding author at: Department of Food Science and Technology, University of Nebraska-Lincoln, 1901 N 21st St., 260 Food Innovation Center, Lincoln, NE 68588, USA.

E-mail address: aramer-tait2@unl.edu (A.E. Ramer-Tait).

<https://doi.org/10.1016/j.jff.2020.104288>

Received 20 July 2020; Received in revised form 4 November 2020; Accepted 11 November 2020

Available online 26 November 2020

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Buckner, Shay, Gu, & Chung, 2016; Kang, Kim, Tomas-Barberan, Espin, & Chung, 2016; Puupponen-Pimia et al., 2013). Uros exhibit anti-inflammatory, anti-oxidative, anti-proliferative, and lipid-lowering properties *in vitro* (Kang, Kim, et al., 2016; Piwowarski, Kiss, Granica, & Moeslinger, 2015). Additionally, daily administration of UroA to mice fed a high fat (HF) diet has been shown to improve systemic insulin sensitivity and attenuate triglyceride accumulation *in vivo* (Toney et al., 2019). However, the metabolic effects of Uros in humans are not fully understood, and results from human intervention studies providing berries rich in ETs, such as strawberries and raspberries are inconsistent (Calvano et al., 2019; Puupponen-Pimia et al., 2013). Variation among these studies could arise from differences in berry processing method, intervention period, and the health status of the participants. Moreover, these variable results may also reflect individual differences in gut microbial transformation of ETs and EA to Uros (Li et al., 2015; Tomas-Barberan et al., 2014). Li et al. reported several healthy participants were unable to produce UroA even after long-term consumption of a pomegranate extract containing ETs (Li et al., 2015). Notable differences between the gut microbiome profiles of UroA producers and non-producers were also observed (Li et al., 2015), suggesting that the gut microbiota may contribute to polyphenol catabolism, which in turn mediates the metabolic benefits of ETs and EA for the host.

In a previous study, we found that supplementation with RR pulp polyphenols significantly limited the development of diet-induced obesity and insulin resistance in C57BL/6 mice (Fan et al., 2019). Furthermore, polyphenols from RR whole fruits and pulp, but not the seeds, decreased adipose tissue macrophage (ATM) recruitment, NLRP3 inflammasome activation, and adipocyte hypertrophy (Fan et al., 2019). In this study, we investigated the relationship between red raspberries, metabolic health, and the gut microbiota by evaluating the effects of polyphenolic extracts isolated from RR whole fruits, pulp or seed on the gut microbiome. We found that supplementation with RR polyphenols induced distinct changes in the composition of the gut microbiota. We also observed strong correlations between obesity-related host parameters improved by pulp polyphenols and gut microbiota profiles, suggesting that specific bacteria may be involved in RR polyphenol catabolism and/or RR-mediated host benefits.

2. Materials and methods

2.1. Animals, diets, and experimental design

C57BL/6 male mice were purchased from the Jackson Laboratory and assigned to one of five treatments as described in our previous study (Fan et al., 2019): low-fat diet (LF, 10% fat, $n = 4$), high-fat diet (HF, 45% fat, $n = 8$), HF diet supplemented with 0.4% by weight RR whole fruit polyphenols (HFwhole, $n = 7$), 0.1% by weight RR seed polyphenols (HFseed, $n = 8$), or 0.3% by weight RR pulp polyphenols (HFpulp, $n = 8$). Polyphenolic extracts from whole fruit, seed and pulp were prepared as described in our previous study from freshly-frozen "WakeField" RR fruits donated by Enfield Farms (Lynden, WA) (Fan et al., 2019). Briefly, pulp and seed fractions were physically separated using a fine sieve. Polyphenols were extracted in acidified methanol (0.5% acetic acid), purified via an ion-exchange column (Ambelite FPX66, Dow Chemical) to remove sugars and fibers, and then lyophilized. The polyphenolic compounds in the extracts were analyzed on an Agilent 1200 HPLC (Agilent Zorbax SB-C18 column). Total phenolic content was determined using Folin-Ciocalteu reagent with gallic acid as a standard. The composition of the lyophilized RR polyphenolic extracts was consistent with previous reports (Sojka, Macierzynski, Zaweracz, & Buczek, 2016) and included quercetin, myricetin, ellagic acid, catechin, epicatechins, and anthocyanins (Table S1).

Diets were formulated based on the AIN-93M purified rodent formula such that the levels of polyphenols present in the fruit fractions reflected those obtained from whole fruit as described in our previous study (Fan et al., 2019). Specifically, we isolated three times more

polyphenols from pulp than seed when starting from the same amount of whole fruit, thus resulting in a whole:seed:pulp ratio of 4:1:3 (Table S2). Based on the amount of extracts added into the diets, the HFpulp diet contained anthocyanin (37.1% of the total GAE), EA (14.7%), and epicatechin (19.9%). The HFwhole diet contained primarily EA (23.7%) and epicatechin (27.0%), and the HFseed diet contained mostly EA (40.7%; Table S2). Mice were fed for 16 weeks ad libitum, and cecal contents were collected and snap-frozen in liquid nitrogen at necropsy and stored at -80°C . All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln (Protocol 1469) and carried out in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2.2. Bacterial DNA extraction, PCR amplification, and 16S rRNA gene sequencing

Bacterial DNA was extracted from mouse cecal contents using the BioSprint 96 workstation (Qiagen, Germantown, MD) and the BioSprint 96 one-for-all Vet kit (Qiagen) as per manufacturer's instructions with the addition of a bead-beating step (Caporaso et al., 2011). Briefly, cecal contents were mixed with 600 μL pre-heated (70°C) ASL buffer in a 2-mL deep-well microplate (Axygen, Union City, CA) containing 250 μL of 0.1 mm zirconium beads (BioSpec Products, Bartlesville, OK). Bacterial cells were disrupted by bead beating using a TissueLyser II (Qiagen) with 3 cycle repeats (4 min of 30 s on with a 10 min off interval for cooling). The mixture was allowed to settle in a 70°C water bath for 10 min. One hundred μL of the mixture was then transferred to an S-block containing 40 μL proteinase K in each well for enzymatic lysis in a 70°C water bath for 10 min. The lysate plate was completed by adding 600 μL RLT mixture (the master mix for 96 wells contained 35 mL buffer RLT, 35 mL isopropanol, 3 mL MagAttract Suspension G, and 310 μL Carrier RNA solution) to each well of the S-block containing lysate. S-Block wash plates and elution microplates were prepared according to the manufacturer's instructions. All plates were loaded onto the workstation according to the on-screen instructions. Protocol BS96 Vet 100 was used for sample processing. The quality and quantity of eluted DNA were tested using Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA).

The V4 region of the bacterial 16S rRNA gene was amplified from each sample using the dual-indexing sequencing strategy (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Amplified PCR products were purified and normalized using a SeqPrep™ Normalization Plate (Applied Biosystems, Waltham, MA). Equal volumes of each of the normalized PCR products were pooled together. Pooled library quality was checked using the Agilent High Sensitivity D1000 ScreenTape system (Agilent, Santa Clara, CA). Library quantification was checked by qPCR using a Library Quantification Kit (Kapa Biosystems, Wilmington, MA). The library was sequenced on an Illumina MiSeq platform using a MiSeq Reagent Kit v3 for 600 cycles (Illumina, San Diego, CA) according to the manufacturer's instructions.

2.3. Sequence processing and data analyses

Paired-end sequences were demultiplexed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) program (version 2). Briefly, sequence quality was checked by FastQC and, based on the quality, sequences were filtered using the DADA2 workflow by removing chimeric sequences and truncating the forward reads to 230 bases and reverse reads to 160 bases. Sequences were de-replicated into unique amplicon sequence variants (ASVs) and a list of representative sequences was generated by DADA2 (Callahan et al., 2016).

For taxonomy analyses, each of the representative sequences was assigned to the taxonomic information using the Greengenes database with the pre-trained RDP Classifier (<http://greengenes.microbio.me>) based on 99% sequence identity. The representative sequences were aligned, highly variable regions in the sequences were masked and then

a phylogenetic tree was created with FastTree. Analyses of the alpha and beta diversity were all performed based on the normalization of using a sample depth of 16,745 sequences (lowest number of sequences per sample) in QIIME 2. Data were visualized using GraphPad Prism 7.0 or RStudio (version 3.4.3). For evaluation of community richness and diversity in each sample, three different alpha diversity metrics were calculated (Observed ASVs, Shannon index and Faith's PD index). Microbial diversity among samples was assessed using four different beta diversity metrics (Jaccard, Bray-Curtis, unweighted Unifrac and weighted Unifrac). Principal coordinate analyses (PCoA) and hierarchical clustering analyses (HCA) plots for all samples were generated to visually depict the differences among treatments. Only taxa with sequence reads numbering more than 0.01% of the total reads were included in relative abundance analyses of the different taxa among treatments. To identify taxa differentially responding to treatments, the linear discriminant analyses (LDA) effect size (LEfSe) algorithm was used with an LDA threshold of 3.5 and adjusted p-value cutoff of 0.05. Random forest analyses were performed using default settings with $n_{tree} = 500$ and $m_{try} = 7$. Hierarchical clustering analyses, heatmap diagrams, LEfSe, and random forest analyses were performed using the packages in Marker Data Profiling (MDP) from the web-based tool MicrobiomeAnalyst (Dhariwal et al., 2017). Spearman's rho non-parametric correlations between gut microbiota (taxa relative abundance) and host metabolic markers were calculated based on the data from HF and HFpulp fed mice by using the package microbiome in R software (R Foundation for Statistical Computing, Vienna, Austria) (R Core Team., Accessed July 2015).

2.4. *Gordonibacter* species-specific primer design and validation

Representative genomic sequences for *Gordonibacter pamelaiae* (*G. pamelaiae*) and *Gordonibacter urolithinfaciens* (*G. urolithinfaciens*) as well as representatives of the family Coriobacteriaceae (Selma, Tomas-Barberan, Beltran, Garcia-Villalba, & Espin, 2014) were downloaded from NCBI database (<http://www.ncbi.nlm.nih.gov/genome>) and can be identified using the following GenBank accession numbers: *G. pamelaiae* 7-10-1-b (NC_021021.1), *G. urolithinfaciens* An234A (NZ_NFJN01000001.1), *Paraeggerthella hongkongensis* RC2/2 A (PPTQ01000001.1), *Eggerthella lenta* DSM2243 (NC_013204.1), *Eggerthella sinensis* DSM16107 (QICC01000001.1 and PPTT01000001.1), *Denitrobacterium detoxificans* NPOH1 (NZ_CP011402.1), *Adlercreutzia equolifaciens* DSM19450 (NC_022567.1), *Asaccharobacter celatus* DSM18785 (QICA01000001.1), *Enterorhabdus caecimuris* B7 (NZ_KE159646.1) and *Enterorhabdus mucosicola* DSM19490 (NZ_KE383895.1). Putative primer pairs that specifically targeted *G. pamelaiae* and/or *G. urolithinfaciens* were designed using RUCS-1.0 (Thomsen, Hasman, Westh, Kaya, & Lund, 2017) (<https://cge.cbs.dtu.dk/services/RUCS/>) with minor modifications of the default settings: k-mer size changed to 22 and product size range changed to 200–500. Specificity of the candidate primer pairs was first checked using NCBI Primer-Blast and representative genomes in the Refseq database. Candidate primer sets were then experimentally validated for specificity and efficiency by quantitative PCR using genomic DNA from *G. pamelaiae* DSM19378, *G. urolithinfaciens* DSM27213, *Eggerthella lenta* JMA32, *Lactobacillus plantarum* ATCC4008, *Bacteroides ovatus* ATCC8483, *Bifidobacterium longum* CR15, *Salmonella enterica*, and *Escherichia coli* 13L.

2.5. Quantitative PCR (qPCR)

The extracted DNA subjected to 16S rRNA gene sequencing was also used for qPCR. DNA was quantified using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA). Bacterial genus- or species-specific primer sequences used include: *Bifidobacterium* spp. F-TCGCGTCYGGTGTGAAAG, R-CCACATCCAGCRTCCAC (Schneeberger et al., 2015); *Bifidobacterium pseudocatenulatum* F-AGCCATCGTCAAGGAGCTTATCGCAG, R-

CACGACGTCTGCTGAGAGCTCAC (Junick & Blaut, 2012); *Gordonibacter* spp. F-GCTGCGTTGAAATATGGCTGT, R-GCAGAAGGACCAACGTCAAC; *G. pamelaiae* F-GCTTTGTATTGGTCGCCTGTG, R-CATC-GACTCTGCATTCAAGCG; *G. urolithinfaciens* F-GCGGTTCTGCTAAGACGAGAA, R-GGAAGGGGTCATTGACACAT. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR reactions were prepared in a 25 μ L volume containing 12.5 μ L 2x SYBR Green, 8.5 μ L nuclease free water, 2 μ L primer mix (a mixture of forward and reverse primer of 5 μ M each), and 2 μ L DNA template (around 10 ng/ μ L). Thermocycling conditions included: (i) an initial denaturation step of 2 min at 95 °C; (ii) 35 cycles of 15 s at 95 °C, 15 s at annealing temperature (60 °C for *Bifidobacterium* genus-specific primers, *Gordonibacter* genus-specific primers, and *G. pamelaiae* species-specific primers; 62 °C for *G. urolithinfaciens* species-specific primers; 68 °C for *Bifidobacterium pseudocatenulatum* species-specific primers), and 20 s at 68 °C; (iii) one cycle of 15 s at 95 °C; (iv) one cycle of 15 s at 60 °C; (v) one 20-min interval to generate a melting curve; and (vi) one cycle of 15 s at 95 °C. The cycle threshold of each sample was then compared to a standard curve made by diluting genomic DNA (five-fold serial dilution) as previously described (Gomes-Neto et al., 2017). The data were expressed as log bacterial colony forming units/g of DNA.

2.6. Statistical analyses

Alpha diversity indices were calculated based on diet type. Pairwise comparisons between different diet treatments were performed using a Kruskal-Wallis test. False discovery rate correction was incorporated for all statistical tests. For beta diversity, Adonis statistical tests were used to investigate whether the differences between diet treatments were statistically significant. To compare the relative abundance of taxa among diet treatments, multiple comparisons to the HF treatment were performed in Prism 7.0 (GraphPad Software, La Jolla, CA). First, the empirical distribution of data with an assumption of normality was tested using Kolmogorov-Smirnov, D'Agostino and Pearson, and Shapiro-Wilk normality tests. Secondly, data determined to be non-normal were analyzed using one-way Kruskal-Wallis testing followed by Dunn's post-test correction for multiple comparisons. For parametric analyses, the multiple comparison test was performed by controlling the false discovery rate in one-way ANOVA analyses according to the Benjamini and Hochberg procedure (Benjamini & Hochberg, 1995). The mean relative abundance of each taxon in each treatment was graphed with corresponding SEM values. In each graph, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus HF treatment were noted above the mean value.

3. Results

3.1. Supplementation with red raspberry (RR) polyphenolic extracts changed the diversity of the gut microbiota

We previously demonstrated that feeding polyphenols from RR whole fruit and pulp, but not the seeds, attenuated the development of HF diet-induced obesity and insulin resistance in mice (Fan et al., 2019). To determine whether supplementation with various polyphenol-containing extracts derived from RR also differentially affected the gut microbiota, we analyzed cecal contents by 16S rRNA gene sequencing from those same mice fed either a low fat (LF) diet, high fat (HF) diet, or HF diet supplemented with polyphenols from either RR whole fruits (HFwhole), RR pulp (HFpulp) or RR seed (HFseed) for 16 weeks (Fig. 1A).

We first assessed the effects of supplementing a HF diet with polyphenolic extracts from RR on microbial community richness and alpha diversity. Feeding mice HFwhole and HFpulp diets, but not a HFseed diet, significantly increased the observed number of ASVs compared to feeding the LF diet but not the HF diet (Figure S1A). No significant effects of diet were observed during Shannon diversity analysis

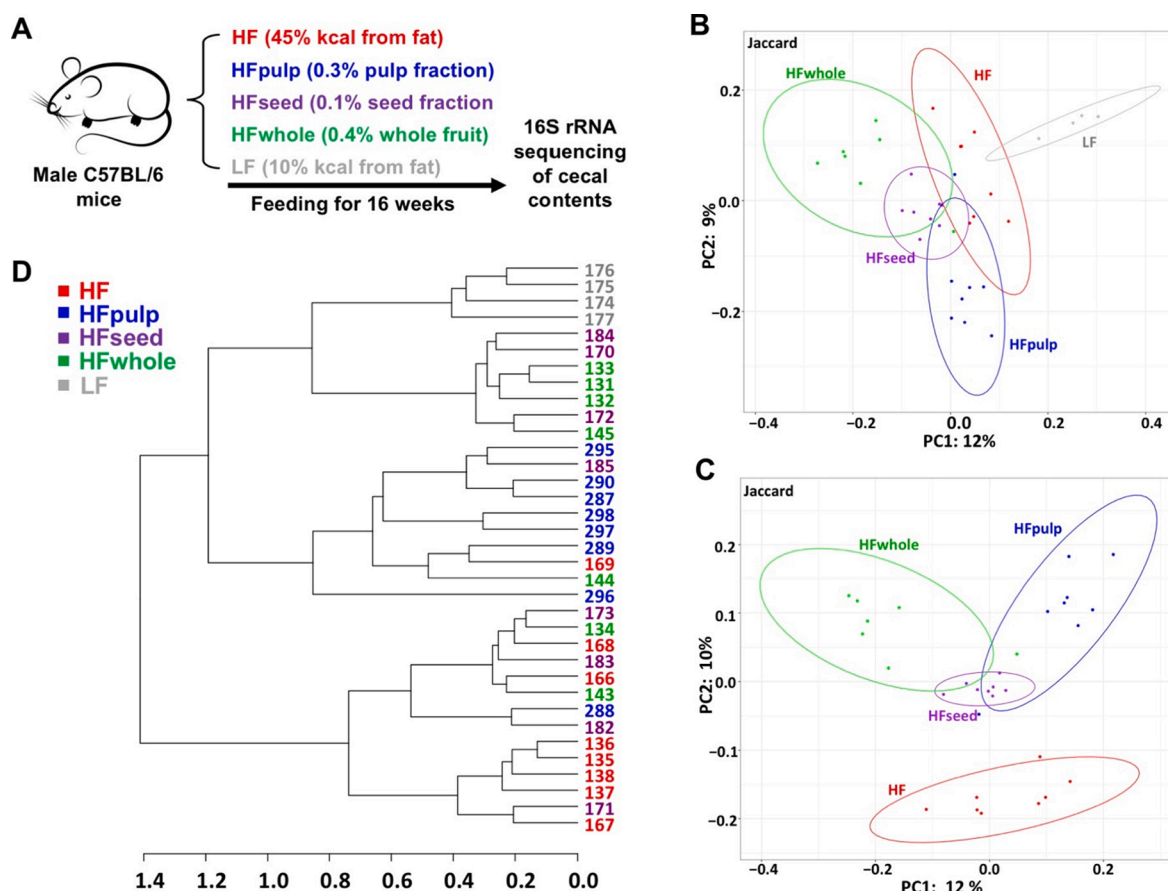


Fig. 1. Supplementation with red raspberry polyphenolic extracts changed the diversity of the gut microbiota. (A) Experimental scheme for mouse feeding study. Principal Coordinate Analyses (PCoA) plot of β -diversity based on binary Jaccard distance for all dietary treatments (B) or high-fat diets supplemented with or without raspberry polyphenols (C). Ellipses represent the 95% confidence interval for each dietary treatment. (D) Ward clustering algorithm analyses using the Jaccard distance matrix. LF = low-fat diet, n = 4; HF = high-fat diet, n = 8; HFwhole = whole raspberry polyphenols, n = 7; HFseed = raspberry seed polyphenols, n = 8; HFpulp = raspberry pulp polyphenols, n = 8.

(Figure S1B). To consider the phylogenetic relatedness of the communities, we performed Faith's PD diversity analysis and observed a significantly higher microbiome diversity value for mice fed a HFwhole diet, but not HFpulp or HFseed diets, as compared to LF- and HF-fed mice (Figure S1C). Overall, these results indicate that supplementation with polyphenols from RR whole fruits had the most significant effects on microbial community richness and phylogenetic diversity, whereas RR pulp polyphenols affected only richness, and RR seed polyphenols had no significant effect on either richness or phylogenetic diversity.

Beta diversity among diet treatments was analyzed by principal coordinate analyses (PCoA) and hierarchical clustering analyses (HCA). PCoA analyses based on Jaccard distance clearly showed microbiomes clustering by diet (Fig. 1B; $p \leq 0.002$ for comparison of HF diet to each RR diet using permutational ANOVA; see Table S3). Considering that HF and LF diets differ notably in their compositions, we next performed PCoA analyses using only HF-based diets. These analyses revealed more distinct clustering and significant differences between microbiomes from HF-fed mice and those of mice fed HF diets supplemented with a RR polyphenol extract regardless of fraction (Fig. 1C; $p \leq 0.002$ for pairwise comparisons, Table S3). Similar results were observed using analyses based on Bray-Curtis and unweighted Unifrac metrics (Figure S2; Table S3). By weighted Unifrac analysis, only microbiomes from HFwhole fed mice were significantly different compared to those from HF control mice (Table S3).

Hierarchical clustering of the data using the Ward method revealed notable segregation between microbiomes from RR-supplemented mice and those from LF-fed mice (Fig. 1D). Microbiomes from mice fed

HFwhole diets were most similar to those from LF controls, followed by microbiomes from mice fed HFpulp and HFseed diets. Together, these data demonstrate that supplementation of HF diets with RR polyphenol extracts induced global changes to the gut microbial community structure when fed to mice.

3.2. Supplementation with RR polyphenols altered gut microbiota composition

To determine how RR polyphenolic extracts fed with a HF diet altered gut microbiota composition, we examined distribution of significant taxa in our data. The most predominant phylum observed in our mice was Firmicutes, which accounted for more than half of all sequencing reads (56.43%), followed by Verrucomicrobia (22.77%), Bacteroidetes (18.05%), Actinobacteria (2.6%) and Proteobacteria (0.15%) (data not shown). The most abundant genus was *Akkermansia*, found to constitute 20–26% of total sequences in samples; however, no differences in the abundance of this genus were observed among treatments (Fig. 2A; Table S4). The second most highly abundant taxon in our mice mapped to a sequence variant that could not be classified below the order Clostridiales (uncl.Clostridiales), which was significantly increased by HF diet feeding compared to a LF diet. Notably, the abundance of uncl.Clostridiales was significantly decreased by feeding polyphenols from RR whole fruits, pulp, and seed compared to a HF control diet (Table S4).

Diet-associated changes were also observed at the genus level (Fig. 2B; Table S4). Compared to a LF diet, a HF diet significantly

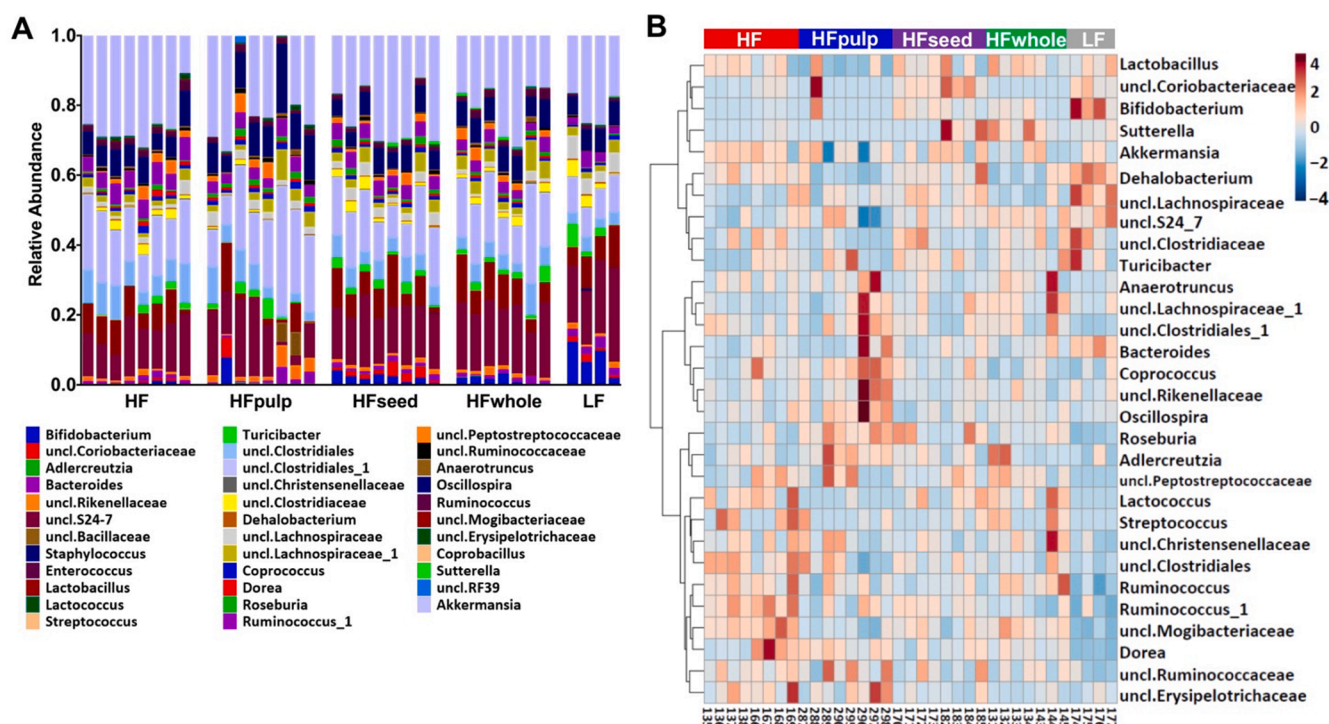


Fig. 2. Supplementation with red raspberry polyphenols altered gut microbiota composition. (A) Stacked column bar graphs depict the relative abundance and the most highly abundant resolved taxa at the genus level for individual mice. (B) Heatmap depicting relative abundance patterns of taxa affected by dietary interventions. The dendrogram clusters genera that frequently occur together. Taxa were filtered with reads numbering less than 0.01% of total reads.

decreased the abundance of *Bifidobacterium*. Importantly, feeding a HFseed diet reversed this trend and significantly increased the abundance of *Bifidobacterium* to levels even greater than those found in LF diet-fed mice. HF diet feeding also significantly decreased levels of *Dehalobacterium*, *Bacteroides*, and an unclassified genus from *Lachnospiraceae* (uncl.*Lachnospiraceae*) compared to mice fed a LF diet. Levels of *Dehalobacterium* were further decreased in mice fed HFwhole and HFpulp diets compared to HF control mice. No additional changes in the abundance of *Bacteroides* or uncl.*Lachnospiraceae* were observed during

RR polyphenol feeding compared to HF diet feeding.

Relative to mice fed a LF diet, mice receiving a HF diet exhibited significant increases in the abundance of *Dorea*, *Ruminococcus*, *Ruminococcus_1*, and an unclassified genus from *Mogibacteriaceae* (uncl.*Mogibacteriaceae*). Supplementing a HF diet with RR polyphenols from whole fruits, pulp, or seed significantly decreased the abundance of *Ruminococcus_1* compared to a HF diet. Also compared to a HF diet, HFseed specifically decreased levels of *Ruminococcus*, and feeding mice either a HFseed or HFpulp diet significantly lowered their abundance of

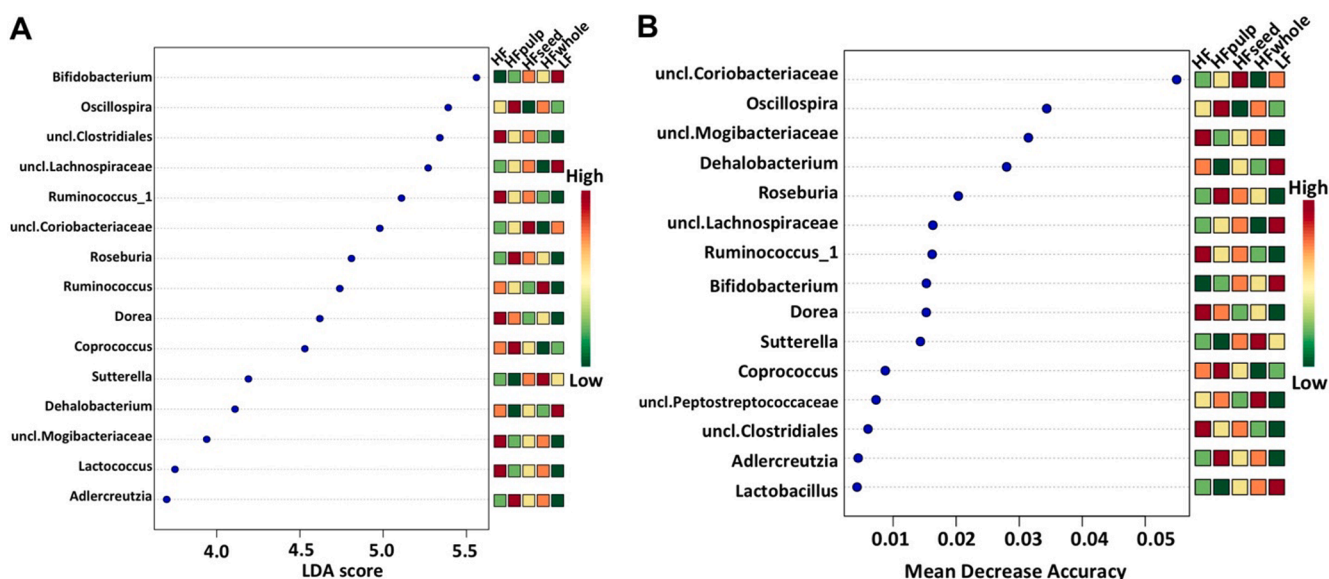


Fig. 3. Prediction of key taxa responding to red raspberry polyphenols. Taxa differentially responding to dietary treatments were identified by LEfSe (A) and random forest (B) analysis. The top 15 candidates are shown here. The color bar represents the relative abundance of taxa, with green corresponding to a low abundance and red corresponding to a high abundance. The average relative abundance for each taxa in individual dietary treatments is represented by a colored square that corresponds to the relationship between relative abundance and the color gradient in the bar.

uncl.*Mogibacteriaceae*. RR polyphenol supplementation did not effect *Dorea* levels compared to the HF control. Other notable changes observed during RR polyphenol supplementation included an increased abundance of *Roseburia* in mice fed either a HFseed or HFpulp compared to those fed a HF diet as well as increased levels of *Lachnospiraceae_1* in mice receiving a HFwhole diet versus the HF control.

3.3. Prediction of key taxa responding to RR polyphenols

To identify the specific bacterial taxa driving differential abundances among the diets supplemented with various RR polyphenols, a LEfSe analysis (with 3.5 as the threshold on the logarithmic LDA score for discriminative features) was performed. Seventeen taxa were identified as significantly different ($p < 0.05$ using Mann-Whitney/Kruskal-Wallis tests and comparisons of all diets), and the top 15 taxa were ranked according to their LDA score (Fig. 3A). Random forest classification corroborated the relevance of a majority of these top candidates (Fig. 3B), with 13 of the 17 taxa identified by LEfSe also included in the list of top 15 predictors given by random forest. The relative abundances for each of these 13 taxa (as ranked by their in LEfSe p -values) are shown in Fig. 4.

LEfSe analysis revealed the genus *Bifidobacterium* to be the strongest taxon driving differences among the various diets. The relative abundance of *Bifidobacterium* was dramatically decreased by HF diet feeding. However, supplementing a HF diet with RR polyphenolic extracts increased *Bifidobacterium* abundance, especially in mice fed the HFseed diet. This observation was confirmed by qPCR using *Bifidobacterium*-specific primers (Figure S3A).

The most notable taxon driving differential abundance among diets identified by random forest was an unclassified genus from the *Coriobacteriaceae* family (uncl.*Coriobacteriaceae*). Notably, the urolithin-producing species, *Gordonibacter pamelaee* and *Gordonibacter urolithinifaciens* have been previously classified in this family (Selma et al., 2014; Tomas-Barberan et al., 2014). Subsequent qPCR analysis showed that *G. pamelaee* was not present in our mice and that only two mice receiving the HFseed diet harbored *G. urolithinifaciens* (Figure S3A-B). These data suggest that *G. pamelaee* and *G. urolithinifaciens* are not the species responsible for the diet-driven changes in abundance observed for the unclassified genus mapping to *Coriobacteriaceae*, and that another bacterial species in this family is likely affected by RR polyphenols.

3.4. Correlations between gut microbiota profiles and obesity-related host parameters improved by RR pulp polyphenols

In our previous study, we found that RR polyphenol supplementation reduced triglyceride accumulation and inflammation in the liver, brown fat, and pancreas of mice, especially those fed with polyphenols from the RR pulp fraction (Fan et al., 2019). To determine whether there was a link between changes in the gut microbiota and the host metabolism improvements observed following RR polyphenol supplementation, we performed Spearman's correlation analyses between the bacterial taxa relative abundances and host metabolic parameters. For mice fed HFpulp and HF control diets, we observed significant positive correlations between the relative abundance of an unclassified genus from *Clostridiaceae* (uncl.*Clostridiaceae*) and brown adipose tissue weight, mesenteric WAT weight, and perirenal WAT weight (Fig. 5A-D). Significant negative correlations were found between the relative abundance of an unclassified genus from *Lachnospiraceae* (uncl.*Lachnospiraceae_1*) and both mesenteric WAT weight and perirenal WAT weight (Fig. 5E-F). In addition, the relative abundance of *Oscillospira* was significantly negatively correlated with brown adipose weight (Fig. 5G). Together, these results suggest that select gut bacterial taxa may be involved in mediating the metabolic benefits of polyphenols derived from the pulp fraction of red raspberries.

4. Discussion

Because the gut microbiota has been linked to metabolic improvements in humans and implicated in the transformation of ETs and EA found in RR (Tomas-Barberan et al., 2014), we determined whether supplementation with various RR polyphenolic extracts differentially affected the gut microbiota. Here, we show that supplementation with RR polyphenols induced changes in the diversity and composition of the gut microbiota. Furthermore, correlation analyses between obesity-related host parameters improved by RR pulp polyphenols (e.g., brown adipose tissue weight, mesenteric WAT weight, and perirenal WAT weight) and microbiome profiles revealed that specific gut bacteria may be involved in polyphenol metabolism and/or mediating the host benefits observed during RR consumption.

The polyphenol EA occurs naturally in many fruits and vegetables, including berries (red and black raspberries and strawberries), tree nuts, pomegranates, and muscadine grapes (Kang, Buckner, et al., 2016). In RR, ETs (including EA conjugates) constitute about 60% of the total phenolic compounds and are mainly found in solid fractions such as pulp and seed (Sojka et al., 2016). A considerable number of ETs and its hydrolyzed product EA are not absorbed in the small intestine and instead reach the colon (Espin et al., 2013; Lipinska et al., 2014). This scenario creates an opportunity for a reciprocal relationship between the gut microbiota and dietary polyphenols that likely yields host health benefits in both directions. Polyphenols are metabolized by the resident microbiota, and the resulting products may have bioactivities that differ from the parent compound and affect host health. Dietary polyphenols can also have prebiotic effects on the gut microbiota, with many reports describing selective enrichment for specific microbiota members known to have beneficial health effects. For example, ETs and condensed tannins have been shown to promote the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* and inhibit the growth of potentially detrimental species from Clostridiales and Enterobacteriales (Duenas et al., 2015). Oral administration of UroA (a byproduct of microbial transformation of ETs and EA) to rats also promoted the growth of *Lactobacillus* and *Bifidobacterium* (Larrosa et al., 2010). We also observed prebiotic effects when feeding RR polyphenols, particularly on *Roseburia* and *Bifidobacterium*. Specifically, polyphenol extracts from RR pulp and seed significantly increased the abundance of *Roseburia*, which were decreased due to HF feeding. *Roseburia* produce short-chain fatty acids, especially butyrate, and loss of these species has been associated with several chronic diseases, including Crohn's disease (Willing et al., 2010) and type 2 diabetes (Gurung et al., 2020). *Roseburia* abundance is often increased when diets are supplemented with fibers, such as resistant starch (Maier et al., 2017), and these increases are sometimes associated with host metabolic improvements, including weight loss and improved glucose tolerance (Ryan et al., 2014). Taken together, these observations indicate that RR polyphenols may represent a previously unappreciated prebiotic for the promotion of *Roseburia* growth and subsequent metabolic benefits for the host.

We also observed a prebiotic effect on *Bifidobacterium* when supplementing diets with RR polyphenols, especially from RR seeds. Moreover, *Bifidobacterium* species are also reported to participate in the conversion of ETs to EA as well as the transformation of EA to Uros and other smaller phenolic acids (Gaya, Peiróten, Medina, Álvarez, & Landete, 2018). *Bifidobacterium pseudocatenulatum* INIA P815 has been isolated from healthy humans capable of producing Uros and is capable of metabolizing EA to UroA and UroB *in vitro* (Gaya et al., 2018). Of note, *B. pseudocatenulatum* was not detected in our present study by qPCR using species-specific primers, suggesting that *Bifidobacterium* species other than *B. pseudocatenulatum* are likely responding to RR polyphenols.

Gordonibacter species are also thought to play a role in polyphenol catabolism. In particular, two urolithin-producing species from this genus of gut bacteria have been isolated from human feces, *G. urolithinifaciens* DSM 27,213 and *G. pamelaee* DSM 19,378 (Selma

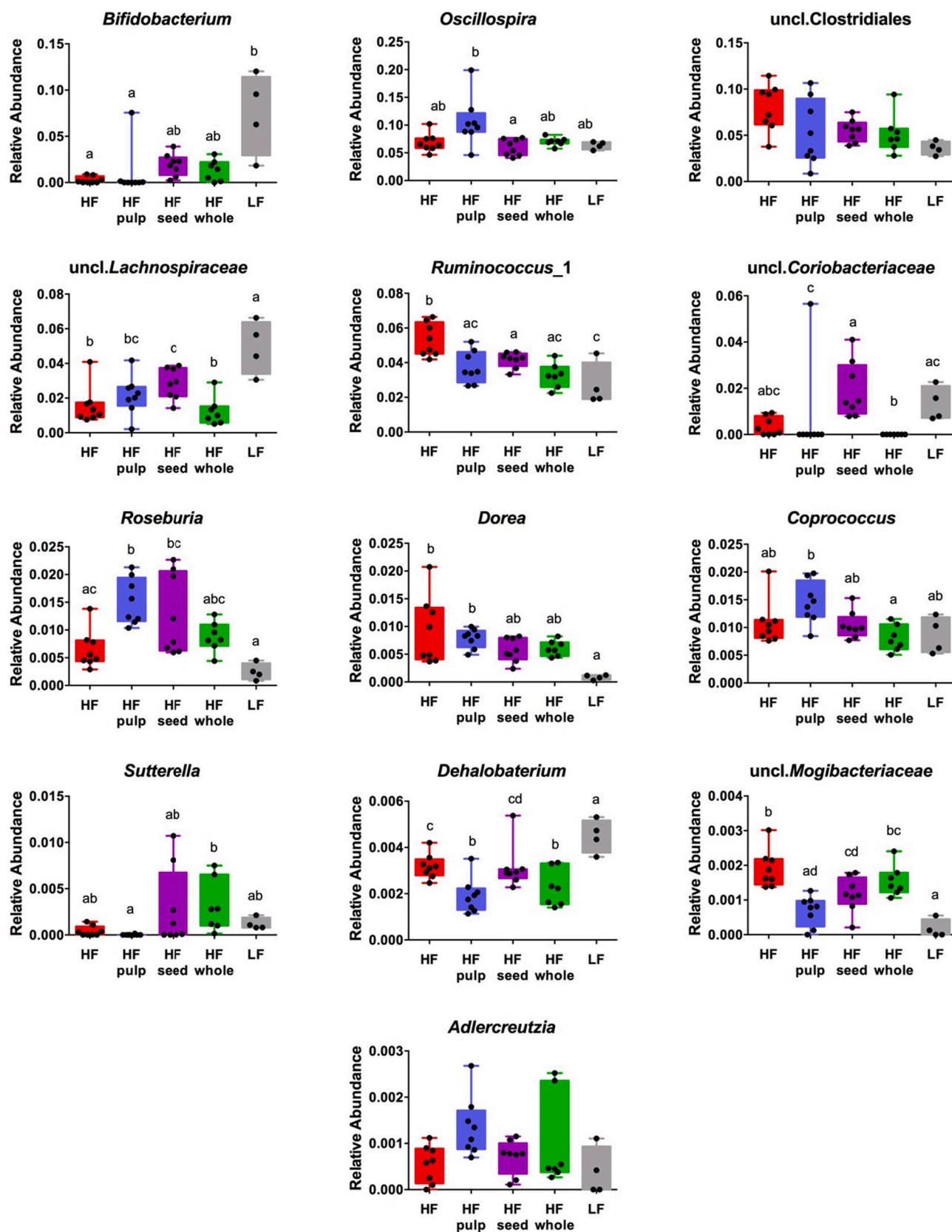


Fig. 4. Abundance of key taxa identified by LEfSe and random forest during supplementation with red raspberry polyphenols. All data were analyzed using a non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn's post-hoc test. Data are presented as mean \pm SEM and treatments with different letters are significantly different from one another at $p < 0.05$. LF = low-fat diet; HF = high-fat diet; HFwhole = whole raspberry polyphenols; HFseed = raspberry seed polyphenols; HFpulp, raspberry pulp polyphenols.

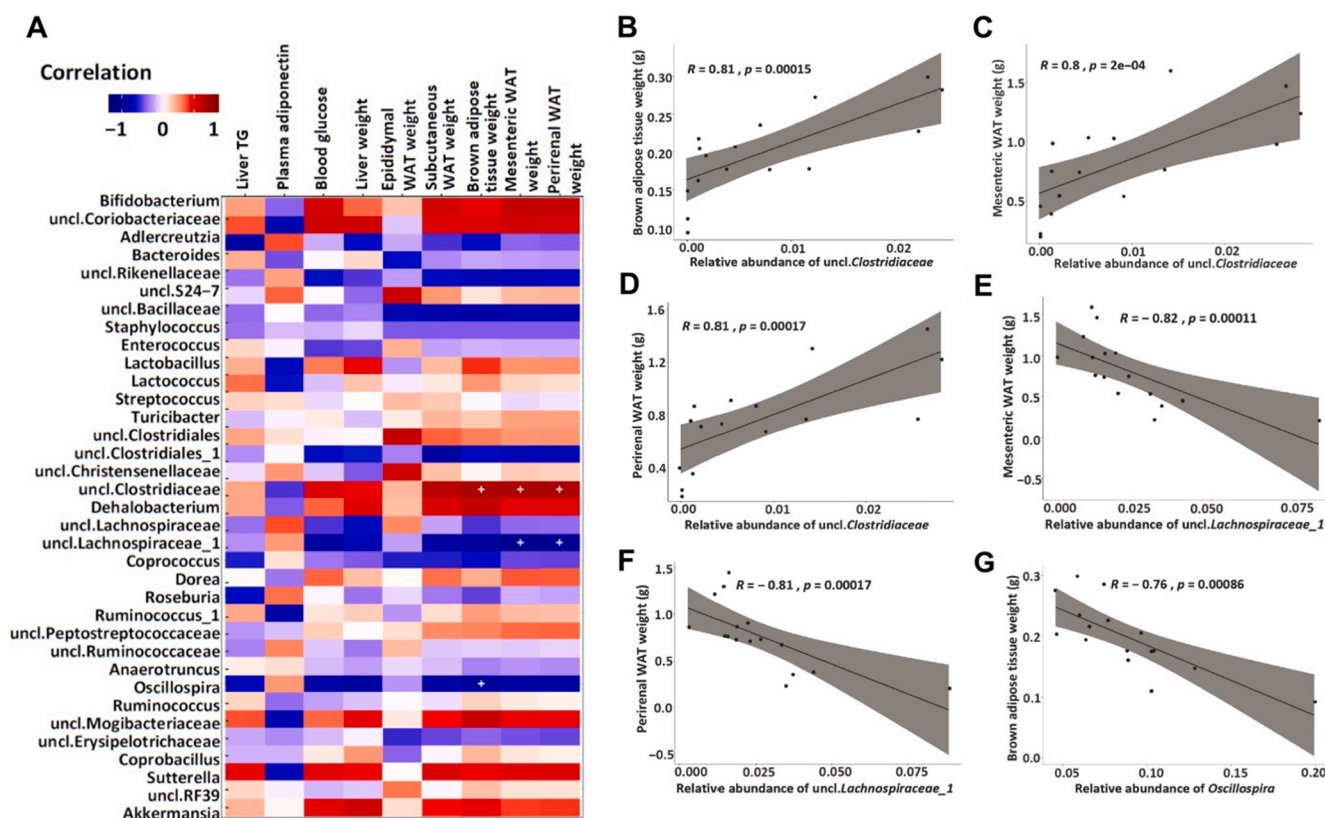


Fig. 5. Correlations between gut microbiota profiles and obesity-related host parameters improved by pulp polyphenols. (A) Heatmap of Spearman's correlations between relative abundance of bacterial taxa and host metabolic markers previously reported in (Fan et al., 2019) for mice fed HF and HFpulp diets. Colors range from blue (negative correlation) to red (positive correlation). Statistically significant correlations are marked by + ($p < 0.05$). The abundance of uncl. Clostridiaceae is significantly positively correlated with brown adipose tissue weight (B), mesenteric WAT weight (C) and perirenal WAT weight (D). The abundance of uncl. Lachnospiraceae_1 is significantly negatively correlated with mesenteric WAT weight (E) and perirenal WAT weight (F). The abundance of Oscillospira is significantly negatively correlated with brown adipose tissue weight (G). HF = high-fat diet; HFpulp = raspberry pulp polyphenols; WAT = white adipose tissue.

et al., 2014; Tomas-Barberan et al., 2014). However, LC-MS analyses only detected production of some intermediary urolithins (such as pentahydroxy-urolithin and tetrahydroxy-urolithin), but not the final urolithins (such as UroA, iso-UroA and UroB), in pure cultures of these strains (Tomas-Barberan et al., 2014). In our study, LEfSe and random forest analyses identified an unclassified genus from the Coriobacteriaceae family as a taxon driving the differential microbiota profiles observed among the various diets tested. The genus *Gordonibacter* was originally classified in the Coriobacteriaceae family (Tomas-Barberan et al., 2014) but has been more recently reassigned to the Eggerthellaceae family (Clavel, Lepage, & Charrier, 2014). Considering that this update was not reflected in the database used for our taxonomic analysis, we performed qPCR tests using species-specific primers for *G. urolithinfaciens* and *G. pamelaeeae*. We did not detect *G. pamelaeeae* in our mice and only detected *G. urolithinfaciens* in two mice receiving the HFseed diet. This result suggests that other *Gordonibacter* species or other genera from the Coriobacteriaceae family are responsible for the altered gut microbiota profiles we observed during RR polyphenol feeding.

Some members of the Eggerthellaceae family, which is in the same class (Coriobacteriia) as the Coriobacteriaceae family, are capable of transforming other polyphenols into bioactive molecules (Tomas-Barberan et al., 2017). For example, *Adlercreutzia equolifaciens* can produce equol from daidzein, a type of isoflavone found in raspberries and other plant sources (Maruo, Sakamoto, Ito, Toda, & Benno, 2008). In our study, LEfSe and random forest analyses identified *Adlercreutzia* as a key taxon explaining the differential microbiome profiles among our diets. The relative abundance of *Adlercreutzia* was enriched during RR polyphenol feeding (for HFwhole, HFpulp, and HFseed diets) compared to

control diets, but the increase was not statistically significant. The importance of *Adlercreutzia* in the microbiome response to feeding RR polyphenol supplemented diets suggests that either this genus contains previously unidentified species involved in RR polyphenol catabolism or that our extracts may have contained trace amounts of isoflavones.

We observed that supplementing HF diets with RR polyphenols did not significantly affect the abundance of *Akkermansia*, which stands in contrast to reports by others describing dramatic increases in *A. muciniphila* when supplementing HF diets with grape (Roopchand et al., 2015) or cranberry polyphenols (Anhe et al., 2015). In our mice, *Akkermansia* accounted for a very large portion of the gut microbiota, even in the mice fed a HF diet. It is possible that we did not observe a prebiotic effect on *Akkermansia* when feeding RR polyphenols because it was already present in high proportions at baseline. However, the lack of a prebiotic effect does not exclude *Akkermansia* from contributing to RR polyphenol catabolism. Indeed, work by Henning et al. showed that the addition of EA to *in vitro* cultures of *A. muciniphila* did not alter its growth, but changes were detected in ET hydrolysis (Henning et al., 2017), suggesting that *A. muciniphila* may play a role in the breakdown of polyphenols for further catabolism by other members of the microbiota.

Finally, it is important to note that the differential effects on the microbiota we observed after feeding polyphenolic extracts derived from either whole RR or RR pulp or seed may have been influenced by the unique polyphenolic profiles found in each of these fractions. Our HFseed and HFwhole diets contained primarily EA (40.7% and 23.7% of the total GAE, respectively). In contrast, the HFpulp diet contained predominantly anthocyanins (40.7%) and a lesser amount of EA (14.7%) compared to the other diets, which may result in a unique gut

microbiota signature. Indeed, anthocyanins have been widely studied for their prebiotic effects (Faria, Fernandes, Norberto, Mateus, & Calhau, 2014), especially on *Bifidobacterium* (Overall 2017), which were increased in our study when mice were fed a diet supplemented with RR seed polyphenols. Considering this unique response, further study of the distinct polyphenolic compositions present in RR fractions could provide a more mechanistic understanding of how specific food components interact with the gut microbiota to influence host health.

5. Conclusion

In summary, our current study demonstrates that supplementing HF diets with RR polyphenols derived from either whole fruits, pulp, or seed has a prebiotic effect on the gut microbiota. Microbiome features were likely influenced by the varying polyphenol concentrations present in the different RR fractions (i.e., seed versus pulp). Correlation analyses between microbiome profiles and host metabolic parameters suggest that specific gut bacteria may be involved in RR polyphenol catabolism and/or mediate the metabolic benefits observed during RR consumption. These microbial taxa are important for future study as relatively few species of intestinal bacteria capable of contributing to polyphenol catabolism have been identified to date.

6. Ethics statement

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln (Protocol 1469) and carried out in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

CRediT authorship contribution statement

Yibo Xian: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Rong Fan:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Jing Shao:** Investigation. **Ashley Mulcahy Toney:** Investigation, Writing - review & editing. **Soonkyu Chung:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **Amanda E. Ramer-Tait:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

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.

Acknowledgments

This study was supported by a USDA-NIFA grant awarded to SC and ART (2017-67017-26781). YX was supported in part by funding from a University of Nebraska-Lincoln (UNL) Jumpstart Seed Grant to ART and the UNL Quantitative Life Sciences Initiative. We thank Mr. David Giraud for assistance with polyphenol extractions and Ms. Kristin Beede for assistance with graphical abstract preparation. We also acknowledge the equipment and technical support provided by the Nebraska Food for Health Center. Analysis was completed utilizing the Holland Computing Center, which receives support from the Nebraska Research Initiative. The graphical abstract was created in part with BioRender.com.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2020.104288>.

References

- Anhe, F. F., Roy, D., Pilon, G., Dudonne, S., Matamoros, S., Varin, T. V., ... Marette, A. (2015). A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. *Gut*, 64(6), 872–883. <https://doi.org/10.1136/gutjnl-2014-307142>.
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*, 57, 289–300.
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7), 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Calvano, A., Izuora, K., Oh, E. C., Ebersole, J. L., Lyons, T. J., & Basu, A. (2019). Dietary berries, insulin resistance and type 2 diabetes: An overview of human feeding trials. *Food & Function*, 10(10), 6227–6243. <https://doi.org/10.1039/c9fo01426h>.
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., ... Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, 108(Suppl 1), 4516–4522. <https://doi.org/10.1073/pnas.100080107>.
- Clavel, T., Lepage, P., & Charrier, C. (2014). The family coriobacteriaceae. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The prokaryotes: Actinobacteria* (pp. 201–238). Berlin, Heidelberg: Springer, Berlin Heidelberg.
- Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, 45(W1), W180–W188. <https://doi.org/10.1093/nar/gkx295>.
- Duenas, M., Munoz-Gonzalez, I., Cueva, C., Jimenez-Giron, A., Sanchez-Patan, F., Santos-Buelga, C., ... Bartolome, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. *BioMed Research International*, 2015, Article 850902. <https://doi.org/10.1155/2015/850902>.
- Espin, J. C., Larrosa, M., Garcia-Conesa, M. T., & Tomas-Barberan, F. (2013). Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: The evidence so far. *Evidence-Based Complementary and Alternative Medicine*, 2013, Article 270418. <https://doi.org/10.1155/2013/270418>.
- Fan, R., You, M., Toney, A. M., Kim, J., Giraud, D., Xian, Y., ... Chung, S. (2019). Red raspberry polyphenols attenuate high-fat diet-driven activation of NLRP3 inflammasome and its paracrine suppression of adipogenesis via histone modifications. *Molecular Nutrition & Food Research*, e1900995. <https://doi.org/10.1002/mnfr.201900995>.
- Faria, A., Fernandes, I., Norberto, S., Mateus, N., & Calhau, C. (2014). Interplay between anthocyanins and gut microbiota. *Journal of Agriculture and Food Chemistry*, 62(29), 6898–6902. <https://doi.org/10.1021/jf501808a>.
- Garcia-Nino, W. R., & Zazueta, C. (2015). Ellagic acid: Pharmacological activities and molecular mechanisms involved in liver protection. *Pharmacological Research*, 97, 84–103. <https://doi.org/10.1016/j.phrs.2015.04.008>.
- Gaya, P., Peiroten, A., Medina, M., Alvarez, I., & Landete, J. M. (2018). *Bifidobacterium pseudocatenulatum* INIA P815: The first bacterium able to produce urolithins A and B from ellagic acid. *Journal of Functional Foods*, 45, 95–99.
- Gomes-Neto, J. C., Mantz, S., Held, K., Sinha, R., Segura Munoz, R. R., Schmaltz, R., ... Ramer-Tait, A. E. (2017). A real-time PCR assay for accurate quantification of the individual members of the Altered Schaedler Flora microbiota in gnotobiotic mice. *Journal of Microbiological Methods*, 135, 52–62. <https://doi.org/10.1016/j.mimet.2017.02.003>.
- Gurung, M., Li, Z., You, H., Rodrigues, R., Jump, D. B., Morgun, A., & Shulzhenko, N. (2020). Role of gut microbiota in type 2 diabetes pathophysiology. *EBioMedicine*, 51, Article 102590. <https://doi.org/10.1016/j.ebiom.2019.11.051>.
- Haslam, D. W., & James, W. P. (2005). Obesity. *Lancet*, 366(9492), 1197–1209. [https://doi.org/10.1016/S0140-6736\(05\)67483-1](https://doi.org/10.1016/S0140-6736(05)67483-1).
- Henning, S. M., Summanen, P. H., Lee, R. P., Yang, J., Finegold, S. M., Heber, D., & Li, Z. (2017). Pomegranate ellagitannins stimulate the growth of *Akkermansia muciniphila* in vivo. *Anaerobe*, 43, 56–60. <https://doi.org/10.1016/j.anaerobe.2016.12.003>.
- Junick, J., & Blaut, M. (2012). Quantification of human fecal *Bifidobacterium* species by use of quantitative real-time PCR analysis targeting the groEL gene. *Applied and Environment Microbiology*, 78(8), 2613–2622. <https://doi.org/10.1128/AEM.07749-11>.
- Kang, I., Buckner, T., Shay, N. F., Gu, L. W., & Chung, S. (2016). Improvements in metabolic health with consumption of ellagic acid and subsequent conversion into urolithins: Evidence and mechanisms. *Advances in Nutrition*, 7(5), 961–972. <https://doi.org/10.3945/an.116.012575>.
- Kang, I., Kim, Y., Tomas-Barberan, F. A., Espin, J. C., & Chung, S. (2016). Urolithin A, C, and D, but not iso-urolithin A and urolithin B, attenuate triglyceride accumulation in human cultures of adipocytes and hepatocytes. *Molecular Nutrition & Food Research*, 60(5), 1129–1138. <https://doi.org/10.1002/mnfr.201500796>.
- Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environment Microbiology*, 79(17), 5112–5120. <https://doi.org/10.1128/AEM.01043-13>.
- Larrosa, M., Gonzalez-Sarrias, A., Yanez-Gascon, M. J., Selma, M. V., Azorin-Ortuno, M., Toti, S., ... Espin, J. C. (2010). Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *Journal of Nutritional Biochemistry*, 21(8), 717–725. <https://doi.org/10.1016/j.jnutbio.2009.04.012>.

- Li, Z. P., Henning, S. M., Lee, R. P., Lu, Q. Y., Summanen, P. H., Thames, G., ... Heber, D. (2015). Pomegranate extract induces ellagitannin metabolite formation and changes stool microbiota in healthy volunteers. *Food & Function*, 6(8), 2487–2495. <https://doi.org/10.1039/c5fo00669d>.
- Lipinska, L., Klewicka, E., & Sojka, M. (2014). The structure, occurrence and biological activity of ellagitannins: A general review. *Acta Scientiarum Polonorum Technologia Alimentaria*, 13(3), 289–299. <https://doi.org/10.17306/j.afs.2014.3.7>.
- Maier, T. V., Lucio, M., Lee, L. H., VerBerkmoes, N. C., Brislawn, C. J., Bernhardt, J., ... Jansson, J. K. (2017). Impact of dietary resistant starch on the human gut microbiome, metaproteome, and metabolome. *MBio*, 8(5). <https://doi.org/10.1128/mBio.01343-17>.
- Maruo, T., Sakamoto, M., Ito, C., Toda, T., & Benno, Y. (2008). *Adlercreutzia equolifaciens* gen. nov., sp. nov., an equol-producing bacterium isolated from human faeces, and emended description of the genus *Eggerthella*. *International Journal of Systematic and Evolutionary Microbiology*, 58(Pt 5), 1221–1227. <https://doi.org/10.1099/ijs.0.65404-0>.
- Overall, J., Bonney, S. A., Wilson, M., Beermann, A., Grace, M. H., Esposito, D., ... Komarnitsky, S. (2017). Metabolic effects of berries with structurally diverse anthocyanins. *International Journal of Molecular Sciences*, 18(2). <https://doi.org/10.3390/ijms18020422>.
- Piowowski, J. P., Kiss, A. K., Granica, S., & Moeslinger, T. (2015). Urolithins, gut microbiota-derived metabolites of ellagitannins, inhibit LPS-induced inflammation in RAW 264.7 murine macrophages. *Molecular Nutrition & Food Research*, 59(11), 2168–2177. <https://doi.org/10.1002/mnfr.201500264>.
- Puupponen-Pimia, R., Seppanen-Laakso, T., Kankainen, M., Maukonen, J., Torronen, R., Kolehmainen, M., ... Oksman-Caldentey, K. M. (2013). Effects of ellagitannin-rich berries on blood lipids, gut microbiota, and urolithin production in human subjects with symptoms of metabolic syndrome. *Molecular Nutrition & Food Research*, 57(12), 2258–2263. <https://doi.org/10.1002/mnfr.201300280>.
- Roopchand, D. E., Carmody, R. N., Kuhn, P., Moskal, K., Rojas-Silva, P., Turnbaugh, P. J., & Raskin, I. (2015). Dietary polyphenols promote growth of the gut bacterium *Akkermansia muciniphila* and attenuate high-fat diet-induced metabolic syndrome. *Diabetes*, 64(8), 2847–2858. <https://doi.org/10.2337/db14-1916>.
- Ryan, K. K., Tremaroli, V., Clemmensen, C., Kovatcheva-Datchary, P., Myronovych, A., Karns, R., ... Seeley, R. J. (2014). FXR is a molecular target for the effects of vertical sleeve gastrectomy. *Nature*, 509(7499), 183–188. <https://doi.org/10.1038/nature13135>.
- Schneeberger, M., Everard, A., Gomez-Valades, A. G., Matamoros, S., Ramirez, S., Delzenne, N. M., ... Cani, P. D. (2015). *Akkermansia muciniphila* inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. *Scientific Reports*, 5, 16643. <https://doi.org/10.1038/srep16643>.
- Selma, M. V., Tomas-Barberan, F. A., Beltran, D., Garcia-Villalba, R., & Espin, J. C. (2014). *Gordonibacter urolithinfaciens* sp. nov., a urolithin-producing bacterium isolated from the human gut. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 7), 2346–2352. <https://doi.org/10.1099/ijs.0.055095-0>.
- Sojka, M., Macierzynski, J., Zaweracz, W., & Buczek, M. (2016). Transfer and mass balance of ellagitannins, anthocyanins, flavan-3-ols, and flavonols during the processing of red raspberries (*Rubus idaeus* L.) to juice. *Journal of Agriculture and Food Chemistry*, 64(27), 5549–5563. <https://doi.org/10.1021/acs.jafc.6b01590>.
- Team, R. D. C. (Accessed July 2015). R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2012. <http://www.R-project.org/>.
- Thomsen, M. C. F., Hasman, H., Westh, H., Kaya, H., & Lund, O. (2017). RUCS: Rapid identification of PCR primers for unique core sequences. *Bioinformatics*, 33(24), 3917–3921. <https://doi.org/10.1093/bioinformatics/btx526>.
- Tomas-Barberan, F. A., Garcia-Villalba, R., Gonzalez-Sarrias, A., Selma, M. V., & Espin, J. C. (2014). Ellagic acid metabolism by human gut microbiota: Consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. *Journal of Agriculture and Food Chemistry*, 62(28), 6535–6538. <https://doi.org/10.1021/jf5024615>.
- Tomas-Barberan, F. A., Gonzalez-Sarrias, A., Garcia-Villalba, R., Nunez-Sanchez, M. A., Selma, M. V., Garcia-Conesa, M. T., & Espin, J. C. (2017). Urolithins, the rescue of “old” metabolites to understand a “new” concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. *Molecular Nutrition & Food Research*, 61(1). <https://doi.org/10.1002/mnfr.201500901>.
- Toney, A. M., Fan, R., Xian, Y., Chaidez, V., Ramer-Tait, A. E., & Chung, S. (2019). Urolithin A, a gut metabolite, improves insulin sensitivity through augmentation of mitochondrial function and biogenesis. *Obesity (Silver Spring)*, 27(4), 612–620. <https://doi.org/10.1002/oby.22404>.
- Willing, B. P., Dicksved, J., Halfvarson, J., Andersson, A. F., Lucio, M., Zheng, Z., ... Engstrand, L. (2010). A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology*, 139(6), 1844–1854 e1841. <https://doi.org/10.1053/j.gastro.2010.08.049>.
- World Health Organization. Obesity and overweight. Fact sheet. Updated October 2018. <http://www.who.int/mediacentre/factsheets/fs311/en/>.