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Impact of ferulic and vanillic acids on soluble and insoluble dietary fiber utilization from maize bran by the human gut microbiota†

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Ferulic (FA) and vanillic (VA) acids are phenolic compounds with antioxidant activity and health benefits. Our previous research indicated that the utilization of maize dietary fiber by the human gut microbiota might be negatively impacted by phenolic compounds. This study investigated the effects of FA and VA at different concentrations (0, 0.3, 3, 30 mg g^{-1}) on soluble and insoluble maize bran fibers during *in vitro* fecal fermentation. High VA (30 mg g^{-1}) reduced insoluble fiber utilization (p = 0.016), increased branched-chain fatty acid production (p = 0.024), and was associated with increased *Veillonellaceae* and *Bacteroidaceae* abundances. Low FA (0.3 mg g^{-1}) improved soluble fiber utilization (p = 0.017) and enhanced propionate production (p = 0.013). High FA (30 mg g^{-1}) elevated propionate (p = 0.015) and butyrate (p = 0.004) production. FA and VA reduced *Streptococcaceae* and *Peptostreptococcaceae* abundances. These findings highlight the complex interplay between phenolic compounds and dietary fiber utilization with implications for dietary strategies promoting gut health.

1. Introduction

Phenolic compounds (PC) are pivotal bioactive components contributing to bitterness, astringency, and pigmentation of plants. They play a key physiological role in plant defense, offering biological protection against ultraviolet radiation, insect herbivory, and pathogenic microorganisms.[1] Consumption of PC have been shown to confer numerous health benefits, potentially mitigating diseases such as cancer, cardiovascular disease, rheumatoid arthritis, depression, and diabetes.[2–4]

PC possess antimicrobial properties and can modulate the microbial population of the gastrointestinal (GI) tract, which impacts both microbiota composition and function.[5] Recent review papers conclude that PC generally promote the growth of beneficial bacteria, thereby helping to maintain human health.[6,7] Thus, some PC have been considered "prebiotics".[8]

Dietary fibers also have strong influences on gut microbiota composition and function. Fermentation of dietary fibers by the microbiome supports the growth of beneficial bacteria while suppressing growth of undesirable microbes that thrive when carbohydrates are limiting.[9,10] Fiber fermentation results in the production of short chain fatty acids, which have pleiotropic local effects on intestinal function as well as systemic roles in insulin secretion, lipid metabolism, and inflammation, among others.[11–13] Thus, dietary fiber fermentation is considered critical for bringing about many of the health benefits dietary fibers.[14]

While both of these compounds—PC and dietary fibers—have demonstrated beneficial effects on the gut microbiota and human health, it is important to consider how these bioactive compounds interact. For example, a recent study found that ferulic acid (FA) in combination with arabinoxylan, the principal PC and dietary fibers in most cereal grains, increased the abundance of *Bifidobacterium* and *Faecalibaculum*, which was accompanied by reduced body weight gain and enhanced glucose tolerance in high-fat diet-fed mice.[15] In contrast, when mice were fed arabinoxylan oligosaccharides in combination with green tea polyphenolics, the bifidogenic effect normally characteristic of arabinoxylan oligosaccharides was abolished.[16]

The PC in maize are primarily hydroxycinnamic and benzoic acid derivatives.[17] As mentioned, FA is the most predominant hydroxycinnamic acid.[17,18] Most of the FA in maize (>90%) is bound to cell wall polysaccharides, where it forms cross-links between arabinoxylan polymers to strengthen the cell wall.[19] In ruminant animals, bound FA cross-links inhibit cell wall polysaccharide utilization by the microbiota.[20,21]

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Thus, supplementation of feed with bacteria that produce feruloyl esterases, which remove FA from its bound state, helps improve animal health and product quality.[22] The effect of bound FA cross-links on cell wall polysaccharide utilization by the human gut microbiota has not been as straightforward. One study found that bound FA cross-links impeded carbohydrate utilization,[23] while another study found that they did not.[24]

Regardless, the gut microbiota contains bacteria that produce feruloyl esterases that can release bound ferulate from its matrix.[25,26] This free FA has been shown to reduce carbohydrate utilization in the rumen microbiota[27–29] by reducing the ability of bacteria (*Bacteroides* and *Ruminococcus* species) to attach to insoluble cell wall particles.[30,31] Free FA also possesses antimicrobial properties, particularly against Gram-positive bacteria, that could contribute to reduced carbohydrate utilization.[30,32,33] To reduce toxicity, rumen microbes transform FA through demethylation, dehydroxylation, and hydrogenation reactions (as do human gut microbes).[30,34–36]

While free FA reduces carbohydrate utilization by the rumen microbiota, supplementing feed with FA has been shown to enhance feedlot performance of lambs.[37] Free FA in feed is rapidly absorbed within the rumen and contributes to relieving inflammation and oxidative stress in the animal.[21,37] This is likely the mechanism of improvement in animal quality with supplementation with free FA. Conversely, free FA released from the cell wall matrix during fermentation either may not reach concentrations high enough to inhibit carbohydrate utilization[38] or it may be rapidly detoxified through the biotransformation reactions mentioned previously.[36] Thus, the effects of free FA on carbohydrate utilization in ruminant animals are dependent on whether it is free or bound and its concentration. These effects have not been reported in human gut microbiota.

Comparatively less is known about vanillic acid (VA), the most predominant hydroxybenzoic acid in maize.[18] Like FA, most (>85%) of the VA in maize is bound.[17] However, unlike FA, bound VA was not significantly correlated with cell wall digestibility of maize stalks.[29] Additionally, free VA does not exhibit antimicrobial activity against cellulosic bacteria like FA does,[30,39] although it is metabolized by demethylation and dihydroxylation by the microbiota.[40] Additionally, free VA is also less toxic to Gram-positive bacteria due to its lower hydrophobicity than FA.[33]

Despite recent research focusing on the health benefits of PC, studies showing a negative influence of PC, particularly FA, on carbohydrate utilization by the rumen microbiota[27–29] suggest there may be an interaction between PC and carbohydrate utilization by the human gut microbiota. Given the structural differences between FA and VA, we hypothesized that FA would inhibit carbohydrate utilization by the gut microbiota due to its higher hydrophobicity and antimicrobial activity, which could disrupt bacterial adhesion and fiber degradation. In contrast, VA, with its lower hydrophobicity and reduced antimicrobial properties, was expected to have minimal impact on carbohydrate utilization.

A prior study by our team compared the *in vitro* fermentation of alkaline-treated maize bran with and without ethanol washing to remove free PC. The results showed that ethanol washing, which primarily removed free PC, enhanced soluble carbohydrate utilization.[41] However, there remains a knowledge gap regarding the influence of PC on carbohydrate utilization and subsequent shifts in the gut microbiota. Therefore, the aim of this research was to investigate how varying PC impact the utilization of soluble and insoluble dietary fiber extracted from maize and to elucidate the role of PC in SCFA production during *in vitro* fermentation by the human microbiome.

2. Materials and methods

2.1. Materials

Maize bran from the dry milling process was obtained from Cargill (High fiber fine bran #88011-00, Cargill, Wayzata, MN, USA). The nutritional information provided by the company is shown in Table S1.† Two PC were used in this study: FA (Thermo, US), and VA (Thermo, US). They were used in this study because they are two of the predominant PC in corn, with free concentrations in yellow corn approximately 0.39 mg g^{-1} and 0.28 mg g^{-1}, respectively, and belonging to two different structure groups: cinnamic and benzoic acids, respectively.[17]

2.2. Dietary fiber extraction

Insoluble and soluble fiber were extracted from maize bran according to a previous study,[42] with modifications. For insoluble fiber extraction, 100 g of maize bran was suspended in 5 mM $CaCl_2$ (1 : 9 w/w, 900 mL), and the pH was adjusted to 7.0 with 1 M NaOH. The mixture was then boiled under constant stirring, after which 4 mL of heat stable α-amylase (150 U, A-3403, Sigma-Aldrich Corp., St Louis, MO) was added and the mixture was incubated for 30 min in a boiling water bath. Upon cooling the mixture to 50 °C, the pH was adjusted to 6.0 with 1 M HCl. Subsequently, 5 mL of neutral protease (4 U, P-1236, Sigma-Aldrich) and 5 mL amyloglucosidase (3260 U, E-AMG, Megazyme) were added, and the mixture was incubated at 50 °C for 4 h. The mixture was then boiled to inactivate the enzymes and cooled in an ice bath. The slurry was centrifuged at 1000*g* for 10 min. The residue was resuspended in excess water, centrifuged at 1000*g* for 5 min, and the wash water was discarded five times. Then the pellet was washed with 100 mL 80% ethanol three times to remove all the free PC, after which the pellet was dried in a forced draft oven at 40 °C for 48 h.

For soluble fiber preparation, 50 g of insoluble fiber were suspended in 500 mL of 1 M NaOH. The mixture was heated to 60 °C, and 42 mL of 30% hydrogen peroxide was gradually added over the course of 4 h while stirring continuously. Additional NaOH was added as needed to maintain the pH between 11 and 12. After 4 h, the pH was adjusted to 7 with 6 M HCl. The supernatant was collected after centrifugation at

1000*g* for 10 min. Three volumes of absolute ethanol were then added to the supernatant, and the mixture was allowed to stand overnight at room temperature. The aqueous ethanol layer was decanted, and the residue was washed five times with 100 mL 80% ethanol to remove all free PC, twice with 100 mL absolute ethanol, and twice with 100 mL acetone. The solvent was allowed to evaporate under atmospheric conditions until no solvent odor was detectable.

2.3. *In vitro* fermentation

Fresh fecal samples from four healthy adults with no history of gastrointestinal abnormalities and no prebiotic, probiotic, or antibiotic consumption within the past 6 months were collected. Fecal samples were collected in commode specimen collection containers (02-544-208, Fisher Scientific, Hampton, NH, USA) and transported in insulated shippers on ice. The fecal slurries were prepared within 2 h of defecation in an anaerobic chamber (Bactron X, Sheldon Manufacturing, Cornelius, OR, USA) containing 5% H_2, 5% CO_2, and 90% N_2. Each fresh fecal sample was mixed by stomacher (BagMixer 400, Saint Nom la Bretèche, France) separately with sterile phosphate-buffered saline, pH 7.0 (1 : 9 wt/vol), containing 10% glycerol as a cryoprotectant for 4 min inside a sterile filter bag (Filtra-Bag, Thomas Scientific, New Jersey, USA). The individual slurries were then pooled together with equal ratio and aliquoted in 15 mL polypropylene centrifuge tubes and frozen at −80 °C until fermentation was performed. All procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska–Lincoln before initiating the study (approval number 20210621206EP). All subjects provided written informed consent before participating in any study protocols.

In vitro batch fecal fermentation was performed as described previously,[43] with some modifications. Briefly, inside the anaerobic hood, 15 mg of the insoluble or soluble fiber was weighed in a 2 mL tube, suspended in 1 mL of sterile fermentation media. The media contained (per L): peptone (2 g; Fisher Scientific, Waltham, MA), yeast extract (2 g; Fisher Scientific, Waltham, MA), bile salts (0.5 g; Oxoid, Cheshire, England), $NaHCO_3$ (2 g), NaCl (0.1 g), K_2HPO_4 (0.04 g), $MgSO_4{\cdot}7H_2O$ (0.01 g), $CaCl_2{\cdot}2H_2O$ (0.01 g), L-cysteine hydrochloride (0.5 g; Fisher Scientific, Waltham, MA), hemin (5 mg dissolved in dimethyl sulfoxide [DMSO]), Tween 80 (2 mL, Fisher Scientific, Waltham, MA), vitamin K (0.5 mL, 0.5 g dissolved in 49.5 mL of ethanol; Alfa Aesar, Haverhill, MA), and 0.025% (w/v) resazurin solution (4 mL, dissolved in water; Alfa Aesar, Haverhill, MA). The fermentation media also contained either FA or VA at four concentrations: 0, 3, 30, and 300 mg L^{-1}. These concentrations were designed to achieve final ratios of 0, 0.3, 3, and 30 mg of phenolic compound per gram of fiber. The concentrations of 0.3 mg g^{-1} and 3 mg g^{-1} represent the range of free FA and VA in maize reported in the literature.[17,44] The high concentration, 30 mg g^{-1}, reflected the concentrations of free PC we found in our recent study where we isolated fibers using alkali extraction.[41] The FA or VA were added to the media *via* filter sterilization (0.2 μm) after dissolution in 0.5 mL of 80% ethanol. After sterilization, media was kept inside the anaerobic chamber until the color of the resazurin disappeared (~24 h) to ensure anaerobicity. Tubes containing fiber and fermentation media were then inoculated with 0.1 mL of fecal slurry, capped, and incubated at 37 °C with orbital shaking (140 rpm) under anaerobic conditions. The blank samples were prepared by mixing 0.1 mL of fecal slurry with 1 mL fermentation media and incubated at the same condition. Samples were collected at 0 and 24 h of fermentation and immediately frozen at −80 °C for further analysis. Separate 2 mL tubes (each in triplicate) were prepared for (1) carbohydrate (CHO) quantification and (2) microbiota composition and SCFA analysis.

2.4. Carbohydrate utilization

Samples were hydrolyzed following the method previously described,[41] with some modifications. Samples containing insoluble fiber were transferred to a 15 mL tube, three times of absolute ethanol was added, and the mixture was incubated at 80 °C for 5 min. After centrifugation at 10 000*g* for 10 min, the subsequent pellets were dried by resuspension and centrifugation, first using twice 1 mL of 80% aqueous ethanol, followed by 1 mL of absolute ethanol, and 1 mL of acetone. After discarding the supernatants, the solvent was allowed to evaporate under atmospheric conditions until no solvent odor remained. Afterwards, 0.2 mL of 12 M sulfuric acid was added to the pellet, and the mixture was incubated in 30 °C for 1 h. After that, 5.8 mL of water containing 2 mg myo-inositol were added to achieve a final concentration of 0.4 M sulfuric acid. For soluble fiber samples after fermentation, 4.8 mL of water containing 2 mg myo-inositol and 0.2 mL 12 M sulfuric acid was added to achieve a final sulfuric acid concentration of 0.4 M. All the tubes were loosely capped and autoclaved at 121 °C for 1 h.

Neutral sugars and uronic acids in the hydrolyzed samples were quantified using HPLC (Model 1260, Agilent, USA) equipped with different columns. Neutral sugars (glucose, galactose, xylose, arabinose, and mannose) were separated by an Aminex HPX-87P column (Biorad, Hercules, CA,USA) preceded by de-ashing and Carbo-P guard columns (1250118 and 1250119, Biorad); uronic acids (glucuronic acid and galacturonic acid) were separated by an Aminex HPX-87H column (Biorad) with a Cation H guard column (1250129, Biorad). The HPLC conditions were the same as previously described.[41] Results were calculated by dividing the peak area of each monosaccharide by that of the internal standard and compared to authentic standards (0–1 mg mL^{-1}). Carbohydrate utilization was calculated as the difference in carbohydrate composition between 0 and 24 of fermentation divided by total carbohydrate content at 0 h.

2.5. Short chain fatty acids

SCFA were extracted and measured by gas chromatography as described previously.[45] In brief, 0.4 mL of fermented sample supernatant, 0.1 mL of 7 mM 2-ethylbutyric acid in 2 M potass-

ium hydroxide, 0.2 mL of 9 M sulfuric acid, and 0.16 g of sodium chloride were mixed. Then, 0.5 mL of diethyl ether was added and vortex mixed. The top layer was injected into a gas chromatograph (Clarus 580; PerkinElmer, MA, USA) equipped with a capillary column (Nukol; 30 m by 0.25 mm [inner diameter] by 0.25 μm [film thickness]; Supelco, Bellefonte, PA) and flame ionization detector (FID). SCFA were quantified by calculating response factors for each SCFA relative to 2-ethylbutyric acid using injections of pure standards and expressed as the difference between the SCFA content at 24 h minus SCFA content at 0 h.

2.6. Microbiota composition

Pellets containing bacterial DNA were recovered from the fermented samples after mechanical and enzymatic bacterial cell lysis using the BioSprint 96 One-for-all Vet Kit (Qiagen, Germantown, MD). TheV4 region of the 16S rRNA gene was amplified using the Ilumina MiSeq (Illumina, San Diego, CA) platform and the MiSeq Reagent kit v2 (2 × 250 bp).[46] Sequences were demultiplexed and barcodes were removed prior to sequence analysis with QIIME 2.[47] Sequence quality control, trimming, chimera removal, and denoising were performed with DADA2.[48] Forward and reverse reads were truncated to 220 and 160 bp, respectively, to maintain sequence qualities above a phred score of 30. Using DADA2, sequences were dereplicated into 100% amplicon sequence variants (ASVs) for exact sequence matching. The taxonomy was assigned using the SILVA database.[49] Samples were rarefied to a sequencing depth of 16 364 reads per sample. Rarefying and diversity calculations (Bray–Curtis dissimilarity, and Shannon index) were performed using the phyloseq and vegan packages in R (version 4.1.3).[50–52]

2.7. Functional composition of the microbiota

Functional composition of the gut microbiota was calculated from 16S rRNA gene sequencing data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2).[53] Pathway data were categorized using classifications designated in the MetaCyc database (**https://metacyc.org/**).[54] Pathways involved in 'degradation/utilization/assimilation of nutrients' or 'generation of metabolites and energy' were analyzed.

2.8. Statistical analysis

All data were analyzed using R (version 4.1.3) and RStudio (2022.02.3 Build 492).[52] Carbohydrate utilization, SCFA production, and α-diversity data were checked for normality using the Shapiro–Wilk test. Some of the SCFA were found to be non-normally distributed ($p < 0.05$ in the Shapiro–Wilk test). Therefore, data for all SCFA were log2-transformed. After the transformation, data for all SCFA were normally distributed. These variables were analyzed by fiber type and phenolic compound type using a one-way ANOVA where phenolic compound concentration was treated as a discrete variable. Significant differences among phenolic compound concentrations were calculated using Tukey's Honestly Significant Difference test. The difference between fibers were analyzed by one-way ANOVA with fiber type as the factor. Differences in the β-diversity with Bray–Curtis distances across metadata variables (PC concentration, PC type, fiber type) were compared using PERMANOVA with the adonis2 function in vegan.[51] Statistical significance was determined as $p < 0.05$. For microbiota composition, DESeq2 was employed to identify taxa and predicted pathways with significant differences in abundance across PC concentrations and PC types after 24 hours of fermentation.[55] Statistical significance was adjusted using the false discovery rate method. Significant taxa were plotted using complexHeatmap.[56]

3. Results

3.1. Impact of phenolic compounds on carbohydrate utilization

FA and VA had different effects on carbohydrate utilization across different fiber types (Fig. 1). VA decreased insoluble dietary fiber utilization at the two higher concentrations (3 and 30 mg g^{-1} fiber), while FA had no effect. In fact, VA seemed to have a negative linear association with insoluble dietary fiber utilization (slope = −0.14426, $p = 0.0086$). Conversely, VA did not have any significant impact on soluble fiber fermentation while FA increased soluble fiber fermentation at the lowest concentration (0.3 mg g^{-1}). Although the lowest concentration of FA treatment increased soluble fiber utilization, higher concentrations did not further increase carbohydrate utilization, but it returned to the same level as the control with no added FA (0 mg g^{-1}).

3.2. Impact of phenolic compounds on short chain fatty acid production

SCFA production from insoluble fiber and soluble fiber were significantly different during *in vitro* fermentation, where soluble fiber resulted in higher acetate ($p < 0.001$), propionate ($p < 0.001$), and butyrate ($p < 0.001$) production and less branch chain fatty acid (BCFA; $p < 0.001$) production than insoluble fiber. FA and VA had similar effects on SCFA production but different significance (Fig. 2; Table S2†). In fermentation with soluble fiber, normal, physiological concentrations (0.3 mg g^{-1}) of FA and VA as well as the highest FA and VA levels (30 mg g^{-1}) significantly enhanced butyrate and total SCFA production relative to none (0 mg g^{-1}). This was also true for propionate in all treatment combinations except vanillic acid at 30 mg g^{-1}. BCFA, and especially iso-valerate, were significantly decreased at 3 mg FA or VA per gram of fiber. With insoluble fiber, iso-valerate and total BCFA increased at the highest level of VA (30 mg g^{-1}) compared to the control (0 mg g^{-1}).

3.3. Impact of phenolic compounds on microbiota composition

The α-diversity of gut bacteria, determined by Shannon's index, was significantly higher when fermented with soluble

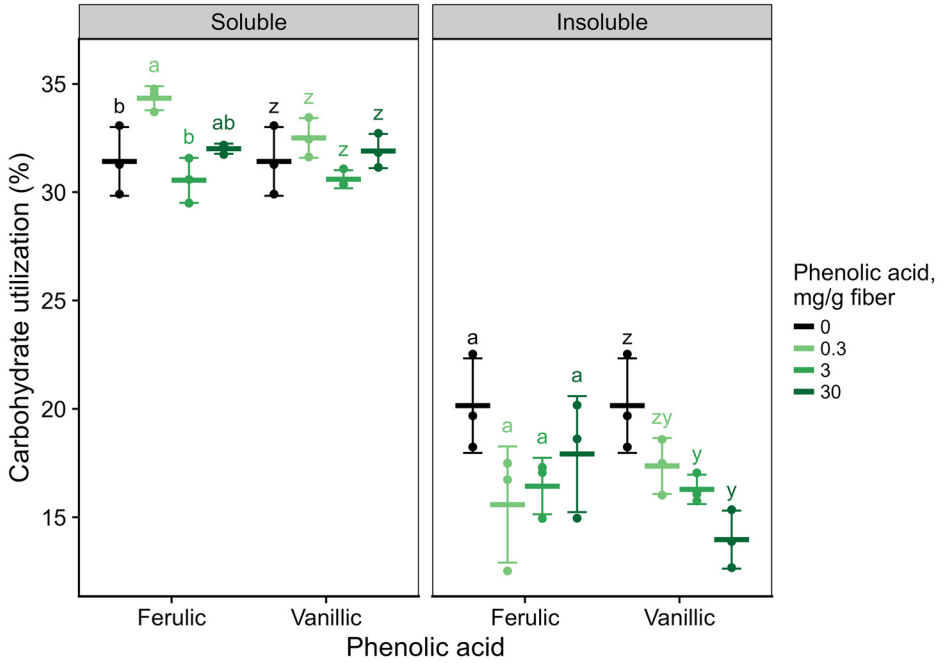

Fig. 1 Impact of phenolic compounds on carbohydrate utilization during 24 h of *in vitro* fermentation of soluble and insoluble fibers. Crossbars show the mean and error bars show standard deviation (N = 3). Significant differences among phenolic acid concentrations within phenolic compound type are indicated by different letters (Tukey's Honestly Significant Difference, $p < 0.05$).

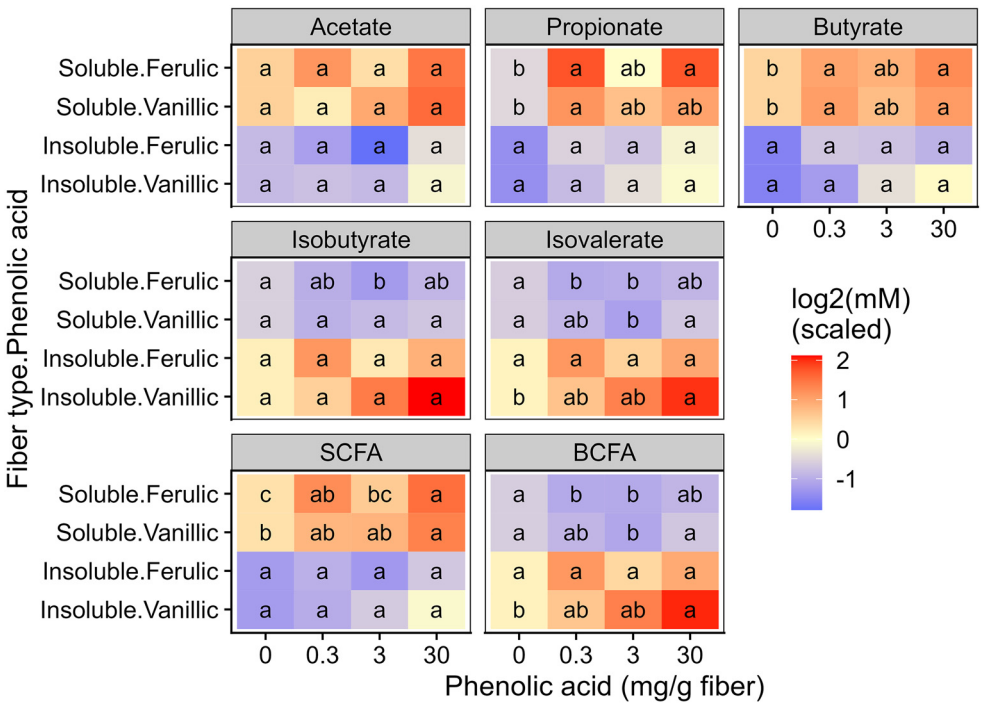

Fig. 2 Impact of ferulic acid and vanillic acid on short and branched chain fatty acid (S/BCFA) production during 24 h of *in vitro* fermentation of soluble and insoluble fibers. Colors represent the average (N = 3) $\log_2$ concentrations (mM) after Z scaling. Significant differences among phenolic acid concentrations within row are indicated by different letters (Tukey's Honestly Significant Difference, $p < 0.05$).

fiber compared with insoluble fiber (Fig. 3A). However, within fiber type, only VA at 30 mg g^{-1} was significantly higher than 0 mg g^{-1} during soluble fiber fermentation.

A principal coordinates biplot of the Bray–Curtis distances among samples showed strong clustering by fiber type (R^2 = 0.28, p = 0.001) (Fig. 3B). Relative to fiber type, PC type and PC

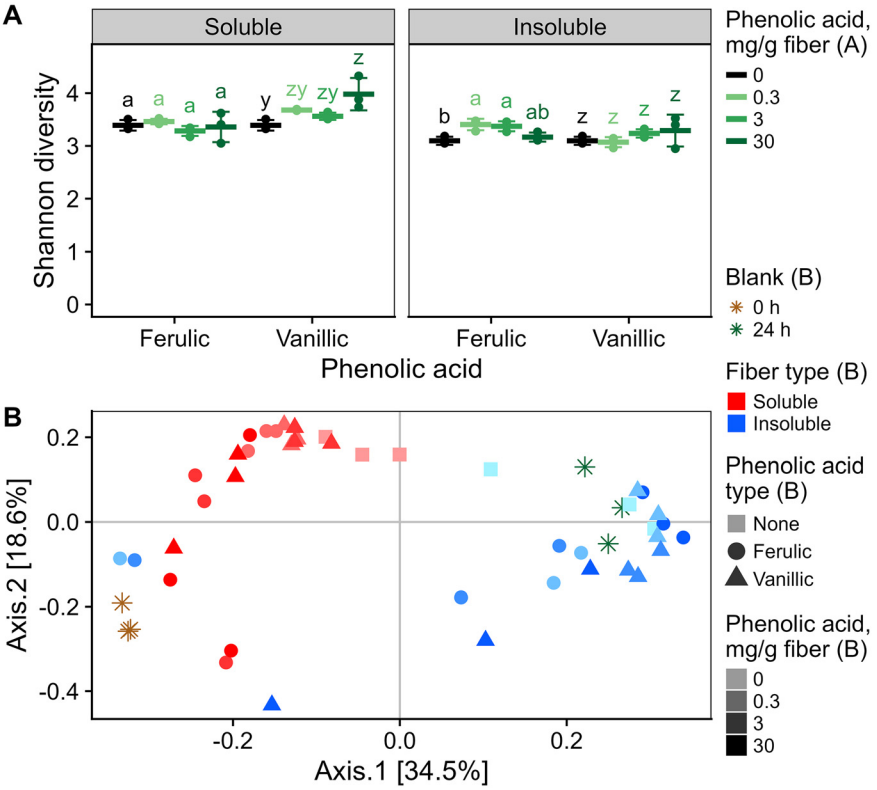

Fig. 3 Diversity of the microbiota during 24 h *in vitro* fermentation. (A) α-Diversity in terms of Shannon's index; (B) β-diversity expressed as principal coordinates analysis biplots of the Bray–Curtis dissimilarity matrix. In panel A, crossbars show the mean and error bars show standard deviation (N = 3). Significant differences among phenolic acid concentrations within phenolic compound type are indicated by different letters (Tukey's Honestly Significant Difference, $p < 0.05$).

concentration had a much smaller impact on global microbiota composition (PC type: R^2 = 0.05, p = 0.063; PC concentration: R^2 = 0.03, p = 0.044). Soluble fiber had a greater impact on microbiota composition compared to insoluble fiber, as the latter shifted microbiota composition in a similar direction to the blank sample, which did not include any fiber or PC.

DESeq2 was used to identify differences in taxon abundances across various PC treatments during the fermentation of both insoluble and soluble fibers. All taxonomic ranks were analyzed and taxa that showed significant differences are reported in Table S3.† Here, family-level differences are shown as they were the most informative (Fig. 4). The fermentation of soluble fiber revealed significant variations across a broad range of taxa. Compared to FA treatment, VA positively affected the abundance of *Christensenellaceae*, *Eggerthellaceae*, *Bifidobacteriaceae*, *Barnesiellaceae*, *Erysipelotrichia*, and *Rikenellaceae*. Additionally, VA at the highest concentration promoted *Marinifilaceae* and *Ruminococcaceae* more than other treatments. Meanwhile, FA treatments increased the abundance of *Tannerellaceae* and *Bacteroidaceae*. Interestingly, *Streptococcaceae* and *Peptostreptococcaceae* significantly decreased across all PC treatments compared with no PC treatment (0 mg g^{-1}).

Among insoluble fiber samples, relatively fewer significant differences were identified across the microbiota. Specifically, the highest concentration of VA (30 mg g^{-1}) increased the abundance of *Veillonellaceae*, *Streptococcaceae*, and *Bacteroidaceae* compared to other treatments.

3.4. Impact of phenolic compounds on functional prediction of the microbiota

PICRUSt2 was used to predict the functional composition of the microbiota after fermentation. The fermentation of soluble or insoluble fiber showed significant differences among treatments across 48 or 34 metabolic pathways related to degradation/utilization/assimilation of nutrient or generation of metabolites and energy, respectively (Fig. 5). For soluble fiber, FA at the higher concentrations (3 and 30 mg g^{-1} fiber) were the most distinct among treatments, showing increases in pathways involved in sugar degradation and SCFA production, among others, relative to the other samples. Although these differences were predicted from the sequencing data, the actual chemical measurements of carbohydrate utilization (Fig. 1) and SCFA production (Fig. 2) did not necessarily reflect this.

In contrast, there were some notable predicted functional differences among the insoluble fiber samples (Fig. 5; ESI Table S4†). In particular, there were several pathways related to aromatic, phenolic, and nitrogen compound degradation that showed significant variation among treatments for insoluble

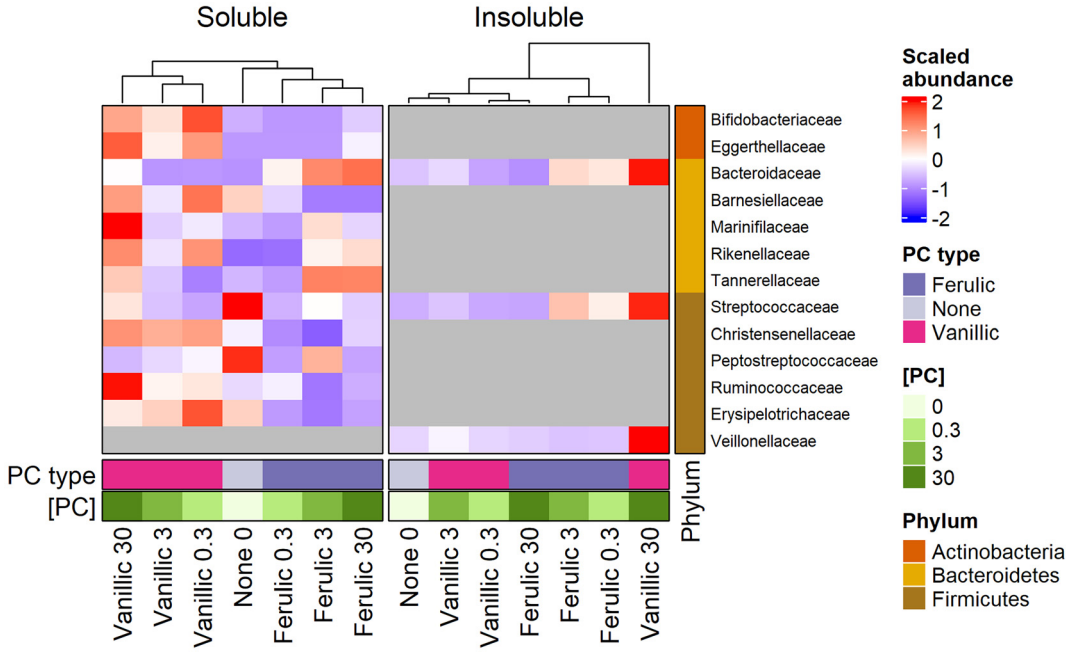

Fig. 4 Bacterial families significantly affected by concentration and type of phenolic compound during 24 h of *in vitro* fermentation of soluble fiber and insoluble fiber. Rows with colors have significant variation among treatments (DESeq2, $p < 0.05$ after Benjamini–Hochberg adjustment), while rows in gray show no significant differences.

and not soluble samples. The pathways involved in phenolic compound degradation were enriched under the highest FA treatment (30 mg g^{-1} fiber) as well as lower concentrations of VA (0.3 and 3 mg g^{-1} fiber). In contrast, the VA at the highest concentration (30 mg g^{-1} fiber) had high aromatic compound degradation and nitrogen compound degradation. The VA at the highest concentration (30 mg g^{-1} fiber) also, along with FA at the lower concentrations (0.3 and 3 mg g^{-1} fiber) and the control, appeared to have diminished phenolic compound degradation.

4. Discussion

The present study aimed to elucidate the impacts of FA and VA on carbohydrate utilization, SCFA production, and gut microbiome composition during the fermentation of insoluble and soluble fibers. Our findings provide valuable insights into the differential responses elicited by these PCs across various fiber types and concentrations.

Our results demonstrated that FA and VA influenced carbohydrate utilization distinctly between insoluble and soluble fibers. For insoluble fibers, VA, but not FA, had a strong inhibitory effect on the microbial degradation of insoluble carbohydrates, which is contrary to previous studies using rumen microbiotas,[27–29] and was contrary to our hypothesis. The differences between rumen and human gut microbiota with respect to the influence of FA and VA on carbohydrate utilization may be due to differences in composition, pH, or other factors. In a recent study comparing the effects of FA and VA on the gut microbiota in weaned piglets, which have a digestive system closer to humans than ruminants, VA was found to have a stronger influence on the gut microbiota than FA, although both acids resulted in reduced inflammation and oxidative stress.[57]

The observed decrease in carbohydrate utilization was accompanied by a shift towards increased BCFA production at higher VA levels. This was associated with increases in *Bacteroidaceae* and *Veillonellaceae*, two bacterial families known to utilize protein metabolic pathways, leading to the production of BCFA.[58–60] Predicted pathway functionality confirmed increased nitrogen compound metabolism at higher VA levels. This suggests a metabolic rerouting from carbohydrate to protein fermentation since BCFA are formed exclusively upon fermentation of branched-chain amino acids.[61–63] Although the fiber samples underwent *in vitro* digestion, the medium contained peptone that could have supplied protein (peptides) for the production of BCFA. Alternatively, the protein could have arisen from dead cells that either could not be cultured in the *in vitro* environment or were susceptible to the antimicrobial effects of VA. Regardless, the shift away from carbohydrate fermentation toward protein fermentation is significant because it indicates that excessive VA could lead to reduced insoluble carbohydrate fermentation, potentially disrupting the gut ability to produce key SCFAs that support colonic health.[64] Interestingly, in the previously referenced piglet study, VA was found to reduce the relative abundance of *Prevotellaceae*.[21] The pooled microbiota that was used in this study was not high in *Prevotellaceae*, but instead had *Bacteroideaceae*, which decreased in the presence of VA. The

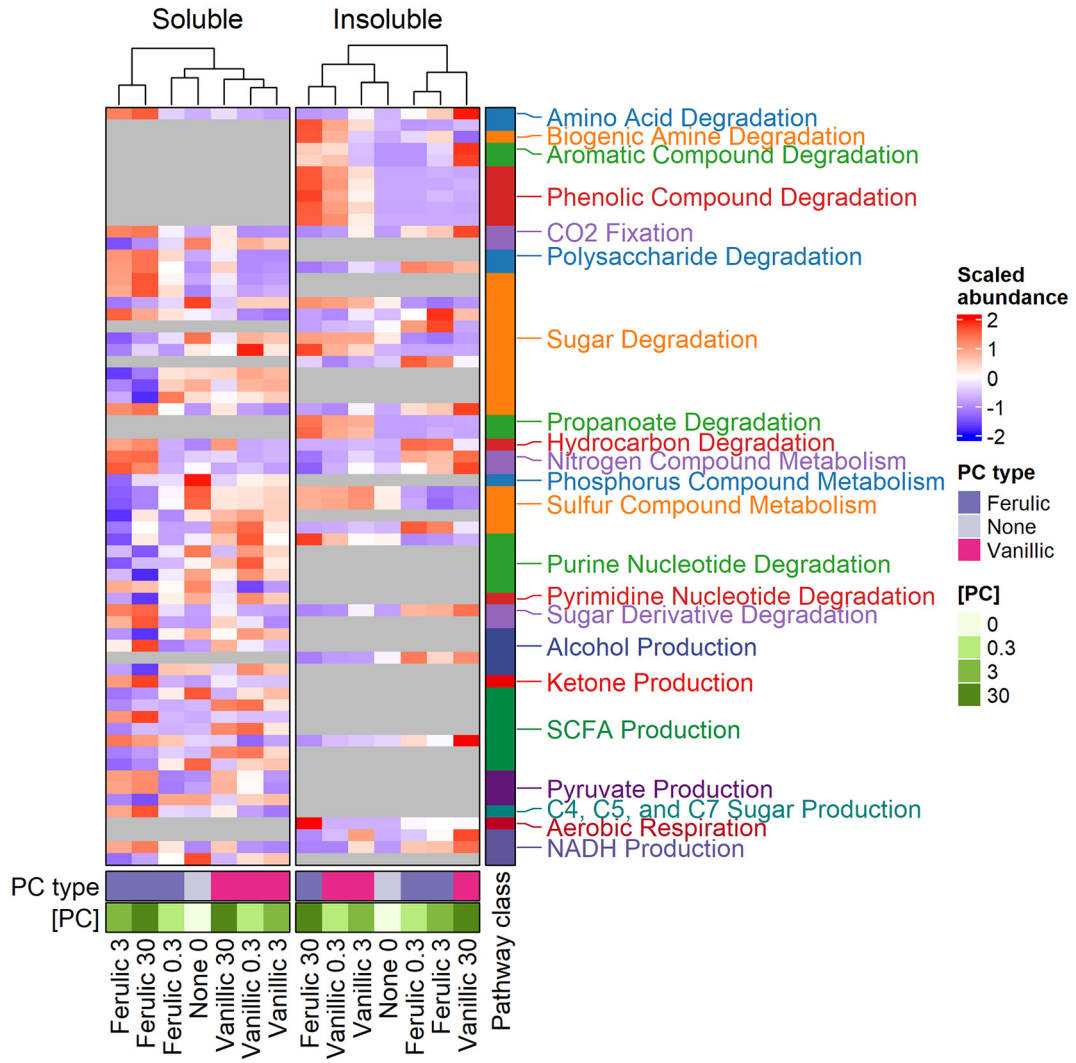

Fig. 5 Predicted functional differences in the microbiota that were significantly affected by concentration and type of phenolic compound during 24 h of *in vitro* fermentation of soluble fiber and insoluble fiber. Rows with colors have significant variation among treatments (DESeq2, $p < 0.05$ after Benjamini–Hochberg adjustment), while rows in gray show no significant differences.

major genera in these families, *Prevotella* and *Bacteroides*, respectively, form the basis for separating microbiotas into two "enterotypes", since these two genera tend to exclude each other.[65] The *Prevotella* enterotype has been associated with fiber-rich diets, while the *Bacteroides* enterotype was associated with low-fiber, Western diets.[66]

The increase in *Bacteroidaceae* and *Veillonellaceae* suggests a possible antimicrobial effect. These bacterial families are Gram negative, and FA and VA have been found to be more toxic to Gram positive bacteria than Gram negative.[33] Although the antimicrobial effect of FA was reported to be greater against Gram positive bacteria than VA in one study,[33] the opposite was reported in an earlier study.[67]

In contrast to VA, FA did not show negative effects on carbohydrate utilization. According to the predicted pathway analysis, the high FA concentration enriched for microbial pathways involved in PC degradation more so than VA. Structurally, FA is more complex than VA due to its propenoic acid side chain, which requires specific enzymatic activation and imposes greater antimicrobial pressure.[33] Thus, the microbiota may have efficiently integrated FA degradation into their metabolism, leading to detoxification and metabolism of FA and effectively maintaining carbohydrate utilization. By comparison, high concentrations of VA were not effectively metabolized, or the microbiota may have attempted to process VA through alternative or less efficient pathways, ultimately leading to reduced carbohydrate utilization.

In contrast to insoluble fiber, soluble fiber demonstrated a more variable response, particular to FA, reflecting a non-linear response that may be influenced by specific microbial community shifts or metabolic pathways. In our previous study, we observed that soluble carbohydrate utilization increased when PC were removed from alkali-treated maize bran.[41] The concentration we used in this study is based on

the content of free FA and VA in yellow corn, which is around 0.3 mg g^{-1}.[17] The free PC concentration in our previous study was 17.5 mg g^{-1}, which falls between the two concentrations we selected in this study: 3 and 30 mg g^{-1}. This suggests that PC at levels around 17.5 mg g^{-1} may have a negative effect on soluble carbohydrate utilization and there may be an optimal PC concentration for maximizing its beneficial effects.

Previous studies have found that insoluble fiber and soluble fiber have different impact on gut microbiome, as also demonstrated in our results.[68,69] Soluble fiber demonstrated better accessibility and a greater effect on shifting gut microbiome composition, which is consistent with findings from previous studies.[70,71] For example, when soluble fiber fermented with PC, FA favored the growth of *Bacteroidaceae* and *Tannerellaceae* families, both of which contain carbohydrate utilizers and SCFA producers.[72–74] Similarly, VA encouraged the growth of *Rumimococcaceae*, *Bifidobacteriaceae*, and *Rikenellaceae*, all contributing to increased SCFA production and improving overall gut health.[73,75–77] These findings align with previous studies that have shown phenolic compounds can promote the growth of *Bacteroidaceae* and Rikenellaceae.[78] *Barnesiellaceae* have shown varied responses to different phenolic compounds in this study, consistent with findings from previous research. For example, they are inhibited by purple tea leaf extract, which contains high anthocyanin content,[79] but carotenoids have been found to increase their growth.[80] On the contrary, the observed decreased in families such as *Peptostreptococcaceae* and *Streptococcaceae* is particularly noteworthy given their association with various diseases, including cancer, chronic radiation proctitis, and other diseases.[81–84] The reduction in their abundance with the addition of PC suggests a potential protective effect of FA and VA against these pathogenic bacteria.

In grains, the majority of PC in insoluble fiber are bound.[17] However, soluble PC has better bioaccessibility and bioavailability, and were the major contributor to the antioxidant activity,[85] are more readily accessible to bacteria than bound PC.[7,32] While our study focused on the effects of soluble PC, it is important to note that both our insoluble and, to some extent, our soluble fiber preparations contained naturally occurring bound phenolics. These bound phenolics, although less bioavailable, may have contributed to the overall microbial accessibility observed in our results.

5. Conclusion

The findings of this study underscore the intricate interactions between phenolic compounds, fiber types, and gut microbiota. The differential effects on carbohydrate utilization and SCFA production highlight the importance of considering both fiber type and PC concentration when evaluating dietary interventions aimed at modulating gut health. The observed variations in microbiome composition further suggest that PCs can selectively enhance or suppress specific microbial taxa, potentially offering a means to tailor gut microbiota towards beneficial configurations. Future research should explore the mechanistic pathways underlying these interactions, including the dose-dependent effects and potential synergistic actions of different PC could pave the way for optimized dietary recommendations and therapeutic strategies targeting the gut microbiome. A deeper understanding of these mechanisms could inform optimized dietary recommendations and therapeutic strategies for gut healthier. Additionally, given the limitations of *in vitro* fermentation models, *in vivo* studies are necessary to assess long-term microbial adaption and the physiological relevance of these findings.

Author contributions

Conceptualization (S. L., D. R.); data curation (S. L., D. R.); formal analysis (S. L.); funding acquisition (D. R.); investigation (S. L., C. P.); methodology (S. L., C. P.); project administration (D. R.); resources (D. R.); supervision (D. R.); visualization (S. L.); writing – original draft (S. L.); writing – review and editing (S. L., C. P., D. R.). All authors have read and agreed to the published version of the manuscript.

Ethical standards disclosure

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of Nebraska–Lincoln (IRB protocol #20210621206EP). All participants provided written informed consent before participating in the research.

Data availability

Raw data are published on the Mendeley Data website at: **https://dx.doi.org/10.17632/z6cht2drtp.1**. Raw sequence reads from 16S rRNA gene sequencing are available in the Sequence Read Archive under PRJNA1201101 (**https://www.ncbi.nlm.nih.gov/sra/PRJNA1201101**). Additional inquiries or clarification should be directed to the corresponding author.

Conflicts of 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.

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