Fecal menaquinone profiles of overweight adults are associated with gut microbiota composition during a gut microbiota–targeted dietary intervention[1,2]

J Philip Karl,[3,4] Xueyan Fu,[3] Xiaoxin Wang,[5] Yufeng Zhao,[5] Jian Shen,[5] Chenhong Zhang,[5] Benjamin E Wolfe,[7] Edward Saltzman,[4] Liping Zhao,[5,6] and Sarah L Booth[3*]*

[3]Vitamin K Laboratory and [4]Energy Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; [5]State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China; [6]Ministry of Education Key Laboratory of Systems Biomedicine, Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai, China; and [7]Department of Biology, Tufts University, Medford, MA

ABSTRACT

Background: Emerging evidence supports novel roles for vitamin K in cardiometabolic health, some of which may be unique to the bacterially synthesized vitamin K forms known as menaquinones. However, factors influencing menaquinone biosynthesis by the gut microbiota and associations with cardiometabolic health have not been examined.

Objective: The objective of this study was to identify associations between fecal menaquinone profiles, gut microbiota composition, and biomarkers of cardiometabolic health.

Design: The menaquinone profile and gut microbiota structure were periodically measured in fecal samples collected from 77 overweight Chinese adults who consumed a prescribed diet previously shown to alter gut microbiota composition and to improve cardiometabolic biomarkers.

Results: Covariance among menaquinones within individual fecal samples partitioned individuals into 2 distinct groups, herein introduced as menaquinotypes of the human gut. Menaquinotypes were characterized by differences in menaquinone (MK) 5 and MK9–MK13 and differences in the relative abundance of several operational taxonomic units (OTUs) delineated at the species level, predominantly within the genera *Prevotella* spp. and *Bacteroides* spp. Fecal MK4, MK6, and MK8 decreased during the intervention ($P < 0.05$); and longitudinal changes in the relative abundance of >100 OTUs were associated with altered fecal content of ≥1 individual menaquinone. The strongest and most consistent relations were between *Prevotella* spp. and MK5 and MK11–MK13, between *Bacteroides* spp. and MK9 and MK10, and between *Escherichia/Shigella* spp. and MK8. Neither individual menaquinones nor menaquinotypes were longitudinally associated with markers of glycemia, insulin resistance, or inflammation.

Conclusions: These findings suggest that variability in fecal menaquinone content is predominantly determined by relatively few genera within the gut microbiota and that diet-mediated alterations in gut microbiota composition may provide a feasible approach for altering gut menaquinone content. This trial was registered at the Chinese Clinical Trials Registry as ChiCTR-TRC-09000353. *Am J Clin Nutr* 2015;102:84–93.

Keywords: phylloquinone, menaquinone, gut microbiota, glycemia, inflammation, vitamin K2

INTRODUCTION

The collective genetic capacity of the human gut microbiome provides diverse metabolic functions not intrinsic to human biology (1). These functions include the biosynthesis of multiple forms of vitamin K known as menaquinones (2, 3). Despite longstanding knowledge that gut bacteria synthesize menaquinones, the impact on human health is not well characterized.

Vitamin K, composed of phylloquinone and the menaquinones, represents a family of structurally and functionally related fat-soluble vitamers (4). These vitamers are discriminated by the length and saturation of a lipophilic isoprenoid side chain attached to a napthoquinone ring shared by phylloquinone and all menaquinones (MKn,[8] with "n" representing the number of isoprenoid units in the side chain). Whereas phylloquinone is synthesized by plants and is the primary dietary form (5), menaquinones are synthesized by bacteria (6), including several genera inhabiting the human gut (2). The exception is MK4, which is endogenously synthesized in mammals (7) and is found in animal products (8). MK5–MK13 are not thought to be abundant in most diets but are found in some fermented food products (8). Emerging evidence suggests novel roles for vitamin K in cardiovascular health, inflammation, glucose homeostasis,

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[2] Supplemental Tables 1–4 and Supplemental Figures 1–3 are available from the "Supplemental data" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

*To whom correspondence should be addressed. E-mail: sarah.booth@tufts.edu (S Booth), lpzhao@sjtu.edu.cn (L Zhao).

[8] Abbreviations used: MK, menaquinone; OTU, operational taxonomic unit; PCA, principal components analysis; SI, Silhouette Index.

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cognitive function, and cancer (9–11), some of which may be unique to menaquinones (12).

The human gut is thought to be a menaquinone reservoir and is estimated to contribute to 10–50% of the human vitamin K requirement (2, 3). The large uncertainty in this estimate reflects important gaps in the current understanding of menaquinone biosynthesis in vivo. Few studies have attempted to measure the menaquinone content of the human gut (13, 14). Moreover, the historical reliance on culture-based techniques to study menaquinone biosynthesis in gut bacteria (15, 16) has ignored the contribution of unculturable bacteria, which comprise a substantial proportion of the gut microbiota (17), to the gut menaquinone pool. The possibility that menaquinone bioavailability and bioactivity vary with side-chain length (18–21), the fact that menaquinone forms synthesized by bacteria vary across species (22), and accumulating evidence that dietary modulation of the gut microbiota influences host health (23) indicate that an improved understanding of relations between the gut microbiota and menaquinone biosynthesis could help develop targeted strategies for improving human vitamin K nutriture.

In this study we aimed to use 16S ribosomal RNA gene sequencing, a culture-independent approach for measuring gut microbiota composition, to determine whether altering gut microbiota composition is associated with changes in fecal menaquinone content, to identify novel relations between gut bacteria and menaquinones, and to conduct exploratory analyses of relations between menaquinone biosynthesis and metabolic health. We hypothesized that interindividual variability in fecal menaquinone content would be associated with variability in gut microbiota composition.

METHODS

Participants

Chinese adults aged 25–55 y with central adiposity (defined as a waist circumference ≥80 cm for women and ≥90 cm for men or a waist-to-hip ratio of ≥0.85 for women and ≥0.90 for men) and a BMI (in kg/m^2) ≥28 were recruited from Taiyuan in Shanxi Province, China, between 2009 and 2010. Exclusion criteria included active or previous history of chronic disease or psychiatric disorders, alcoholism, use of antibiotics within the previous 3 mo, active infectious disease, anemia, and recent weight loss by surgery or pharmaceutical intervention. This study was approved by the Ethics Committee of the Chinese Clinical Trials Registry (no. ChiECRCT-000011) and the Tufts University Health Sciences Institutional Review Board. All participants provided written informed consent before participation.

Study design

The study design and intervention were previously reported and are described in detail elsewhere (24). Briefly, all participants received the same 23-wk intervention, which was separated into 2 phases: a 9-wk weight-loss intervention phase (phase 1) and a 14-wk maintenance phase (phase 2). During phase 1, participants consumed a prescribed, hypocaloric 1000- to 1600-kcal/d diet designed to increase the abundance of beneficial gut bacteria (e.g., *Bifidobacterium*), decrease the abundance of endotoxin-producing gut bacteria (e.g., *Enterobacteriaceae*), and to promote weight loss. Members of this group previously reported weight loss in response to this diet in a pilot study (25). The primary component of the phase 1 diet was 3–4 cans of a custom-made precooked porridge/d (Shanghai Meilin Meida Food Company) which consisted of a variety of whole grains and traditional Chinese medicinal plant foods (**Supplemental Table 1**). Participants were allowed limited amounts of fruit, vegetables, and legumes during phase 1. Two powdered supplements were also consumed during phase 1 (Supplemental Table 1). The first supplement, a powdered blend of bitter melon and oligosaccharides, was consumed before breakfast and lunch each day by reconstituting 20 g in warm water. The second supplement, a powdered blend of prebiotic soluble and fermentable fibers, was consumed once weekly before breakfast by reconstituting 50 g in water. During phase 2, consumption of the porridge was discontinued, and participants were instead instructed by the study dietitian on following a high-fiber, low-meat (<50 g/d) diet. Participants were also instructed to continue consuming 20 g of the bitter melon and oligosaccharide supplement twice daily, but the intake of the other fiber supplement was reduced to 50 g once every 2 wk. All volunteers were asked to maintain their usual pattern of daily activities throughout the study and to avoid taking medications that could affect the gut microbiota (e.g., antibiotics).

Anthropometric measurements were obtained by trained study staff. Fecal, blood, and urine samples were collected 30 d before beginning the study (baseline) and at the end of each study phase (study weeks 9 and 23) for the assessment of study outcomes as described below. Study activities took place at Shanghai Jiao Tong University, Shanghai, China, and the Shanxi High-tech Medical Testing Center, Shanxi Province, China.

Vitamin K

Aliquots of fecal samples were freeze-dried to a constant weight and homogenized by using a mortar and pestle. The porridge supplement was homogenized before analysis. Approximately 40 mg dried fecal sample, 600 mg wet weight of the porridge supplement, and 50 mg of the powdered bitter melon and oligosaccharide supplement were then analyzed for vitamin K content. The powdered fiber supplement was not available for analysis. The protocol for extracting and purifying vitamin K vitamers proceeded as previously described (26). After extraction and purification, 5 μL of a suspension of the sample residue in 170 μL methanol and 30 μL methylene chloride was injected into an Agilent 1200 HPLC (Agilent Technologies)–AB SCIEX Triple Quad 5500 mass spectrometry system for identification and quantification of vitamers. Vitamers were separated before mass spectrometry by HPLC by using a reverse-phase C_{18} analytic column (Kinetex: 2.6 μm, 150 mm × 3.0 mm; Phenomenex), as previously described (26). Phylloquinone and MK4–MK13 were detected at the following mass-to-charge ratios: *m/z* 451 (phylloquinone), *m/z* 445 (MK4), *m/z* 513 (MK5), *m/z* 582 (MK6), *m/z* 650 (MK7), *m/z* 718 (MK8), *m/z* 786 (MK9), *m/z* 854 (MK10), *m/z* 923 (MK11), *m/z* 991 (MK12), and *m/z* 1059 (MK13).

Deuterium-labeled phylloquinone was used as an internal standard in all samples, and calibration standards for phylloquinone and MK4 were used to quantify phylloquinone and MK4 concentrations in food and fecal samples as previously described

(26). Because calibration standards for MK5–MK13 were not available at the time of analysis, we could not confidently quantify precise fecal or diet supplement MK5–MK13 concentrations. Fecal vitamer amounts were therefore statistically analyzed by using the AUC derived from the HPLC–mass spectrometry chromatogram.

Gut microbiota profiling

Gut microbiota analysis was reported in detail previously (24). Briefly, fresh stool samples were collected into portable plastic containers while volunteers stayed in a hotel near the testing center. Collected samples were immediately put on ice and transported to the testing center. Aliquots were obtained on arrival at the testing center and stored at −80°C until analysis. Bacterial DNA was extracted from stool samples by using bead beating and the InviMag Stool DNA kit (KFml; Invitek GmbH). The V3 region of the 16S ribosomal RNA gene was amplified and sequenced by using the Roche GS FLX platform (Roche 454 Life Sciences).

Quality control of raw data and taxonomic assignment of operational taxonomic units (OTUs) were completed as previously described in detail (24). Briefly, high-quality pyrosequencing readings were clustered at 98% similarity by using the Cluster Database for High Identity with Tolerance. The Greengenes database, the Arbor Project Program Package (27), and the Distance-Based OTU and Richness program (28) were successively used for sequence alignment, distance calculations, and OTU-binning, respectively. Taxonomy was assigned by using the Ribosomal Database Project classifier (29). Rarefaction was used to correct for differences in sequencing depth between samples. The relative abundance of OTUs in each sample was calculated as the total number of readings for each OTU within a sample divided by the total number of readings in that sample. Relative abundances were then used for taxa-based analyses.

Blood sample analysis

Bioclinical variables were analyzed from blood samples as reported previously (24). Briefly, blood samples were collected after an overnight fast. Serum was assayed for glucose and ultrasensitive C-reactive protein by using an automated biochemical analyzer (Sysmex Chemi-180; Sysmex) and for insulin by using an immunoassay system (Immunolite 1000; Siemens Health Care Diagnostics). Fasting glucose and insulin were used to calculate the HOMA-IR. Plasma was assayed for LPS binding protein by using ELISA (USCN Life Science and Technology) according to the manufacturer's instructions. Plasma TNF-α, IL-1β, IL-6, and adiponectin were also measured by using ELISA (R&D Systems) according to the manufacturer's instructions. Minimum and maximum detectable concentrations for each assay were previously reported (24). Intra- and interassay CVs were $<5\%$ and $<10\%$, respectively, for all assays. Serum phylloquinone and menaquinone concentrations were not measured because insufficient sample volume remained after analysis of primary study outcomes.

Gut permeability

Gut permeability was assessed as previously described (24). Briefly, volunteers drank 5 g lactulose and 2 g mannitol dissolved in 50 mL water. Urine samples were collected for the subsequent 5 h, placed into aliquots, and stored at −80°C until analysis. Urine lactulose and mannitol concentrations were quantified by using an ion chromatograph (DX-600; Dionex). The ratio of lactulose to mannitol measured in urine collected 5 h after consumption of the sugar substitutes is a marker of small intestinal permeability (30).

Statistical analysis

Analyses were conducted by using SPSS version 21.0, XLSTAT version 2014 (Addinsoft; New York, NY), MultiExperiment Viewer version 1.9 (31), and R version 3.0.3. Vitamin K vitamer and OTU data were $\log_{10}$-transformed for analysis. Bonferroni corrections were used to control the familywise error rate in analyses that did not include OTU data. When OTU data were included in analyses, the false discovery rate (Q) was controlled by using the Benjamini-Hochberg correction. Significance was set at $P < 0.05$ or $Q < 0.05$. Data are presented as mean ± SD unless otherwise noted.

Interrelations between vitamin K vitamers

Relations between vitamin K vitamers were examined by using principal components analysis (PCA) and partitioning around medoids clustering based on the Spearman correlation metric. The Spearman correlation metric was used because of the bimodal distributions of several vitamers (MK5, MK11, MK12, and MK13) after $\log_{10}$-transformation, the presence of outliers in the data set, and because of substantial differences in mean AUCs of several vitamers. In addition to standardizing the differences in AUC, Spearman correlation does not require normally distributed data and is more robust to outliers than are other metrics such as Pearson correlation. Partitioning around medoids clustering was used because the number of clusters can be predefined, thereby allowing the observed bimodal distributions and our PCA results to inform the clustering model and because the algorithm is more robust to outliers than other methods in which cluster number is predefined, such as K-medians clustering (32). The Silhouette Index (SI) was calculated to measure cluster quality and used to determine the number of clusters that optimally described the variation in the data. An SI of 0.50–0.75 was considered indicative of moderately strong clustering, and an SI of $\geq$0.75 was indicative of strong clustering (33). Clusters with an SI of $<$0.50 were considered weak and not included as clusters in the final results. PCA was completed by using XLSTAT, and clustering was completed by using the R package "cluster."

Gut microbiota composition and fecal vitamin K content

Procrustes analysis and redundancy analysis were used to explore relations between gut microbiota composition and fecal vitamin K content. Specifically, Procrustes analysis was used to superimpose and assess congruence between PCAs of OTU relative abundances and vitamin K vitamers. To remain consistent with the approach used for the vitamer analysis, Spearman correlation was used as the distance metric for PCA of OTU-level data. Redundancy analysis was used to provide a measure of covariance between OTU relative abundances and fecal vitamin K vitamer content. Procrustes and redundancy analyses were completed by using the R package "vegan."

Linear discriminant analysis of effect size (34) was used to identify OTUs that differed in relative abundance between vitamin K vitamer clusters identified by partitioning around medoids clustering. Bivariate OTU-vitamer relations were examined by using the following steps: *1*) Spearman rank correlation was used to calculate *P* values for bivariate correlations between individual vitamin K vitamers and OTU relative abundances at each time point separately; *2*) *P* values were adjusted by using the Benjamini-Hochberg correction, and each OTU with a *Q* value <0.05 for ≥ 1 vitamer at any single time point was retained for further analysis; *3*) mixed-models ANOVA was then used to identify longitudinal associations between individual vitamers and the relative abundances of the selected OTU. To reduce both the influence of uncommon OTUs on microbiota-vitamer associations and noise in the visualizations, OTUs were filtered for a prevalence of at least 20% before analyses.

Biological outcomes and fecal vitamin K content

Longitudinal changes in fecal vitamin K content were analyzed by mixed-models ANOVA. When a significant main effect of time was observed, post hoc comparisons between time points were examined by Student's *t* test and *P* values adjusted by using Bonferroni corrections. Differences in markers of inflammation and glycemia between vitamin K clusters were examined by Student's *t* test. Longitudinal associations between these outcomes and vitamin K clusters were examined by using generalized estimating equations. Pearson correlation and Spearman rank correlation were used as appropriate to examine correlations between individual vitamers and markers of inflammation and glycemia at baseline. Longitudinal associations between individual vitamers or OTU relative abundances and markers of inflammation and glycemia were assessed by mixed-models ANOVA.

RESULTS

The primary outcomes of the trial, which include changes in gut microbiota composition and cardiometabolic risk factors, were previously reported (24). Briefly, average weight loss at 23 wk was 6.6% ± 4.9% of initial body weight ($P < 0.01$). Weight loss was accompanied by improvements in gut permeability ($P < 0.05$) and improvements in several markers of cardiometabolic health and inflammation to include HOMA-IR ($P < 0.01$), blood pressure ($P < 0.01$), and concentrations of C-reactive protein ($P < 0.05$), TNF-α ($P < 0.05$), and IL-6 ($P < 0.01$) (24). The relative abundance of several endotoxin-producing gut bacteria phylotypes, including *Enterobacteriaceae* and *Desulfovibrionaceae*, was reduced at 23 wk, whereas the relative abundance of *Bifidobacterium*, a beneficial genus, increased ($P < 0.05$ for all) (24). The present analysis includes data from 77 of the 93 volunteers who completed the trial, from whom archived fecal samples at each of the baseline, 9-wk, and 23-wk time points were available for fecal vitamin K analysis (**Table 1**). The 16 excluded individuals did not differ by sex ($P = 0.93$), age ($P = 0.17$), or BMI ($P = 0.11$) relative to individuals included in the analysis.

The measured phylloquinone and MK4 concentrations of the porridge were 0.7 ± 0.1 and 0.2 ± 0.0 μg/100 g, respectively. MK9 and MK10 were also detected at estimated concentrations of <1 μg/100 g. The measured phylloquinone and MK4 concentrations of the bitter melon and oligosaccharide supplement were 61.2 ± 3.2 and 1.4 ± 0.4 μg/100 g, respectively. MK10 and MK11 were also detected at estimated concentrations of <1 μg/100 g. The fiber supplement was not available for vitamin K analysis.

TABLE 1
Participant characteristics[1]

	Baseline value
Sex (M/F), *n*	28/49
Age, y	41 ± 7
Height, cm	163 ± 8
Weight, kg	86.2 ± 12.8
BMI, kg/m^2	32.3 ± 2.9
HOMA-IR	2.6 (2.1)

[1]Values are means ± SDs or medians (IQRs) unless otherwise indicated.

Covariance among vitamin K vitamers

PCA indicated that 62% of the variability in fecal vitamin K vitamer content across all time points could be explained by 2 principal components (**Figure 1**A). The vitamers MK5, MK6, MK11, and MK12 were particularly influential to the variance explained by the first principal component, whereas MK9, MK10, MK12, and MK13 were particularly influential to the variance explained by the second principal component. Visual inspection of the PCA plot indicated a partitioning of individuals into 2 separate clusters, independent of intervention time point. Partitioning around medoids analysis was then used to further investigate and substantiate this observation.

Consistent with PCA, partitioning around medoids analysis provided strong support for partitioning individuals into 2 distinct clusters, or menaquinotypes, on the basis of variability in fecal vitamin K content (SI = 0.78; Figure 1B). Similarly, moderate support for 2 menaquinotypes was observed when Euclidean distance was used in place of Spearman correlation in the clustering algorithm (SI = 0.52; **Supplemental Figure 1**). Menaquinotypes were discriminated by MK5 and MK9–MK13 contents ($P < 0.001$), with samples belonging to the highest-density cluster ($n = 145$ samples) being enriched in MK9 and MK10 and samples belonging to the lower-density cluster ($n = 86$ samples) being enriched in MK5 and MK11–MK13. Support for >2 menaquinotypes was low, as indicated by a decrease in the SI to 0.49 when data were partitioned into 3 clusters (SI = 0.34 when the Euclidean metric was used). Strong support for 2 menaquinotypes was also evident when partitioning around medoids analysis was used to analyze vitamin K data at each time point separately (Figure 1C–E). Menaquinotype membership was relatively stable during the intervention, with only 6 (8%) individuals changing menaquinotype membership during the initial 9-wk weight-loss phase, one of whom reverted back to her baseline menaquinotype membership by the end of the study and only 3 (4%) additional individuals changing menaquinotype membership during the subsequent 14-wk weight-loss maintenance phase.

Interrelations between fecal vitamin K content and gut microbiota composition

Redundancy analysis indicated that variability in OTU-level gut microbiota composition explained 99.7% of the variability in

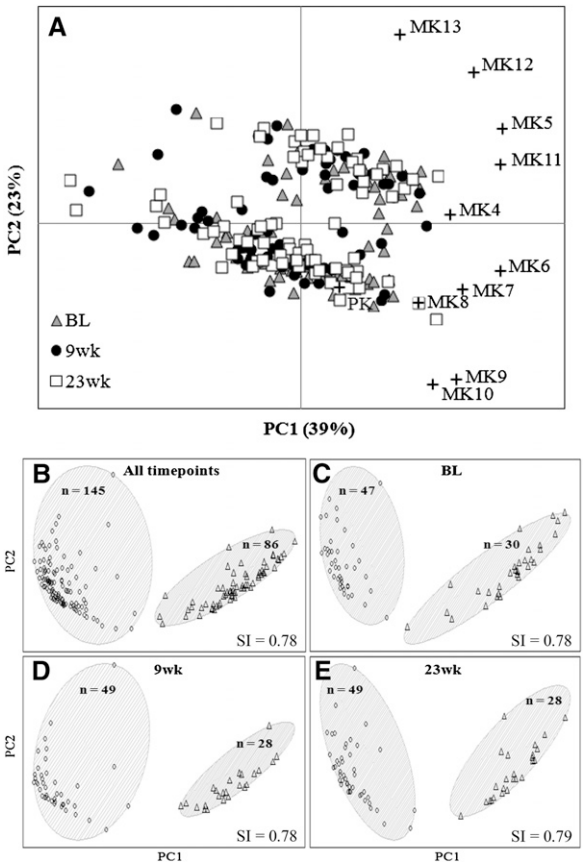

FIGURE 1 Fecal vitamin K content partitions individuals into menaquinotypes. (A) Principal components analysis of the vitamin K content of individual fecal samples before (BL), during (9 wk), and after (23 wk) the diet intervention (n = 77). Spatial location of the "+" symbol indicates the relative contribution of each vitamin K vitamer to the variance explained by the first and second principal components, with greater distance from the (x, y) origin indicating a larger contribution of that vitamer to variance in fecal vitamin K content. (B–E) Partitioning around medoids clustering based on Spearman correlation matrix of fecal sample vitamin K vitamer content at all time points (B) and at baseline (C), 9 wk (D), and 23 wk (E) of the diet intervention. In panels B–E, menaquinotypes are indicated by gray ellipses; open circles indicate MK9–MK10-enriched samples, and open triangles represent MK5/MK11–MK13-enriched samples. BL, baseline; MK, menaquinone; PC, principal component; PK, phylloquinone; SI, Silhouette Index.

fecal vitamin K content across all time points (Monte Carlo P = 0.08). Several OTUs representing unclassified *Ruminococcaceae, Bacteroides* spp., *Prevotella* spp., *Alistipes* spp., *Oscillibacter*, *Bilophila*, *Odoribacter* spp., and *Barnesiella* were particularly influential in explaining variability in fecal MK6 and MK8–MK13 contents (**Figure 2**A). Procrustes analysis revealed congruence between the PCAs of fecal vitamin K content and gut microbiota composition across all time points (R = 0.65, Monte Carlo P = 0.002; Figure 2B) and at each time point separately (R = 0.68, Monte Carlo P = 0.002 for all; Figure 2C–E), suggesting that the menaquinotypes suggested by PCA and confirmed by partitioning around medoids analysis were associated with gut microbiota composition.

Linear discriminant analysis of effect size identified >50 OTUs differing in relative abundance between samples assigned to the MK9–MK10-enriched menaquinotype relative to the MK5/MK11–MK13-enriched menaquinotype (**Figure 3**). The MK9–MK10-enriched menaquinotype was characterized by a higher relative abundance of several OTUs belonging to the *Firmicutes* and *Proteobacteria* phyla and 17 OTUs classified as *Bacteroides*. In contrast, the MK5/MK11–MK13-enriched menaquinotype was characterized by a higher relative abundance of 15 OTUs classified as *Prevotella.* Effect size estimation indicated that OTUs belonging to the *Prevotella* and *Bacteroides* genera were the most influential taxa underlying menaquinotypes (**Supplemental Table 2**).

Fecal vitamin K response to modulation of gut microbiota composition

Fecal phylloquinone (P = 0.01), MK4 (P = 0.001), MK6 (P = 0.01), and MK8 (P = 0.004) content decreased during the 9-wk weight-loss phase (**Figure 4**). The reduction in phylloquinone (P = 0.002) and MK8 ($P < 0.001$) remained significant at 23 wk. Although no significant changes were documented for any other menaquinones, interindividual variability was observed. Therefore, mixed-models ANOVA was used to more clearly define individual OTU-vitamer relations and to determine the extent to which changes in the relative abundance of individual OTUs were associated with changes in individual vitamin K vitamer concentrations.

A total of 164 OTUs were significantly associated ($Q < 0.05$) with ≥1 vitamer at ≥1 time point during the intervention. Changes in 131 of these OTUs were significantly associated ($Q < 0.05$) with changes in ≥1 vitamer during the intervention (**Figure 5**, **Supplemental Figure 2**). Several consistent longitudinal relations between changes in the relative abundance of multiple OTUs within the same phylotype and changes in individual vitamer concentrations were observed (Figure 5). For example, positive longitudinal associations between all 15 OTUs classified as *Prevotella* and fecal MK5, MK11, MK12, and MK13 contents ($Q < 0.05$) were observed. Similarly, positive longitudinal associations between 19 of 21 OTUs classified as *Bacteroides* and fecal MK9 and MK10 contents ($Q < 0.05$), and between all OTUs classified as *Escherichia/Shigella* and fecal MK8 content ($Q < 0.05$), were observed. Finally, positive longitudinal associations between several different OTUs and fecal MK5, MK6, MK7, MK9, and MK10 contents were observed. These OTUs included 5 classified as *Alistipes* and several classified as belonging to the *Ruminococcaceae* family.

Associations between fecal vitamin K content and gut microbiota composition with glycemia and inflammation

At baseline, fecal MK9 content was inversely associated with BMI (P = 0.05), serum insulin (P = 0.01), and HOMA-IR (P = 0.009); and fecal MK10 content was inversely associated with lactulose:mannitol ratio (P = 0.01; **Supplemental Table 3**). In longitudinal analyses, fecal MK4 content was positively associated with BMI (P = 0.01). However, no additional longitudinal associations between individual menaquinones with BMI or biomarkers of glycemia or inflammation were detected (data not shown). Individuals within the MK9–MK10-enriched menaquinotype had lower baseline IL-6 concentrations relative to individuals in the MK5/MK11–MK13 menaquinotype (P = 0.01), but otherwise markers of glycemia and inflammation did not differ between menaquinotypes at baseline (**Supplemental Table 4**). No longitudinal associations between menaquinotype,

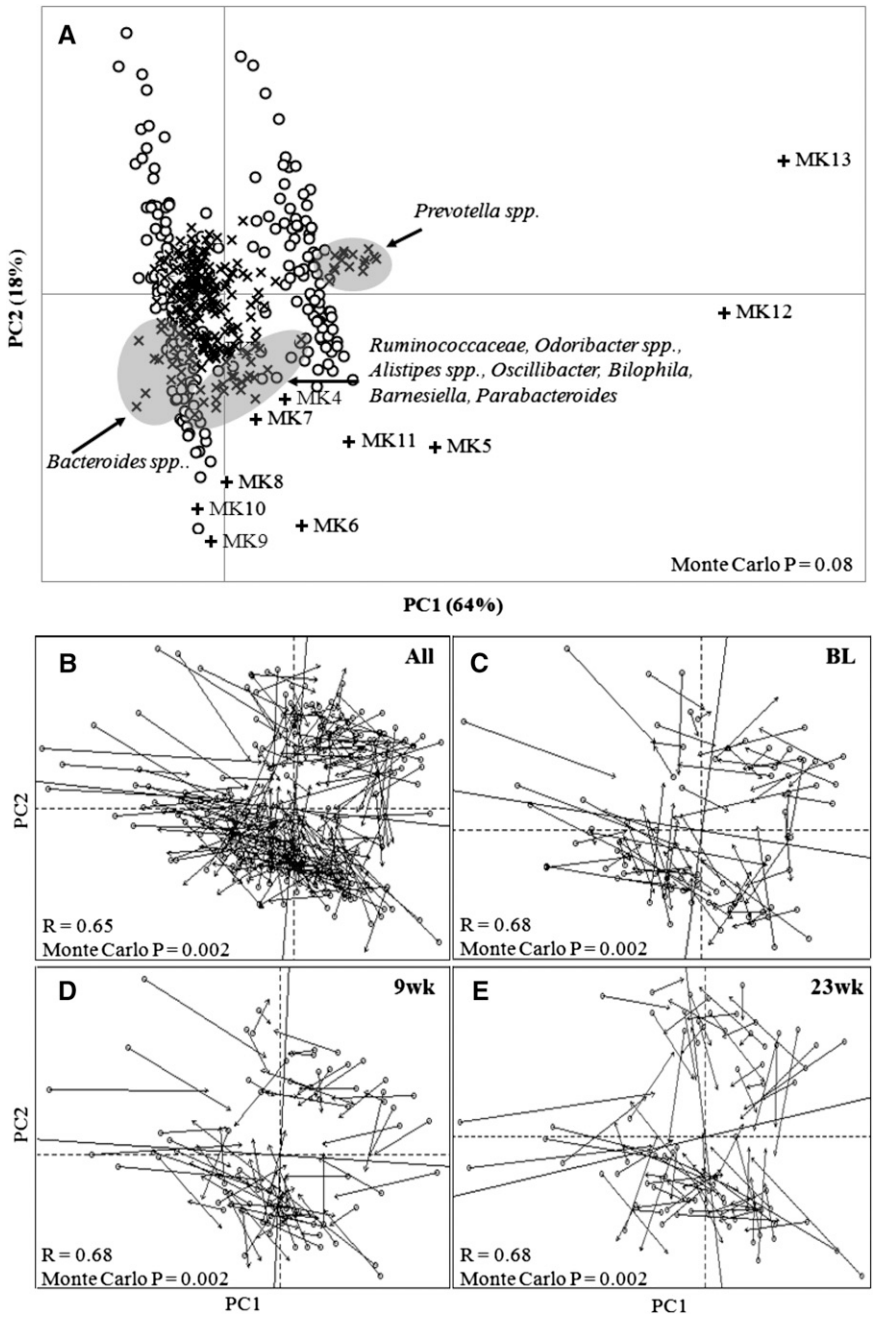

FIGURE 2 Gut microbiota composition is associated with fecal menaquinone content. (A) Redundancy analysis map of relations between fecal vitamin K vitamer content and OTU-level gut microbiota composition. First and second ordination axes, explaining 82% of total variability in vitamer content, are plotted. Spatial location of the "x" and "+" symbols represents associations between OTUs (x) and vitamin K vitamers (+). Greater distance of "+" symbols from the (x,y) origin indicates a larger contribution of that vitamer to the variance in fecal vitamin K content. Gray ellipses highlight OTUs with the greatest contribution to explaining variability in fecal vitamin K content. Open circles represent individual fecal samples collected before (BL), during (9 wk), and after (23 wk) the diet intervention (n = 77). (B–E) Procrustes analysis combining principal coordinates analysis based on Spearman distance of OTU-level gut microbiota composition (arrowheads) with principal coordinates analysis of fecal vitamin K vitamer content (open circles) of fecal samples at all time points (B) and at baseline (C), 9 wk (D), and 23 wk (E) of the diet intervention. Longer vectors indicate greater intraindividual dissimilarity. Monte Carlo P values represent 500 permutations. MK, menaquinone; OTU, operational taxonomic unit; PC, principal component.

BMI, any marker of inflammation, or glycemia were observed (data not shown).

Procrustes analysis indicated a weak association between menaquinone-associated OTUs and metabolic health biomarkers (**Supplemental Figure 3**). To further investigate this association, OTUs that were significantly associated with ≥1 vitamers in longitudinal analyses were investigated as predictors of glycemia and inflammation. With few exceptions, these OTUs were not significantly associated with BMI or markers of glycemia or inflammation (data not shown). Exceptions were limited to 1 OTU classified as *Prevotella* that was positively associated with serum insulin concentrations (Q = 0.004) and HOMA-IR

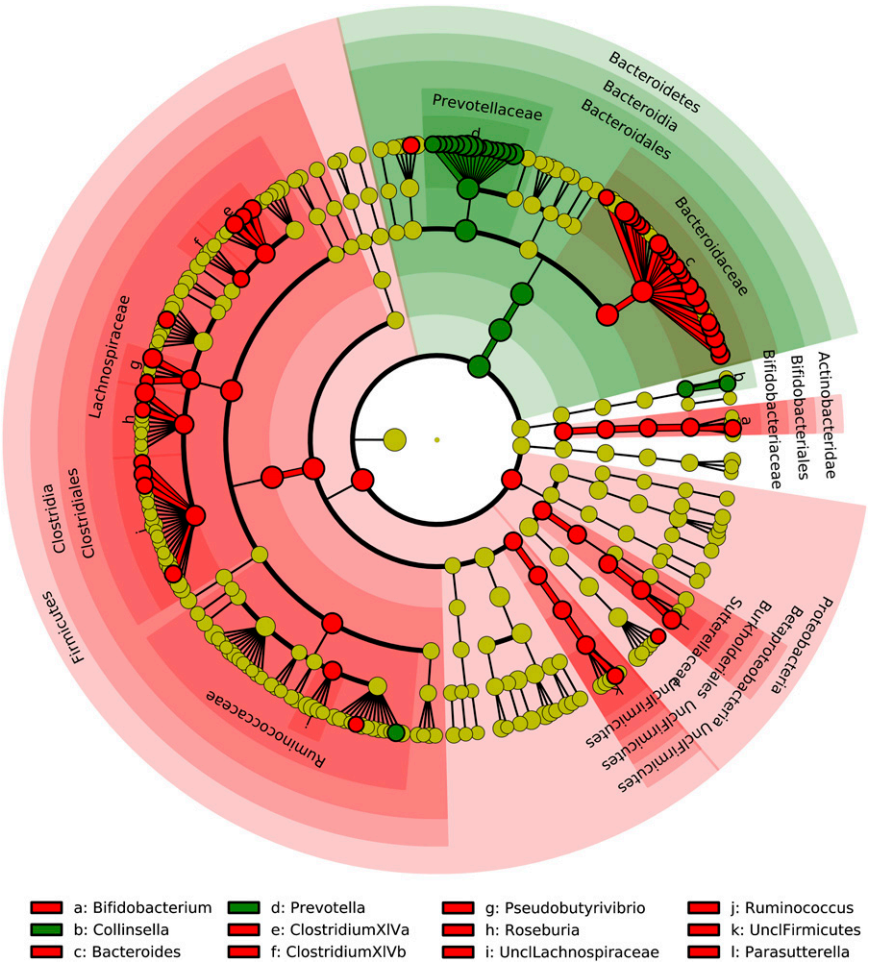

FIGURE 3 Multiple phylotypes are differentially abundant between menaquinotypes. Linear discriminant analysis of effect size for phylotypes differing in relative abundance between menaquinotypes before, during, or after the diet intervention (n = 77). Levels within the cladogram correspond to different taxonomic ranks from phyla (innermost ring) to operational taxonomic unit (outermost ring). Colored circles represent individual taxa. Red shading represents higher abundance in the MK9–MK10-enriched menaquinotype (effect size ≥3.0, $P < 0.00025$); green shading represents greater abundance in the MK5/MK11–MK13-enriched menaquinotype (effect size ≥3.0, $P < 0.00025$). Lowercase letters are differentially abundant genera between menaquinotypes. Both yellow coloring and no shading indicate no difference in relative abundance. MK, menaquinone; Uncl, unclassified.

(Q = 0.04) and 1 OTU classified as *Cronobacter* that was positively associated with IL-1β (Q = 0.04).

DISCUSSION

To the best of our knowledge, this study is the first to examine interrelations between the gut microbiota and menaquinone biosynthesis in vivo and the first to examine the extent to which diet-mediated modulation of the gut microbiota alters fecal menaquinone content. We showed that intra- and interindividual variability in fecal menaquinone content appears to be explained by relatively few genera within the gut microbiota. Furthermore, we observed associations between diet-mediated changes in the relative abundance of multiple individual OTUs with altered fecal content of individual menaquinones. Collectively, these findings suggest that diet-mediated alterations in gut microbiota composition may provide a feasible approach for altering gut menaquinone content.

Our findings extend those of previous studies that were limited by sample sizes too small to comprehensively describe intra- and interindividual variability in fecal vitamin K content and that did not examine gut microbiota composition in combination with fecal menaquinones (13, 14). Within our cohort, variability in fecal vitamin K content was not due to differences in the predominant dietary vitamin K forms (i.e., phylloquinone and MK4) but instead was due to interindividual differences in the fecal content of several bacterially synthesized menaquinones. Specifically, MK5, MK11, MK12, and MK13 covaried within samples and in an inverse manner to MK9 and MK10. This within-sample covariance partitioned individuals into 2 distinct groups, described as menaquinotypes of the human gut. The observation that several phylotypes differed between menaquinotypes and our identification of >100 individual OTU-menaquinone associations strongly suggest that variability in fecal vitamin K content was attributable to the gut microbiota and not to dietary vitamin K intake.

Our findings also suggest that fecal menaquinone content can be influenced by dietary factors that alter gut microbiota composition. Members of our team previously reported reductions in the relative abundances of several genera, including *Escherichia/Shigella* and *Klebsiella*, within this cohort during the intervention (24). Both *Escherichia* and *Klebsiella* are known to

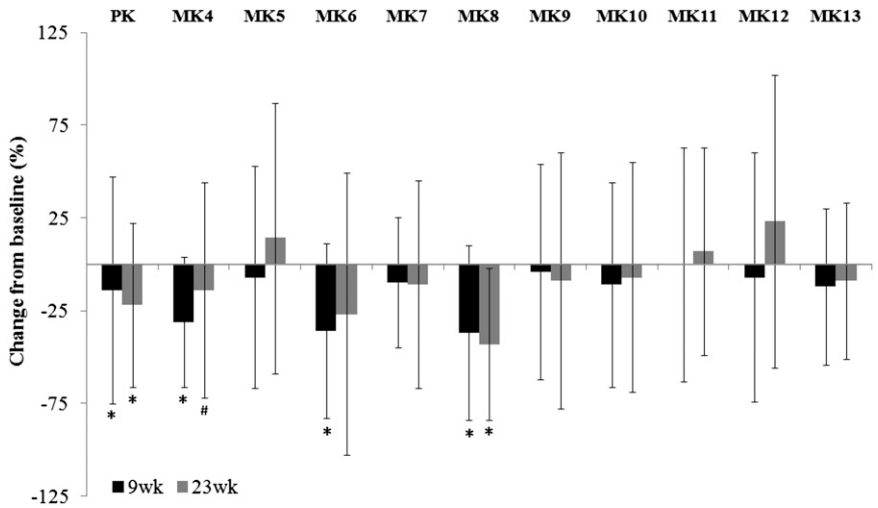

FIGURE 4 A gut microbiota–targeted dietary intervention alters fecal vitamin K content. Changes in fecal vitamin K content during the diet intervention (n = 77). Bars represent median (±IQR) percentage changes from baseline. *,#Different from *baseline and #9 wk (mixed-model repeated measures ANOVA, $P < 0.05$). MK, menaquinone; PK, phylloquinone.

produce MK8 (16, 22), and OTUs belonging to both genera were among the few positively associated with MK8 in longitudinal analyses. Therefore, our findings implicate the diet intervention–mediated effects on the relative abundances of these genera as underlying the observed decrease in fecal MK8 content and provide evidence suggesting that diet-mediated alterations in gut microbiota composition may provide a feasible approach for altering gut menaquinone content.

Diet interventions targeting *Prevotella* and *Bacteroides* could have robust effects on fecal menaquinone content given that OTUs within these genera showed the most consistent and strongest associations with fecal menaquinone content. These findings are consistent with genomic data indicating that MK9–MK10 biosynthetic pathways are predominantly found in *Proteobacteria*, *Firmicutes*, and *Bacteroides*, whereas MK11–MK13 biosynthetic pathways are predominantly found in *Actinobacteria* and *Prevotella* (35). Moreover, differential menaquinone biosynthesis by *Bacteroides* and *Prevotella* was previously reported with MK9–MK11 and MK11–MK13, composing the menaquinone profiles of *Bacteroides* and *Prevotella* strains, respectively, isolated from the human gut (16, 36). Substantial interindividual variability in MK9–MK13 is also consistent with several studies that reported that *Prevotella* abundance in human fecal samples follows a bimodal distribution (i.e., low or high relative abundance) (33), is inversely associated with *Bacteroides* abundance (33), and, in part, drives partitioning of individuals into clusters representing defined microbial communities commonly known as "enterotypes" (33, 37, 38). Although previous metagenomic analyses suggest that the genetic capacity for menaquinone biosynthesis does not differ between these enterotypes (37), our findings suggest that there may nonetheless be important differences resulting from

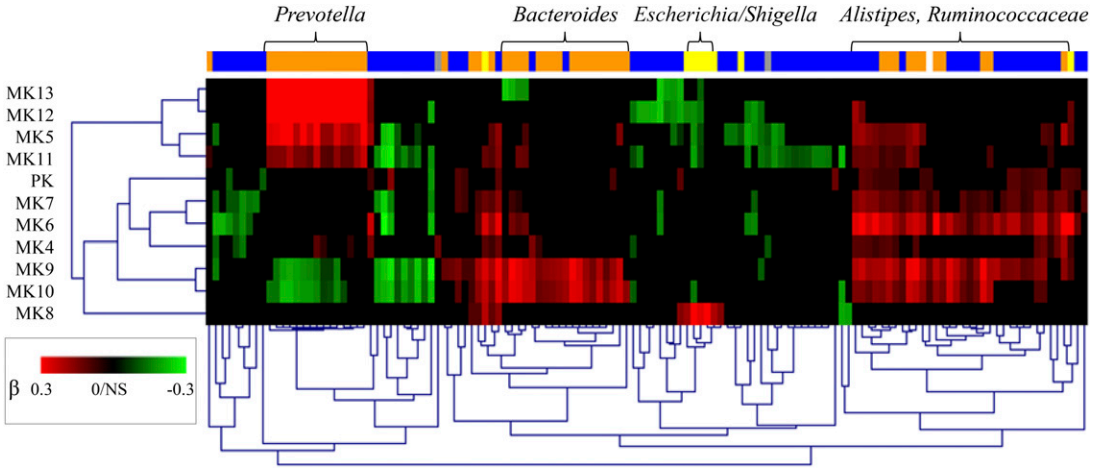

FIGURE 5 Longitudinal associations between fecal vitamin K content and OTUs. Heat map of significant ($Q < 0.05$) β coefficients from mixed-model ANOVA examining longitudinal associations between vitamin K vitamers and OTU-level relative abundances during the diet intervention (n = 77). β coefficients were subjected to hierarchical average-linkage clustering using Pearson correlation. The taxa indicated in the figure represent taxa having the greatest number of positive OTU-menaquinone associations. OTUs are color-coded by phyla: orange, *Bacteroidetes*; blue, *Firmicutes*; yellow, *Proteobacteria*; gray, *Actinobacteria*; white, unclassified. Black shading indicates a nonsignificant association ($Q > 0.05$). MK, menaquinone; OTU, operational taxonomic unit; PK, phylloquinone.

the forms of menaquinone synthesized (i.e., MK9–MK10 vs. MK5/MK11–MK13). In addition to differences in fecal MK9–MK13 content between menaquinotypes, the observed association between *Prevotella* and MK5, which to our knowledge has not been previously reported, may be highly relevant to vitamin K nutriture given a possible inverse relation between isoprenoid side-chain length and menaquinone bioavailability (21).

To our knowledge, this study is the first to examine associations between fecal menaquinone content and metabolic health outcomes. The observed inverse cross-sectional associations between MK9 and insulin resistance are intriguing but should be interpreted with caution. Serum insulin and HOMA-IR did not differ between menaquinotypes, despite differences in fecal MK9 content, and the cross-sectional associations were not corroborated in longitudinal analyses. The absence of longitudinal associations between fecal menaquinone content and metabolic biomarkers suggests that the study duration may have been too short or the magnitude of effect size on fecal menaquinones too small to detect associations. It is also possible that the substantial intervention-induced weight loss (24) masked associations or that fecal menaquinones are not related to the measured outcomes. Regardless, menaquinones may have greater bioactivity than phylloquinone (19, 39) and a growing body of evidence suggests beneficial effects of dietary vitamin K on cardiovascular health (40–43), inflammation (44, 45), and insulin sensitivity (46, 47), some of which may be unique to menaquinones (40–42). Collectively, this evidence suggests that small amounts of menaquinones derived from gut bacteria biosynthesis could have relevant health effects in situ or if absorbed. Modulating gut bacteria menaquinone biosynthesis therefore warrants continued investigation as a novel target for attenuating chronic disease risk.

An important question to address in future studies is to what extent menaquinones excreted in stool reflects the amounts and forms of gut bacteria–derived menaquinones available for absorption. Because of their integral role in prokaryotic electron transport chains, the majority of menaquinones in the gut are thought to be embedded in bacterial cytoplasmic membranes and therefore likely not readily bioavailable. In addition, although not well characterized, menaquinone absorption is thought to occur through a bile acid–dependent pathway (8). As such, any absorption of gut bacteria–derived menaquinones likely predominantly occurs in the ileum where the presence of bile acids could facilitate menaquinone absorption. Although bacteria density is lower and composition differs in the ileum relative to the colon (48), menaquinone-synthesizing bacteria are present in the ileum (49). Moreover, bacterially synthesized menaquinone forms not currently thought to be abundant in the diet are present in human hepatic tissue (50), which suggests that at least some gut bacteria–derived menaquinones are absorbed.

Several limitations should be considered when interpreting the results of this study. First, daily vitamin K intake from nonintervention foods was not assessed. To our knowledge, no information with regard to the menaquinone content of the Chinese diet has been previously reported. However, the observed decrease in fecal phylloquinone concentrations likely reflects reduced consumption of vegetable and vegetable oil sources of phylloquinone, and the decrease in fecal MK4 concentrations may reflect reduced intake of animal products, which can contain MK4 (8). Any contribution of dietary intake to the fecal content of bacterially synthesized menaquinones (MK5–MK13) is likely minimal because consumption of known food sources of these menaquinones (e.g., fermented cheeses) is not common in Chinese adults. A second limitation is the lack of sensitive markers of vitamin K status, which should be included in future studies to more clearly elucidate the relevance of menaquinone biosynthesis by gut bacteria to vitamin K nutriture. Finally, the generalizability of our findings may be limited by the relatively homogeneous study cohort, and replication in additional populations is needed.

In summary, this analysis provides a comprehensive assessment of in vivo relations between gut bacteria and fecal menaquinone content. Findings suggest that interindividual differences in gut microbiota composition and diet-mediated effects on gut microbiota composition have a measurable impact on fecal menaquinone content. Whether interactions between dietary intake and the gut microbiota have a clinically relevant impact on vitamin K nutriture warrants further investigation.

The authors' responsibilities were as follows—JPK, XF, JS, LZ, and SLB: designed the research; JPK, XF, XW, YZ, JS, and CZ: conducted the research; JPK and CZ: analyzed the data; BEW and ES: critically reviewed the manuscript content and guided data interpretation; JPK, LZ, and SLB: wrote the manuscript and had primary responsibility for final content; and all authors: read and approved the final manuscript. None of the authors reported a conflict of interest.

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