Effects of Gut Bacteria Depletion and High-Na\(^+\) and Low-K\(^+\) Intake on Circulating Levels of Biogenic Amines

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Scope: High-sodium and low-potassium (HNaLK) content in Western diets increases the risk of hypertension and cardiovascular disease (CVD). It is investigated if the dietary minerals interact with gut bacteria to modulate circulating levels of biogenic amines, which are implicated in various pathologies, including hypertension and CVD.

Methods and results: Using a metabolomic approach to target biogenic amines, the effects of gut bacteria depletion and HNaLK intake on circulating levels of biogenic amines in rats are examined. Forty-five metabolites whose plasma levels are significantly altered by gut bacteria depletion (\(p < 0.05\)) are found, indicating their regulation by gut bacteria. Many of them are not previously linked to gut bacteria; therefore, these data provide novel insights into physiological or pathological roles of gut bacteria. A number of plasma metabolites that are altered both by gut bacteria and HNaLK intake are also found, suggesting possible interactions of the diet and gut bacteria in the modulation of these metabolites. The diet effects are observed with significant changes in the gut bacterial taxa Porphyromonadaceae and Prevotellaceae (\(p < 0.05\)).

Conclusion: The dietary minerals may regulate abundances of certain gut bacteria to alter circulating levels of biogenic amines, which may be linked to host physiology or pathology.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of death in Western countries; the high-fat content of Western diets is a dominant environmental factor for increased CVD prevalence.\[1\] In addition, high-sodium (Na\(^+\)) and low-potassium (K\(^+\)) content in Western diets increases the risk of CVD.\[2,8\] Epidemiological studies demonstrated that high-Na\(^+\) intake increases blood pressure, endothelial dysfunction, and cardiovascular morbidity and mortality in the general population.\[3-4\] In addition, low-K\(^+\) intake was shown to increase blood pressure and risk of stroke and CVD.\[5-7\] Recent studies demonstrated that the dietary Na\(^+\) to K\(^+\) ratio is more strongly associated with the risk of hypertension or CVD than dietary Na\(^+\) or K\(^+\) alone.\[2,3,5,8\] suggesting that the combination of high-Na\(^+\) and low-K\(^+\) intake may contribute to hypertension or CVD more than either alone.\[2,8\]

Several mechanisms for sensing dietary Na\(^+\) intake have been suggested,\[^9\] including Na\(^+\) sensing by a specific part (i.e., subfornical organ) of the brain,
which elicits subsequent neural or neuroendocrine responses,[10,11] or the kidney, which modulates Na⁺ excretion. However, the extracellular Na⁺ level is usually unaltered by Na⁺ intake due to rapid and effective osmoregulation that causes parallel increases in extracellular volume. Therefore, the effect of Na⁺ intake on blood pressure or CVD may involve Na⁺-sensing mechanisms independent of circulating Na⁺ level (e.g., gut–renal axis).[12]. Extracellular K⁺ level is also tightly regulated due to renal (excreting K⁺) and extrarenal (buffering K⁺) mechanisms, and beneficial (or detrimental) effects of K⁺ supplementation (or deficiency) often occur without changes in extracellular K⁺ level.[13–16] These data suggest that dietary K⁺ effects on blood pressure or CVD may be mediated by mechanisms independent of circulating K⁺ level, possibly those involving gut sensing of dietary K⁺ (i.e., “gut factor”), a concept gleaned from our previous studies of K⁺ homeostatic mechanisms.[13,14] Thus, evidence has accumulated for the gut sensing of dietary Na⁺ and K⁺ intake,[12,15,16] but the exact nature of this sensing is unknown.

Previous studies provided ample evidence for a major role of gut microbiota in host physiology or pathology.[17,18] We previously suggested that gut bacteria may be involved in the gut sensing of K⁺,[19] as K⁺ plays an important role in the growth, colonization, or function of bacteria.[20,21] Although altered K⁺ intake does not markedly alter circulating K⁺ level due to extracellular K⁺ homeostatic mechanisms, it may substantially impact gastrointestinal K⁺ levels, which may acutely or chronically alter gut bacterial functions, which can, in turn, affect host physiology. Biogenic amines, which derive from amino acids, occur in a wide range of foods. Biogenic amines are also produced from gut bacterial metabolism of nutrients and have been implicated in various pathological processes.[22–24] For example, gut bacteria convert dietary nutrients, such as choline and l-carnitine, to trimethylamine (TMA), which is then absorbed into the blood, converted to trimethylamine oxide (TMAO) by the liver, and cause atherosclerosis.[25] Choline and l-carnitine are rich in animal-based high-fat diets, and TMAO and TMAO can provide a link between high-fat diets and CVD.[26,27] The present study was designed to test the effects of gut microbiota and high-Na⁺ and low-K⁺ intake on circulating levels of biogenic amines, including TMAO, and other polar metabolites with particular interest in finding biogenic amines (or other polar metabolites) that are altered by gut microbiota and high-Na⁺ and low-K⁺ intake, as a potential mediator of cardiovascular effects of dietary minerals. We also tested whether high-Na⁺ and low-K⁺ intake alters gut bacterial composition.

2. Experimental Section

2.1. Animal Experiments

Male Wistar rats weighing 280–300 g (approximately 9 weeks old) were obtained from Envigo Laboratories (CA, USA). Animals were individually caged under controlled temperature (22 ± 2 °C) and lighting (12-h light, 6 AM–6 PM; 12-h dark, 6 PM–6 AM) with free access to water and standard rat chow or experimental diets (see below). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Animals were fed for 1 week with diets containing different amounts of Na⁺ and K⁺ (n = 7 each, determined based on our previous experience with metabolomic studies in rats). The diets were prepared from K⁺-deficient powdered rat diet (TD.88239.PWD; Envigo Teklad) with or without supplementation with KCl or NaCl. The control (“healthy”) diet contained 0.29% Na⁺, which is the level in normal rat diets, and 2% K⁺, which is higher than the normal 1%. In the high-Na⁺ and low-K⁺ diet, Na⁺ content was increased from 0.29% to 0.79%, a level similar to those in Western diets,[28] and K⁺ content was decreased to 0.1%, which was selected to be low, but not low enough to deplete plasma K⁺.[29] The diets were gelled by heating and dissolving 30 g agarose in 500 mL of deionized water and adding to 500 g of powdered diet supplemented with KCl and NaCl.[30] Gelled diets were cut in small blocks and stored at −4 °C until use. After the 1-week feeding, animals were anesthetized with isoflurane at ≈7 AM, and blood samples for the analysis of biogenic amines were collected using a heparinized syringe through the abdominal aorta. Blood samples were rapidly spun, and plasma was isolated and frozen immediately in liquid N₂. After blood sampling, fecal samples for the analysis of gut bacterial composition were collected from the rectum and frozen immediately in liquid N₂. The plasma and fecal samples were stored at −80 °C until analysis.

The above feeding experiment was also conducted in animals treated with antibiotics to deplete gut bacteria. Animals were maintained on drinking water (autoclaved tap water) containing vancomycin, metronidazole, neomycin, and ampicillin (0.5 mg mL⁻¹ for vancomycin and 1 mg mL⁻¹ for the others) for a week. During the antibiotic treatment, food intake and weight gain decreased during the initial 2–3 days but returned to normal values thereafter. In addition, the antibiotic-treated rats showed signs of minor diarrhea (e.g., crumbly fecal pellets). After 1-week treatment, fecal DNA content decreased to 3% of control (data not shown), indicating that the antibiotic treatment was effective to remove most gut bacteria. After the antibiotic treatment, the animals were fed either the control or the high-Na⁺ and low-K⁺ diet (n = 7 each) for an additional week, while being continuously treated with antibiotics. After the feeding, blood samples were collected and subjected to analysis on biogenic amines, as described above.

2.2. Chromatographic and Mass Spectrometric Conditions for Biogenic Amines

Plasma aliquots (15 µL) were extracted and derivatized as reported previously[31] using 1 mL of degassed acetonitrile/isopropanol:water (3:3:2; v/v/v) at −20 °C, centrifuged and decanted with subsequent evaporation of the solvent to complete dryness. A clean-up step with 500 µL acetonitrile/water (1:1; v/v) removed membrane lipids and triglycerides and the supernatant was dried down again. Hydrophilic interaction liquid chromatography (HILIC) method was used for the analysis of biogenic amines. Five microliters of re-suspended sample was injected onto a Waters Acquity UPLC BEH Amide column.
previously described. Briefly, Golay-barcoded primers 515F with manufacturer’s protocols. Microbiome profiling was performed by the AllPrep DNA/RNA Mini Kit (Qiagen) in accordance with manufacturer’s protocols. DNA was extracted from fecal samples using Lysing Matrix E beads on a TissueLyser II (Qiagen, Hilden, Germany) system followed by the Illumina MiSeq platform using 2 × 150 bp v2 chemistry. Sequences were demultiplexed with Golay error correction using QIIME 1.9.1. Divisive amplicon denoising algorithm version 2 (DADA2) was used for error correction, exact sequence inference, and chimera removal. Taxonomic assignment was done using RDP trainset 16. Statistical analyses of microbiome data were performed using the phyloseq (v1.19.1), pscl (v1.4.9), and vegan (v2.4-6) packages in the R statistical environment.

### 2.5. Statistical Analysis

All data are expressed as means ± SEM. The significance of differences in plasma metabolites and gut bacterial abundances were assessed by a two-way ANOVA and zero-inflated negative binomial (ZINB) regression, respectively. p-values were adjusted for multiple comparisons using the Benjamin–Hochberg method. A p-value less than 0.05 was considered to be statistically significant.

### 3. Results

#### 3.1. Effects of High-Na⁺ and Low-K⁺ Diet and Antibiotic Treatment on Food Intake, Weight Gain, and Plasma Na⁺ and K⁺ Levels

Food intake and weight gain were not different between the two diet groups in normal rats (food intake = 46 ± 1 and 48 ± 2 g per day; weight gain = 5.6 ± 0.3 and 6.4 ± 0.9 g per day for the control and the high-Na⁺ and low-K⁺ [HNaLK] diet groups, respectively; p > 0.05 for both). Na⁺ intake was threefold greater in the HNaLK than the control diet group, but plasma Na⁺ concentration was not different between groups (135 ± 1 and 136 ± 1 mEq L⁻¹ for the control and the HNaLK groups, respectively; p > 0.05). K⁺ intake was only 1/20 in the HNaLK, compared to the control diet group, but plasma K⁺ concentration was not significantly different between groups (5.1 ± 0.1 vs 4.7 ± 0.3 mEq L⁻¹ for the control and the HNaLK groups, respectively; p > 0.05). To identify metabolites whose plasma levels are altered by gut microbiota, we treated animals with antibiotics to deplete gut bacteria (see Section 2) and subsequently fed these animals with either the control or the HNaLK diet (n = 7 each) for 1 week while continuing the antibiotic treatment. Food intake, weight gain, and plasma Na⁺ and K⁺ levels in antibiotic-treated animals were not different from those in untreated, normal animals (Table S1, Supporting Information).

#### 3.2. Plasma Metabolites That Were Altered by Antibiotic Treatment

Profiling of polar metabolites, targeting biogenic amines that retain well on hydrophilic interaction chromatography, detected a total of 1271 metabolites, including 315 known metabolites. We first identified metabolites whose plasma levels were significantly altered by gut bacteria by comparing plasma metabolite levels between untreated and antibiotic-treated animals. A two-way ANOVA was performed to detect significant differences between untreated and antibiotic-treated animals (on two different diets). Of the total 1271 polar metabolites, 105 metabolites were significantly different between the two animal groups (p < 0.05). However, after correcting for multiple comparisons, only 45 metabolites (10 known and 35 unknown compounds) retained statistical significance (adjusted p < 0.05; Table 1). Among these metabolites are metronidazole and hydroxymetronidazole. The former is one of the antibiotics used in the antibiotic treatment (see Section 2), and the latter is a metabolite of metronidazole.
Table 1. Plasma metabolites that were significantly altered by antibiotic treatment.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>p-value</th>
<th>Adjusted p-value</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAO</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td>↓</td>
</tr>
<tr>
<td>N-nitrosodiethylamine</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td>↑</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>&lt;0.0001</td>
<td>0.0017</td>
<td>↑</td>
</tr>
<tr>
<td>1-Acetilimidazole</td>
<td>0.0001</td>
<td>0.0040</td>
<td>↑</td>
</tr>
<tr>
<td>Hydroxymetronidazole</td>
<td>0.0001</td>
<td>0.0054</td>
<td>↑</td>
</tr>
<tr>
<td>3-Indolepropionic acid</td>
<td>0.0003</td>
<td>0.0094</td>
<td>↑</td>
</tr>
<tr>
<td>DL-normetanephrine</td>
<td>0.0003</td>
<td>0.0094</td>
<td>↓</td>
</tr>
<tr>
<td>DBPM</td>
<td>0.0004</td>
<td>0.0125</td>
<td>↓</td>
</tr>
<tr>
<td>3-Hydroxybutyrylcarnitine</td>
<td>0.0005</td>
<td>0.0126</td>
<td>↓</td>
</tr>
<tr>
<td>4-Piperidinecarboxamide</td>
<td>0.0006</td>
<td>0.0161</td>
<td>↓</td>
</tr>
<tr>
<td>35 Unknown metabolites</td>
<td>&lt;0.0005</td>
<td>&lt;0.005</td>
<td></td>
</tr>
</tbody>
</table>

The significance of differences between untreated and antibiotic-treated animals (on two different diets) was assessed by two-way ANOVA. p-values were adjusted for multiple comparisons using the Benjamini–Hochberg method (see Section 2). DBPM, 6H-dibenzo[b,d]pyran-9-methanol,3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-, (6aR,10aR)-.

3.4. Plasma Metabolites That Were Altered Both by Antibiotic Treatment and HNaLK Diet

We next attempted to find plasma metabolites that were altered by antibiotic treatment as well as the HNaLK diet. Because of the relatively high testing burden and exploratory nature of this study, we used the following strategies. First, we focused on metabolites altered by antibiotic treatment in animals maintained on the control diet (unadjusted p < 0.05, without correcting for multiple comparisons). This allowed us to reduce the metabolome to 144 relevant metabolites. Among these, we next focused on those that were also altered by the HNaLK diet in untreated animals (unadjusted p < 0.05). Eleven metabolites (four known and seven unknown) met both criteria. Interestingly, all these metabolites decreased and none increased their plasma levels with the diet (Figure 3), suggesting that most of these effects are real, not false positives randomly arising from multiple comparisons. Some of these metabolites were completely depleted by antibiotic treatment (Figure 3F–I), suggesting they derive entirely from gut bacteria.

3.5. Annotation and Classification of the Unknown Metabolites

In order to improve the low identification rates in untargeted metabolomics, we used a multilevel annotation approach with state-of-the-art tools and software such as NIST Hybrid search[37] and ClassyFire.[38] NIST Hybrid search is a mass spectral library search algorithm that annotates compounds that differ from reference library compounds and instead looks for the spectra of “nearest neighbor”. ClassyFire is a fully automated tool that uses chemical structures to assign all known chemical compounds to a taxonomy consisting of several levels (Kingdom, SuperClass, Class, SubClass, etc.). First, we exported the spectra of significant unknown metabolites in the .mgf format. Spectra were then imported into NIST MS PepSearch GUI, hybrid search option was activated, and spectra were matched against NIST17 Hybrid Tandem Library. Second, for each unknown metabolite, top ten ranks were exported (Table S2, Supporting Information). Third, InChIKeys were used for automated classification hosted by the Fiehn laboratory (http://cbf.fiehnlab.ucdavis.edu/), as shown in Table 2. By using NIST Hybrid search in combination with ClassyFire, metabolite annotations of level 3 according to Metabolomics Standards Initiative (MSI 3)[39] were possible for all the unknown metabolites in Figure 3, but not the unknown metabolite of Figure 2 (metabolite 260), which did not trigger fragmentation in auto MS/MS mode.

3.6. Effects of HNaLK Diet on Gut Bacterial Community

We next tested whether high-Na+ and low-K+ intake alters the gut bacterial community. Fecal samples collected from the animals fed the two different diets (without antibiotic treatment; n = 7 for each diet) were subjected to 16S rRNA gene sequencing. High-Na+ and low-K+ diet did not significantly alter diversity or composition of the gut bacterial community (Figure 4A). However, zero-inflated negative binomial (ZINB) regression revealed
4. Discussion

Targeting biogenic amines in the metabolomic approach presented here, we identified numerous plasma metabolites whose plasma levels were significantly altered by gut-bacteria depletion, indicating their regulation by gut bacteria. Many of these metabolites were not previously linked to gut bacteria. Therefore, the present data provide novel insights into...
physiological or pathological roles of gut bacteria (see below). In addition, the present study identified a number of plasma metabolites that were altered by gut-bacteria depletion and by a high-Na\(^+\) and low-K\(^+\) diet. Interestingly, all these metabolites showed decreases, and none showed increases, in their plasma levels after antibiotic treatment or high-Na\(^+\) and low-K\(^+\) diet (Figure 3), suggesting they may be produced by gut bacteria, and this production may be inhibited by high-Na\(^+\) and low-K\(^+\) diet. We also found one unknown metabolite whose plasma level was increased by high-Na\(^+\) and low-K\(^+\) diet similarly in untreated and antibiotic-treated rats (Figure 2). These effects of the diet were observed with significant changes in the gut bacterial taxa Porphyromonadaceae and Prevotellaceae, raising the possibility that the dietary minerals may regulate abundances of certain gut bacteria to alter circulating metabolite levels in the host.

The metabolites that were significantly altered by antibiotic treatment showed three patterns of changes. One group of plasma metabolites was almost completely depleted by antibiotic treatment. These metabolites may be produced exclusively by gut bacteria. To support this idea, TMAO that derives from gut bacteria was depleted in plasma after antibiotic treatment. Also included in this group are normetanephrine (see below) and DBPM, which were not previously linked to gut bacteria. A second group of plasma metabolites was partially depleted by antibiotic treatment, including 3-hydroxybutyrylcarnitine and 4-piperidinecarboxamide. These metabolites may be produced by gut bacteria but may also be produced by the host metabolism or derive from food. Interestingly, a third group of metabolites significantly increased plasma levels after antibiotic treatment. Included in this group is 3-indolepropionic acid, known to be

Figure 2. An unknown metabolite significantly altered by the HNaLK diet. Data are means ± SEM in arbitrary scales for seven animals. *Adjusted \(p < 0.05\) versus control diet.

Figure 3. A–I) Plasma metabolites that were significantly altered both by antibiotic treatment (in animals maintained on control diet; unadjusted \(p < 0.05\)) and HNaLK diet (in untreated animals; unadjusted \(p < 0.05\)). Data are means ± SEM in arbitrary scales for seven animals.
Table 2. Plasmametabolites putatively annotated with NIST Hybrid search and ClassyFire.

<table>
<thead>
<tr>
<th>ID</th>
<th>NIST-Hybrid search</th>
<th>InchiKey</th>
<th>Kingdom</th>
<th>Superclass</th>
<th>Class</th>
<th>Subclass</th>
<th>Parent level 1</th>
<th>Parent level 2</th>
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</thead>
<tbody>
<tr>
<td>2018</td>
<td>Dimethoxycurcumin</td>
<td>HMJSBVCDPKODEX-7408</td>
<td>Organic compounds</td>
<td>Phenylpropanoids</td>
<td>Isoflavonoids</td>
<td>Isoflavanols</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>306</td>
<td>1-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine</td>
<td>PTGWMXDISA-N</td>
<td>Organic compounds</td>
<td>Lipids and lipid-like</td>
<td>Glycerophospholipids</td>
<td>Lysophosphatidylcholines</td>
<td>1-Acyl-sn-glycero-3-glycerophosphocholine</td>
<td>Unknown</td>
</tr>
<tr>
<td>3124</td>
<td>2-Phosphonobutyric acid</td>
<td>UHFFFAOYSA-N</td>
<td>Organic compounds</td>
<td>Lipids and lipid-like</td>
<td>Fatty acids and conjugates</td>
<td>Fatty acids and conjugates</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>3796</td>
<td>PyroGlu-Ile-Arg</td>
<td>ZXOFAKATRUWOBW</td>
<td>Organic compounds</td>
<td>Organic acids and carboxylic acids and amino acids</td>
<td>Peptides and peptides</td>
<td>Oligopeptides</td>
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<td>Unknown</td>
</tr>
<tr>
<td>3748</td>
<td>Equol</td>
<td>ADFCQWZHKCXPAJ-7240</td>
<td>Organic compounds</td>
<td>Phenylpropanoids</td>
<td>Isoflavonoids</td>
<td>Isoflavanols</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

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to changes in norepinephrine metabolism to normetanephrine rather than in plasma levels of its precursor norepinephrine. Seasonal variation in plasma normetanephrine levels were reported.\textsuperscript{[46]} Although this variation was small (i.e., 21% higher in winter than in summer), it resulted in a twofold higher prevalence of false-positive elevations of normetanephrine in winter than in summer. Our data suggest that the use of antibiotics (and dietary minerals) may substantially impact plasma normetanephrine levels. Future studies are warranted to test whether this is also the case in humans; that is, whether plasma normetanephrine levels are altered by antibiotics or Na\textsuperscript{+} or K\textsuperscript{+} intake. If so, the use of antibiotics and diet should be carefully controlled in the diagnosis of pheochromocytoma that is based on plasma normetanephrine concentrations.

Western diets are characterized by high-Na\textsuperscript{+} and low-K\textsuperscript{+} contents. Numerous studies have demonstrated that high-Na\textsuperscript{+} and low-K\textsuperscript{+} intake is associated with increased risk of hypertension or CVD. Diet is known to have a profound impact on gut bacterial composition.\textsuperscript{[47]} However, very few studies have investigated whether dietary Na\textsuperscript{+} or K\textsuperscript{+} influences the gut microbiota. As discussed in Section 1, we have reasons to suspect effects of high-Na\textsuperscript{+} and low-K\textsuperscript{+} diet on gut microbiota. Our microbiome profiling data suggest that high-Na\textsuperscript{+} and low-K\textsuperscript{+} intake does not drastically reshape gut bacterial communities, although we cannot exclude the possibility that exposure to the diet for an extended period, much longer than the 1 week in the present study, may significantly impact the gut bacterial community. However, our findings of increased Prevotellaceae in the high-Na\textsuperscript{+} and low-K\textsuperscript{+} group corroborate a recent study of mice fed a high-salt diet, and further reinforces the importance of this bacterial taxon in mediating diet-induced disease states.\textsuperscript{[48]} Additionally, we found a number of plasma metabolites (known and unknown) that seem to be altered by antibiotic treatment and by high-Na\textsuperscript{+} and low-K\textsuperscript{+} diet, suggesting possible interaction of the diet and the gut microbiota in the regulation of these metabolites. One of the unknown metabolites (ID 7481, Figure 3) was annotated to be an equol-like compound (Table S2, Supporting Information). This is quite interesting, as equol is exclusively a product of daidzen metabolismin specific gut bacteria.\textsuperscript{[49,50]} If this unknown metabolite is an equol metabolite, our data might indicate that high-Na\textsuperscript{+}
and low-K⁺ diet decreases bacterial production of equol. Equol is known to bring about various health beneficial effects, including those on hypertension and CVD.\(^{[40,50]}\) Taken together, our data suggest the provocative idea that one mechanism by which high-Na⁺ and low-K⁺ diet improves hypertension or CVD may involve modulation of equol production (or metabolism) in specific gut bacteria. Further studies are warranted to verify high-Na⁺/low-K⁺ diet effects on plasma metabolites (Figure 3) and to elucidate the chemical structures of unknown metabolites. Such studies would provide new insights into the roles of gut bacteria and dietary Na⁺ and K⁺ in host physiology and pathology, possibly including hypertension or CVD.

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

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
antibiotics, cardiovascular disease, dietary minerals, gut bacterial profiling, metabolomics

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