RESEARCH ARTICLE | Gut Microbiota in Health and Disease

Alterations in the gut microbiota can elicit hypertension in rats

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¹University of Houston, Houston, Texas; ²Department of Anesthesiology, Baylor College of Medicine, Houston, Texas; ³Department of Virology and Microbiology, Baylor College of Medicine, Houston, Texas; and ⁴Department of Neurology, University of Texas Health Sciences Center, Houston, Texas

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Adnan S, Nelson JW, Ajami NJ, Venna VR, Petrosino JF, Bryan RM Jr, Durgan DJ. Alterations in the gut microbiota can elicit hypertension in rats. Physiol Genomics 49: 96-104, 2017. First published December 23, 2016; doi:10.1152/physiolgenomics.00081. 2016.—Gut dysbiosis has been linked to cardiovascular diseases including hypertension. We tested the hypothesis that hypertension could be induced in a normotensive strain of rats or attenuated in a hypertensive strain of rats by exchanging the gut microbiota between the two strains. Cecal contents from spontaneously hypertensive stroke prone rats (SHRSP) were pooled. Similarly, cecal contents from normotensive WKY rats were pooled. Four-week-old recipient WKY and SHR rats, previously treated with antibiotics to reduce the native microbiota, were gavaged with WKY or SHRSP microbiota, resulting in four groups; WKY with WKY microbiota (WKY g-WKY), WKY with SHRSP microbiota (WKY g-SHRSP), SHR with SHRSP microbiota (SHR g-SHRSP), and SHR with WKY microbiota (SHR g-WKY). Systolic blood pressure (SBP) was measured weekly using tail-cuff plethysmography. At 11.5 wk of age systolic blood pressure increased 26 mmHg in WKY g-SHRSP compared with that in WKY g-WKY (182 \pm 8 vs. 156 \pm 8 mmHg, P = 0.02). Although the SBP in SHR g-WKY tended to decrease compared with SHR g-SHRSP, the differences were not statistically significant. Fecal pellets were collected at 11.5 wk of age for identification of the microbiota by sequencing the 16S ribosomal RNA gene. We observed a significant increase in the Firmicutes:Bacteroidetes ratio in the hypertensive WKY g-SHRSP, as compared with the normotensive WKY g-WKY (P = 0.042). Relative abundance of multiple taxa correlated with SBP. We conclude that gut dysbiosis can directly affect SBP. Manipulation of the gut microbiota may represent an innovative treatment for hypertension.

microbiota; dysbiosis; hypertension; spontaneously hypertensive rat; short chain fatty acids

IN THE PAST DECADE it has become increasingly apparent that an imbalance of the microbiota (dysbiosis) in the gut has pathological effects beyond the gastrointestinal system. For example, gut dysbiosis has been shown to be an underlying cause or strongly associated with obesity, insulin resistance, cancer, and central nervous system disorders to include anxiety, depression, autism spectrum disorders, and multiple sclerosis (3, 11, 15, 22, 24, 29, 32). While the evidence linking gut dysbiosis to pathological states is convincing, the mechanism(s) for this link are not well understood in most cases. One well-charac-

terized association involves gut microbiota in the development of atherosclerosis. Bacteria in the gut metabolize choline and L-carnitine in food to trimethylamine (14, 21). The trimethylamine crosses the gut-epithelial barrier where it is carried via the portal circulation to the liver. In the liver, trimethylamine is subsequently metabolized to trimethylamine N-oxide, a proatherogenic molecule. Thus, a "diet-microbe-host interaction" can act to promote atherosclerosis and potentially other cardiovascular diseases (4).

Although numerous articles have speculated or discussed a potential role for gut dysbiosis in the development of other cardiovascular diseases, relatively few studies have addressed this issue directly. Several animal models of hypertension and a small cohort of humans suggests that gut dysbiosis is associated with hypertension (16, 17, 23, 33). Durgan et al. (8) demonstrated a causal role of the gut microbiota in the development of obstructive sleep apnea-induced hypertension in rats fed a high-fat diet. One potential link between dysbiosis and hypertension can involve bacterially produced short chain fatty acids (SCFAs), which appear to have a role in regulating blood pressure by acting on various G protein coupled receptors (27). In addition the SCFA butyrate has beneficial effects in the gut wall including maintaining gut barrier integrity and influencing intestinal inflammation (2, 6). Interestingly, bacteria that produce butyrate are decreased in animal models of hypertension (8, 33). Further evidence for a dysbiosis-hypertension link comes from studies showing that supplementing the diet with probiotics (beneficial microorganisms found in the gut) can have modest effects on blood pressure, especially in hypertensive models (12, 20).

One of the most highly studied animal models of hypertension is the spontaneously hypertensive rat (SHR), a strain developed by breeding Wistar-Kyoto rats (WKY) with high blood pressure (25, 26). The SHR begins to develop hypertension around 6–8 wk of age and plateaus near 200 mmHg systolic blood pressure by ~15 wk. As mentioned above Yang et al. (33) reported that the SHR demonstrate gut dysbiosis when compared with the WKY parent strain. If dysbiosis is a component of this genetic model for hypertension, we reasoned that hypertension could be induced in a normotensive strain (WKY) of rats or attenuated in a hypertensive strain (SHR) of rats by exchanging the gut microbiota between the two strains. Thus, we tested the hypothesis that hypertension can be induced or attenuated by controlling the gut microbiota in SHR and WKY rats.

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Table 1. Treatment groups

Recipient Strain	10-Day Treatment	4-Day Treatment Followed by Weekly Treatment	Group Name
WKY	antibiotic cocktail antibiotic cocktail PBS antibiotic cocktail antibiotic cocktail PBS	supernatant from WKY cecal and colon suspension	WKY g-WKY
WKY		supernatant from SHRSP cecal and colon suspension	WKY g-SHRSP
WKY		PBS	WKY
SHR		supernatant from WKY cecal and colon suspension	SHR g-WKY
SHR		supernatant from SHRSP cecal and colon suspension	SHR g-SHRSP
SHR		PBS	SHR

MATERIALS AND METHODS

All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals, 8th edition*, published by the National Institutes of Health (NIH) and were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine, Houston, TX. All rats had ad libitum access to normal rat chow (LabDiet 5V5R, St. Louis, MO) and water throughout the study.

Gut microbiota transplant. Cecal and colon contents were collected and pooled from 4 WKY rats (9 wk of age) after isoflurane anesthesia and decapitation. Similarly, cecal and colon contents were collected and pooled from 4 stroke-prone spontaneously hypertensive rats (SHRSP) (19 wk of age). We used stroke-prone rats, a substrain of the SHR, as donors since we have an established in-house colony (derived from Charles Rivers stock). We reasoned that gut dysbiosis should be fully developed by 19 wk of age, a time when hypertension has plateaued and stabilized. The WKY donor rats were also bred in-house. Each of the pooled samples was diluted 1:20 in sterile PBS and centrifuged at 1,000 rpm for 5 min, and the supernatant from each pooled sample was aliquoted and frozen at -80°C .

Recipient WKY and SHRs, received from Charles Rivers (Houston, TX) at 3.5 wk of age, were housed in a satellite facility with a 12 h light (6 AM–6 PM): 12 h dark (6 PM–6 AM) cycle. To reduce the native microbiota load, allowing for easier colonization of gavaged microbiota, recipient WKY and SHRs (4.5 wk of age) were orally gavaged with 1 ml of a broad spectrum antibiotic cocktail consisting of ampicillin, gentamycin, metronidazole, neomycin (each at 0.25 mg/ml), and vancomycin (0.125 mg/ml) once daily for 10 consecutive days (8). Following antibiotic treatment, quantitative PCR measurement of the 16S rRNA gene was performed to ensure that the effects of antibiotics on the microbiota was similar between WKY and SHRs. 16s rRNA gene copy number was found to be $4.4 \times 10^6 \pm 2.5 \times 10^5$ and $4.9 \times 10^6 \pm 3.1 \times 10^5$ for WKY and SHR, respectively, and not significantly different between strains. Two days after the last antibiotic administration, 750 μ l of the cecal/colonic supernatant was

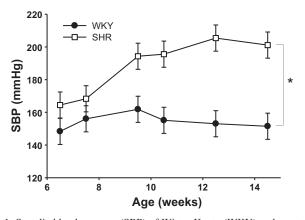
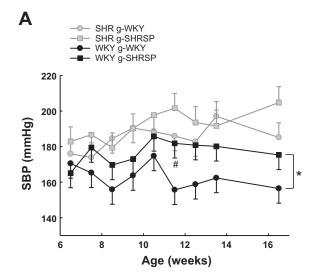


Fig. 1. Systolic blood pressure (SBP) of Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) gavaged with PBS by age. Data are shown as means \pm SE, n=6-7, *P=0.006 for WKY vs. SHR.

gavaged into the recipient rats daily for 4 consecutive days and weekly thereafter. Cecal/colonic supernatant from the SHRSP donors was gavaged into WKY and SHRs. In a similar manner, cecal/colonic supernatant from the WKY donors was gavaged into WKY and SHRs (n=6 to 7/group). In addition to the above rats, 6 SHR and 6 WKY rats were gavaged as described above but the antibiotic cocktail and the donor supernatant was replaced by PBS. The groups of rats involved in the study are shown in Table 1.

Blood pressure measurements. Beginning at 6.5 wk, before the first microbiota gavage treatment, systolic blood pressure (SBP) was measured using a six channel CODA high-throughput (Kent Scien-



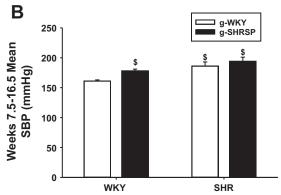


Fig. 2. Stroke-prone spontaneously hypertensive rat (SHRSP) microbiota increases SBP in WKY rats. A: SBP of 4 microbiota gavage treatment groups over time. Three-way repeated measures ANOVA, P < 0.001 for main effects of strain, gavage, and age. Two-way repeated measures ANOVA, *P = 0.028 for main effect of gavage, *P < 0.05 for WKY g-WKY vs. WKY g-SHRSP. *B: mean SBP from 7.5 to 16.5 wk (all time points during microbiota gavage treatments). Two-way repeated measures ANOVA, *P < 0.05 relative to WKY g-WKY. Data are shown as means \pm SE, *n = 6-7.

tific, Torrington, CT) tail-cuff blood pressure system. SBP was assessed every 7–10 days for 7 wk, and a final measurement taken at 16.5 wk old. Rats were acclimatized to the system for 2 wk before the initial measurement. While tail-cuff measurement does not provide the same resolution as telemetry, we have demonstrated that SBP values obtained using the CODA tail-cuff system are highly comparable and not significantly different than direct arterial measurements made in the same rat at the same time of day (Durgan DJ, unpublished observations).

Gut microbiota analysis. The gut microbiota was analyzed as previously described (8). In brief, fecal samples were collected in sterile tubes at 11.5 wk of age and stored at -80° C. DNA was extracted using MO BIO PowerMag Soil DNA Isolation Kit (MO BIO Laboratories), according to the manufacturer's protocol. 16S rRNA gene sequence libraries were generated using the V4 primer region on the Illumina MiSeq platform by the Center for Metagenomics and Microbiome Research at the Baylor College of Medicine. Using the quality trimming features in QIIME (v.1.7.0), we removed 16S rRNA gene sequences with ambiguous base calls or having quality scores <20 (7). After barcodes and primers were trimmed, all remaining reads were clustered into operational taxonomic units (OTUs) by closed-reference OTU-picking with a 97% similarity threshold using

the UCLUST algorithm and Greengenes reference database (v13.5) as implemented in QIIME (9). OTU identities were assigned using the Greengenes (v13.5) database and a confidence score of \geq 97%. All 16S rRNA gene sequence libraries were randomly subsampled to 20,700 sequences per sample and singletons removed before downstream analysis, including the calculation of diversity indices and comparison of relative abundances.

Linear discriminate analysis effect size. Linear discriminate analysis effect size (LEFSe) analysis was performed through the Huttenhower laboratory galaxy site (https://huttenhower.sph.harvard.edu/galaxy) using subsampled 16S rRNA gene sequence data (without prescreening) isolated from fecal samples (described above). The LEFSe algorithm was used to identify taxa characterizing the differences between two groups (e.g., WKY g-WKY vs. WKY g-SHRSP) (30).

Targeted metabolomics analysis. Feces from 10 wk old WKY g-WKY, WKY g-SHRSP, SHR g-WKY, and SHR g-SHRSP rats were submitted to the Metabolomics Core at Baylor College of Medicine for processing and analysis. A panel of 11 metabolites, including SCFAs, neurotransmitters, and amino acids was measured. SCFAs in feces were quantitated using a standard curve generated by labeled SCFAs, while neurotransmitters and amino acids are presented as fold change relative to WKY g-WKY. All samples were

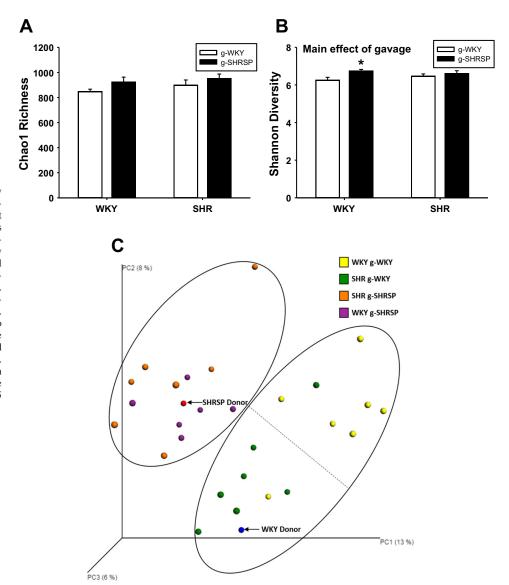


Fig. 3. Measures of alpha- and beta-diversity vary by strain and microbiota gavage treatments. A: strain and gavage treatment did not affect the microbial community richness (Chao1 index). B: SHRSP microbiota increases the microbial community diversity (Shannon index) of WKY rats. C: principal coordinate analysis of WKY and SHRs gavaged with WKY or SHRSP cecal contents. Unifrac analysis was used to generate distance measurements between each sample. Two clusters were formed corresponding to the gavaged microbiota. Also shown is the 3-dimensional localization of the WKY and SHRSP cecal content used for gavages. Dashed line denotes separation between WKY g-WKY and SHR g-WKY. Data are shown as means \pm SE n = 6-7, *P < 0.05for WKY g-WKY vs. WKY g-SHRSP.

measured by LC-MS (Agilent LC-QQQ-MS system), targeted metabolites were identified by their unique multiple reaction monitoring transition, and analysis carried out using MassHunter (Agilent).

Statistics. Line and bar plot data are expressed as means \pm SE. For analyzing the change in blood pressure, we used a three-way ANOVA and a two-way repeated-measures ANOVA. The later was followed by a Holm-Sidak test for individual comparisons when appropriate. Spearman rank order correlation was used to examine potential relationships between taxa relative abundance and SBP. For microbiome analysis, alpha- and beta-diversity indexes were calculated in QIIME. Normality was evaluated by the Shapiro-Wilk test. Two-way analysis of variance was performed followed by Holm-Sidak post hoc analysis when main effects were found to be significant. Differences were considered statistically significant if $P \le 0.05$.

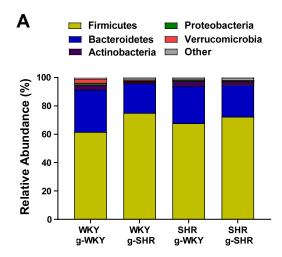
RESULTS

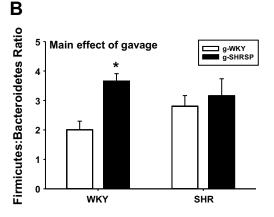
Figure 1 shows SBP as a function of age in SHR and WKY rats gavaged with only PBS (see Table 1 for groups). As expected SHRs developed hypertension that plateaued between 11 and 13 wk. SBP in the WKY rats increased slightly over the course of 10 wk but remained significantly less than SHRs. Figure 2 demonstrates changes in SBP of WKY and SHRs gavaged with the two donor contents. A three-way ANOVA of all four groups during the 10 wk represented in Fig. 2A revealed significant differences in strain (P < 0.001), donor gavage (P < 0.001), and age (P < 0.001). When we considered SBP in the WKY rats from 7.5 wk to 16.5 wk (i.e., all time points with microbiota gavage treatment), a two-way repeatedmeasures ANOVA revealed a significant increase in SBP in WKY g-SHRSP compared with WKY g-WKY (Fig. 2, P =0.028). The mean SBP in WKY rats between the ages of 7.5 and 16.5 wk was 178 ± 3 and 161 ± 2 mmHg for those gavaged with SHRSP and WKY contents, respectively, with the greatest difference (26 mmHg) occurring at 11.5 wk (Fig. 2, P = 0.02). Thus, hypertension developed in WKY g-SHRSP compared with WKY g-WKY.

Figure 2 also shows the SBP of SHRs with different gavages. While there was a tendency for SBP to decrease in SHR g-WKY (with the exception of 13.5 wk) this decrease was not statistically significant. The mean SBP for SHR between 7.5 and 16.5 wk was 194 ± 7 and 186 ± 7 for those gavaged with SHRSP and WKY contents, respectively (Fig. 2B).

We next sought to examine the microbiota composition of WKY and SHR gavaged with native or transplanted microbiota. Figure 3 demonstrates the effects of genotype and gavage contents on measures of alpha (Fig. 3, A and B) and beta diversity (Fig. 3C). The Chao1 richness index, a measure of the total number of distinct genera in a sample, was not significantly different between genotypes or altered by the type of gavage (Fig. 3A). The Shannon diversity index, which takes into account richness and abundance, was significantly different depending on the type of gavage and significantly elevated in WKY g-SHRSP as compared with WKY g-WKY (Fig. 3B). UniFrac and principle coordinate analyses were used to calculate and visualize measurements of distance between each sample, with more similar samples clustering closely together. The three-dimensional scatter plot in Fig. 3C illustrates a separation of two groups that is dependent on the gavaged microbiota. WKY g-WKY cluster closely with SHR g-WKY, while WKY g-SHRSP microbiota cluster more closely with SHR g-SHRSP microbiota. It is worth noting that the two groups receiving SHRSP microbiota cluster more tightly as compared with those receiving the WKY microbiota (Fig. 3*C*). Also shown is the location of the pooled cecal contents supernatant from WKY and SHRSP used for gavage treatments.

An increase in the Firmicutes:Bacteroidetes (F:B) ratio, resulting from increased abundance of the phylum Firmicutes along with a decrease in Bacteroidetes, is a well-established marker of gut dysbiosis and associated with numerous pathologies (10). Figure 4A illustrates the relative abundance of the major phyla present in the gut of WKY and SHRs after microbiota gavage treatments. Compared with WKY g-WKY, WKY g-SHRSP had a significantly decreased abundance of Bacteroidetes, increased Firmicutes abundance, and increased F:B ratio (Fig. 4B). The F:B ratio of WKY g-SHRSP was not significantly different from the SHR g-SHRSP. These data

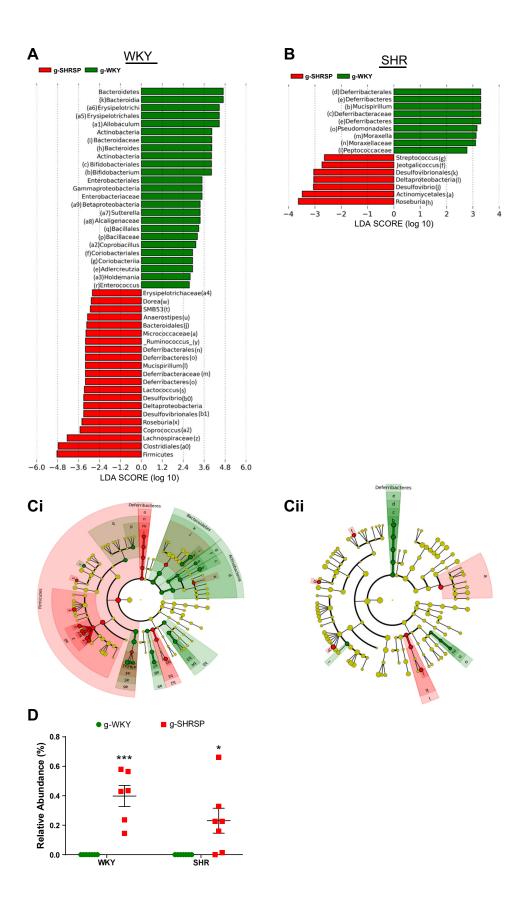




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Phyla	WKY g-WKY	WKY g-SHRSP	SHR g-SHRSP	SHR g-WKY	
Bacteroidetes	31.7	20.8*	24.9	26.2	
Firmicutes	59.6	75.0*	69.7	67.8	

Relative Abundance (%)

Fig. 4. Comparison of phyla between WKY and SHR strains receiving WKY or SHRSP gavage treatments. A: relative abundance of the major phyla of the gut microbiota. B: increased Firmicutes:Bacteroidetes ratio in WKY g-SHRSP, as compared with WKY g-WKY, is due to expansion of Firmicutes as well as contraction of Bacteroidetes. Data are shown as means \pm SE n = 6–7, *P < 0.05 for WKY g-WKY vs. WKY g-SHRSP.



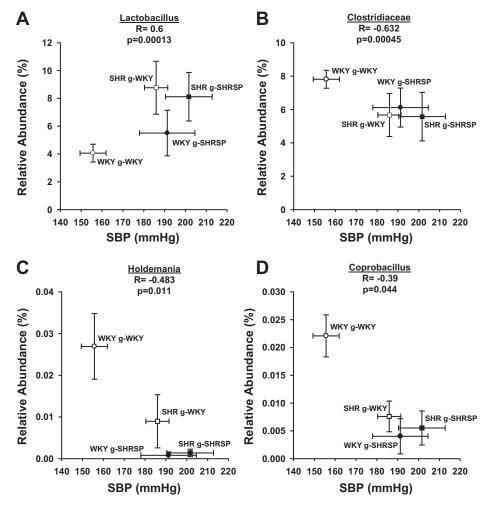


Fig. 6. Shifts in taxon abundance that correlate with SBP. A: the lactate-producing genus Lacto-bacillus positively correlates with SBP. B-D: the butyrate-producing family Clostrideaceae and acetate-producing genera Holdemania and Co-probacillus negatively correlate with SBP. Data are shown as means \pm SE n = 6-7.

suggest that, at the phylum level, the microbiota of WKY rats could be "switched" to resemble an SHRSP. However, transplantation of the WKY microbiota into the SHR genotype appeared less effective, with the F:B ratio of SHR g-WKY not significantly different than SHR g-SHRSP (Fig. 4B).

Figure 5 shows the bacterial taxa that were altered by gavage treatment in the WKY (Fig. 5*A*) and SHR (Fig. 5*B*) genotypes, calculated by LEFSe analysis (30). The fold change in relative abundance (to the log₁₀), vs. the alternative gavage treatment of statistically significant taxa are depicted on the horizontal axis. On the WKY background WKY and SHRSP gavages resulted in 25 and 21 taxa that were highly characteristic of the respective gavage (Fig. 5*A*). On the SHR background WKY and SHRSP gavages resulted in 9 and 7 taxa that were characteristic of the respective gavage (Fig. 5*B*). In Fig. 5*C* the taxa found to be significantly different due to gavage treatments (Fig. 5, *A* and *B*) are visualized in cladograms. In each

cladogram the hierarchal position of taxa can be seen, with the inner circle of nodes representing phyla and the outer nodes representing genera. Visualizing the taxa characteristic to WKY g-WKY (green) or WKY g-SHRSP (red) in a cladogram illustrates a distinct separation of gut microbiota composition on the WKY background (Fig. 5Ci). On the SHR background not only were fewer taxa characteristic of gavage treatment (Fig. 5B), but those that were did not clearly segregate on the cladogram (Fig. 5Cii). Further analysis of genera that were highly characteristic of either WKY or SHRSP gavage treatment led us to examine the relative abundance of the sulfurreducing bacteria Desulfovibrio. Desulfovibrio was found to account for 0.4 and 0.2% of the 16S rRNA sequences in WKY g-SHRSP and SHR g-SHRSP, respectively. Interestingly, Desulfovibrio appears unique to the SHRSP microbiota as it was undetectable in either strain receiving WKY gavage treatments (Fig. 5D).

Fig. 5. Gavage treatments alter the relative abundance of multiple bacterial taxa. Linear discriminate analysis effect size (LEFSe) analysis was used to calculate a linear discriminate analysis (LDA) score for taxa that characterize WKY vs. SHRSP gavage treatment of WKY rats (A), WKY vs. SHRSP gavage treatment of SHR rats (B). Positive LDA scores indicate the enrichment of taxa in g-WKY (green) relative to g-SHRSP (red), and negative LDA scores indicate the depletion of taxa in g-WKY relative to g-SHRSP. Given this relationship, the negative LDA scores can also be interpreted as enrichment in g-SHRSP (red) relative to g-WKY (green). C: taxa found to be significantly different by LEFSe analysis (from A and B) are shown in cladograms to illustrate the phylogenetic relationship between altered taxa. Nodes labeled alpha-numerically in C correspond to the labels in parenthesis in A and B. D: relative abundance of the genus Desulfovibrio in WKY and SHRs gavaged with WKY or SHRSP microbiota. *P < 0.05 relative to WKY g-WKY, p = 6–7.

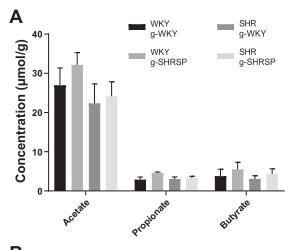
We next examined any potential relationships between bacterial taxon abundance and SBP at 11.5 wk, the time point when fecal samples were collected. While there are only a limited number of studies examining the role of the microbiota in hypertension, our laboratory and others have demonstrated increased lactate-producing bacteria present in various models of hypertension (8, 33). Consistent with previous studies we observed a strong positive correlation (R = 0.6, P = 0.00013) between the lactate-producing genus Lactobacillus and SBP, with SHR g-SHRSP exhibiting a twofold increase in Lactobacillus as compared with WKY g-WKY (Fig. 6A). Previous studies have also demonstrated diminished acetate- and butyrate-producing taxa in numerous models of hypertension (8, 33). We observed a strong negative correlation between abundance of the family Clostridiaceae (R = -0.632, P =0.00045), known to include numerous butyrate-producing genera, and SBP (Fig. 6B). Similarly, relative abundance of the acetate-producing genera *Holdemania* (R = -0.483, P =0.011) and Coprobacillus (R = -0.39, P = 0.044) were negatively correlated with SBP, with greatest abundance in WKY g-WKY (Fig. 6, C and D). Of interest, it is obvious in each panel of Fig. 6 that WKY g-SHRSP clusters closely with SHR g-SHRSP, while SHR g-WKY more closely associates with SHR g-SHRSP; further evidence that shifting the microbiota was more proficient on the WKY background.

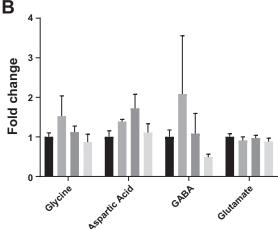
In an effort to identify metabolites that may be linked to hypertension we examined a panel of 11 metabolites in feces isolated from WKY and SHR gavaged with WKY or SHRSP microbiota. This targeted metabolomics panel included SCFAs, neurotransmitters, and amino acids. Despite observed differences in 16S rRNA sequences that would suggest alterations in SCFA production [Fig. 5 and (33)], two-way ANOVA revealed no significant differences in acetate, propionate, or butyrate due to genotype or gavage treatment (Fig. 7A). Similarly, there were no significant differences in the measured neurotransmitters or amino acids due to genotype or gavage treatment (Fig. 7, B and C).

DISCUSSION

The influence of the gut microbiota extends well beyond the gastrointestinal tract. In the current study we tested the hypothesis that the gut microbiota of SHRs contributes to the hypertensive phenotype. To address this hypothesis we utilized Koch's third postulate, which states that if a microorganism(s) contributes to disease, then transferring the microorganism(s) to a healthy organism should induce disease. In these studies we demonstrate that, indeed, transferring microorganisms (i.e., cecal contents) induced the disease state, hypertension. We report three major findings in the present study: 1) The gut microbiota of SHRSP is dysbiotic and significantly different than the microbiota of WKY rats; 2) The SHRSP microbiota is capable of increasing SBP in otherwise normotensive rats; 3) the efficiency by which a microbiota can be altered by gavage transplants varies depending on the host.

Through the use of gut microbiota transplantations we have previously demonstrated a causal role for gut dysbiosis in obstructive sleep apnea (OSA)-induced hypertension (8). Using a similar strategy in this study, we demonstrate that transplantation of the SHRSP microbiota into WKY rats results in significant increases in blood pressure, as compared with





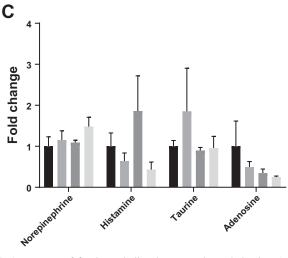


Fig. 7. Assessment of fecal metabolites by targeted metabolomics. A: short chain fatty acid concentrations. B and C: selected neurotransmitters and amino acids were not significantly different between WKY and SHRs gavaged with WKY or SHRSP microbiota. Data are shown as means \pm SE n = 6-7.

WKY rats transplanted with their native WKY microbiota (Fig. 2). We have now demonstrated, in two separate models (i.e., SHR and OSA), that the gut microbiota plays a causal role in the development of hypertension. It is worth noting that while the SBP of WKY g-SHRSP is significantly greater than WKY

g-WKY, it is still lower than SHR g-SHRSP. This suggests that numerous mechanisms, one of which appears to be gut dysbiosis, are influencing the hypertensive phenotype of SHR and SHRSP.

We also tested the hypothesis that hypertension would be attenuated in SHR gavaged with WKY microbiota. While there was a slight trend for lower blood pressure in SHR gavaged with WKY microbiota, as compared with SHR gavaged with SHRSP microbiota, this did not reach statistical significance. Efficiency of the microbiota transplantations (discussed below) may have contributed to the lack of SBP improvement in SHR gavaged with a WKY microbiota.

We demonstrate that the composition of the WKY and SHRSP donor contents are clearly different as noted by the UniFrac and principal coordinate analysis (red and blue points in Fig. 3C). Furthermore, principle coordinate analysis demonstrates that gavaging WKY rats with SHRSP microbiota successfully "switched" the WKY microbiota to resemble that of an SHRSP (Fig. 3C). However, switching the microbiota of SHR to resemble the WKY donor proved less successful. The inability to alter the microbiota of SHR to resemble the WKY is shown in Figs. 3–5, but most obvious in Fig. 3C, where there is still separation between WKY and SHR recipients gavaged with WKY microbiota (noted by dashed line). Therefore we cannot draw conclusions that rely on SHR with a WKY microbiota.

An increase in the F:B ratio is considered a hallmark of gut dysbiosis and observed in multiple disease states. We observe an increased F:B ratio in WKY g-SHRSP, as compared with WKY g-WKY, caused by an increase in the relative abundance of Firmicutes and a decrease of Bacteroidetes (Fig. 4). Additionally, on the WKY background a number of taxa were found to be characteristic of either g-WKY or g-SHRSP (Fig. 5). The genera Bacteroides and Bifidobacterium, which are generally considered beneficial taxa, were associated with the normotensive WKY microbiota. Of interest, the genus Adlercreutzia, showed an increased abundance in the WKY microbiota. Adlercreutzia metabolizes epigallocatechin gallate (EGCG) to metabolites that inhibit angiotensin-I converting enzyme, increase endothelial nitric oxide production, and decrease blood pressure (13, 28, 31). Additionally, the genus Desulfovibrio, which reduces sulfate to hydrogen sulfide (H₂S), was present in both groups receiving the SHRSP microbiota and absent in groups receiving WKY microbiota (Fig. 5). Notably, H₂S inhibits epithelial oxidation of butyrate, the primary energy source for colonic epithelium, which has been shown to lead to impaired gut barrier function and inflammation (1, 19).

When examining potential relationships between taxon abundance and SBP, we observed a strong positive correlation between SBP and the lactate-producing genus *Lactobacillus* (Fig. 6A). While a potential link between lactate and blood pressure regulation is not fully understood, plasma lactate levels have been shown to be associated with an increase in blood pressure (18). We also observed negative correlations between SBP and the abundance of the SCFA-producing Clostrideaceae (butyrate producers), *Holdemania*, and *Coprobacillus* (acetate producers) (Fig. 6, *B*–*D*). Similar shifts in SCFA and lactate-producing bacteria have been reported in the SHR, ANG II infusion, and OSA-induced hypertension models (8, 33). Given the importance of SCFA production in maintaining gut barrier function and reducing gut wall inflammation, we

measured SCFA concentrations in feces from WKY and SHR rats gavaged with WKY or SHRSP microbiota (6). However, no significant differences were observed in acetate, propionate, or butyrate in the feces of any groups (Fig. 7A). Additionally, there were no significant differences between any groups in a panel of eight selected neurotransmitters and amino acids (Fig. 7, B and C). However, it is worth noting that fecal concentrations of SCFAs, and likely other bacterial metabolites, do not reflect concentrations in the cecum or other intestinal regions (5). Therefore, we cannot rule out that intestinal SCFA concentrations, or other metabolites, correlate with changes in SBP.

In summary, the gut microbiota in a hypertensive strain of rats (SHRSP) is sufficient to produce increased blood pressure in its normotensive parent strain (WKY). Thus, our studies provide strong evidence that the gut microbiota has a causal role in the development of hypertension. The fact that hypertension can be induced by altering the microbiome in the WKY rats provides further evidence for the continued study of the microbiota in the development of hypertension in humans and supports a potential role for probiotics as treatment for hypertension (12, 20).

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.A., J.W.N., N.J.A., and D.J.D. performed experiments; S.A., J.W.N., N.J.A., V.R.V., J.F.P., R.M.B.J., and D.J.D. edited and revised manuscript; S.A., J.W.N., N.J.A., V.R.V., J.F.P., R.M.B.J., and D.J.D. approved final version of manuscript; R.M.B.J. and D.J.D. analyzed data; R.M.B.J. and D.J.D. interpreted results of experiments; R.M.B.J. and D.J.D. prepared figures; R.M.B.J. and D.J.D. drafted manuscript; D.J.D. conceived and designed research.

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