Supplemental Digital Content 1.

Genetically determined hypertensive phenotype affects gut microbiota composition, but not vice versa.

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Extended Materials and Methods:

Surgical implementation of the telemetric transmitter

The inguinal region and underbelly were shaved, disinfected and covered with a sterile surgical drape. After palpation of the pulse at the femoral artery, the skin was cut (0.5 - 1 cm) along the limb's long axis. A vascular-nervous bunch was prepared, and the femoral artery was dissected. The transmitter catheter was inserted to a depth of 3-4 cm through the incision in the vessel and stabilized by two single sutures. Subsequently, the dissection of subcutaneous tissue was carried out by forming a subcutaneous pocket where the telemetric transmitter was subsequently placed. The muscle layer and skin were closed with a suture. After recovering from the anesthesia, the rats were returned to the local animal facility and were kept in the conditions described above. Rats were housed individually in cages to avoid damaging the sutures and the catheter.

Antibiotic treatment

Fecal transplant recipients were treated with Neomycin (POLFA S.A. Poland), a wide-spectrum antibiotic, to decontaminate the gastrointestinal tract before the fecal transplantation. Neomycin, rather than a mixture of various antibiotics, was used since the neomycin does not cross the gut-blood barrier. Specifically, the rats received neomycin (1 g/L) dissolved in tap water for 5 consecutive days (8-12 day of the experiment). 48 hours before the fecal transplantation, neomycin treatment was discontinued to prevent the effect of the antibiotic on the transplant.

Stool sample collection and processing

For genomic analysis (of the 16s rRNA), one sample of 0.5 mL of fresh stool was collected from the removed colon and was immediately frozen at -80°C. The remaining samples were weighed and homogenized with 1 mL of 0.9% NaCl in a closed 2 mL laboratory tube by vortexing for 5 min. Next, the sample was centrifuged for 12 minutes at 8000 RPM, and 1 mL of the obtained supernatant was transferred to a laboratory tube and centrifuged again, as mentioned previously. All procedures were performed at a temperature of 2–5 °C. The supernatants were collected into the Eppendorf tubes and frozen at −20 °C.

16S Metagenomic Library

To generate NGS (next-generation sequencing) libraries, 25 ng of DNA was used. Amplification of the V3-V4 region of the 16S rRNA gene was performed according to the 16S Metagenomic Sequencing Library Preparation protocol designed by Illumina (San Diego, USA). Briefly, the V3-V4 region of the 16S rRNA was amplified from bacterial DNA using a KAPA HiFi HotStart Ready Mix (Wilmington; USA) with the following primers: (forward) 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and (reverse) 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'. Each NGS library was tagged by sequencing adapters using the Nextera XT Index Kit, pooled in equimolar amounts and sequenced on an Illumina MiSeq (300 nt, paired-end reads).

Bioinformatics

The NGS Reads were evaluated based on their quality measured by phred quality score (>30) using the FastQC software. Reads not fulfilling the quality criteria and adapters were removed from the set using Trimmomatic [1]. The remaining reads were analyzed using the QIIME 1 – a bioinformatics pipeline for performing microbiome analysis. In the study, as an OTU (operational taxonomic units) picking strategy, we employed an open-reference OTU protocol against the Greengenes database (97% similarity threshold) [2]. Chimeric sequences were identified by a ChimeraSlayer and removed from the dataset, similar to the identified singletons [3]. In the study, the OTU matrix was normalized by a metagenomeSeq's CSS (cumulative sum scaling) transformation [4]. Statistical tests, beta diversity analysis to compare group-to-group population differences (examined by the Principal Coordinate Analysis (PCoA) based on weighted and unweighted Unifrac distance matrixes) and bacterial abundance were assessed using scripts provided by the QIIME. Alpha diversity metrics were measured using the Chao1 and Shannon diversity index from the QIIME and, based on data, paired and unpaired t-tests with $p < 0.05$ were applied. Results were visualized by a phyloseq R package and PhyloToAST software [5,6].

Histopathology of the colon

Tissues sections fixed in 10% buffered formalin were dehydrated using graded ethanol and xylene baths and embedded in paraffin wax. Sections 3-4 μm thick were stained with hematoxylin and eosin (HE). General histopathological examination was evaluated at a magnification of 10x, 40x and 100x (objective lens) and 10x (eyepiece) and images were taken. The mucosa and submucosa of the colon, crypts and their cell composition, blood vessels of mucosa and submucosa, enterocytes with brush border and goblet cells were examined. The morphometric analysis included: the height of the mucosa - measured at a magnification of 10x (objective lens) and 10x (eyepiece), and the height of enterocytes - measured at a magnification of 40x (objective lens) and 10x (eyepiece). Goblet cells and intraepithelial lymphocytes (IELs) were counted in the epithelium and scored per 100 enterocytes in 6 fields of view at a magnification of 20x (objective lens) and 10x (eyepiece).

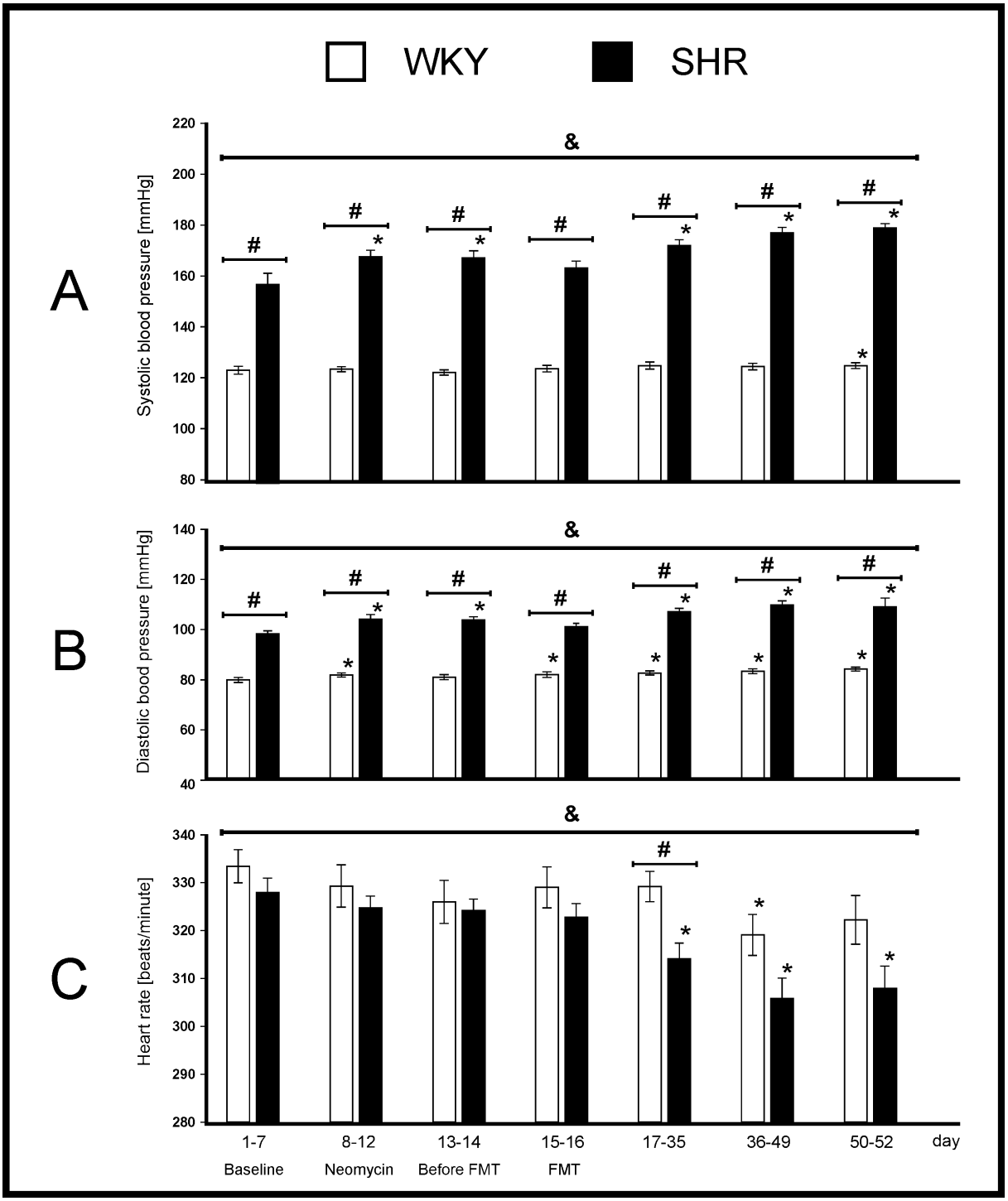

Figure S1. Changes in (A) systolic, (B) diastolic blood pressure and (C) heart rate in WKY (n=13) and SHR (n=10). * $p<0.05$ vs. baseline; # between groups at given time points; & - $p<0.05$ between groups (repeated measure ANOVA) Means ± SE are presented

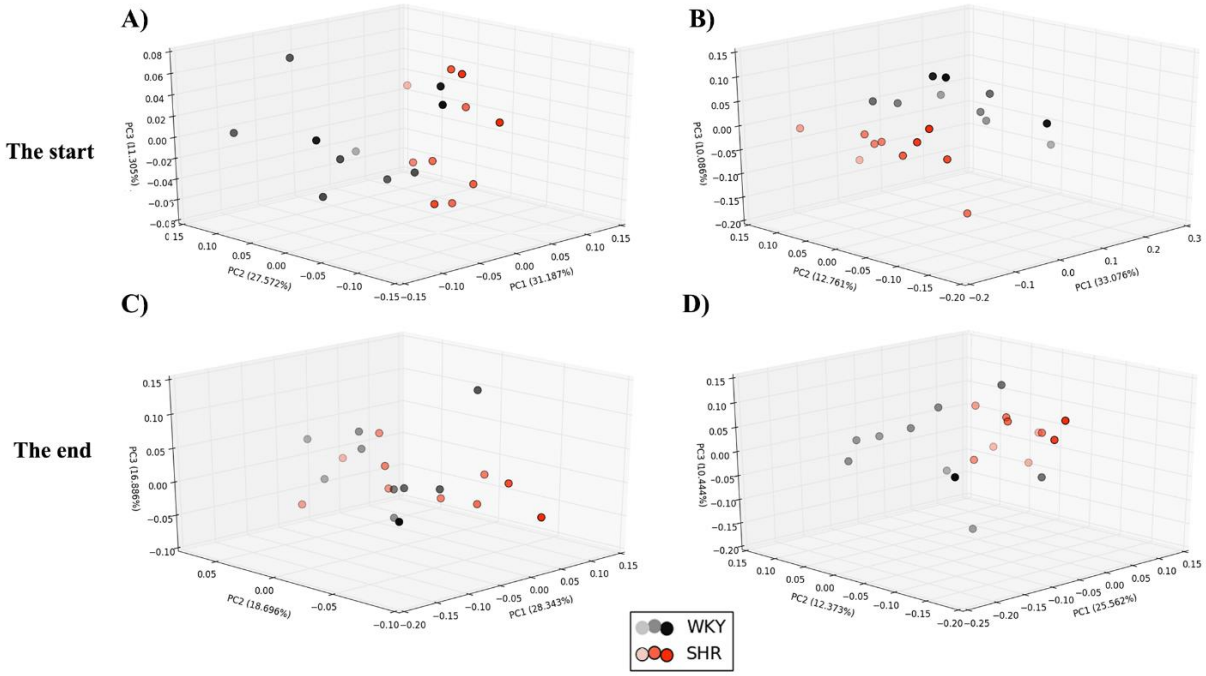

Figure S2. Comparison of gut microbiota composition between WKY and SHR rats at the start (A, n=20, B, n=20) and the end of the experiment (C, n=20, D, n=20). The analysis is based on weighted UniFrac and unweighted UniFrac distances. The color intensity is correlated with the distance from the PC1 axis.

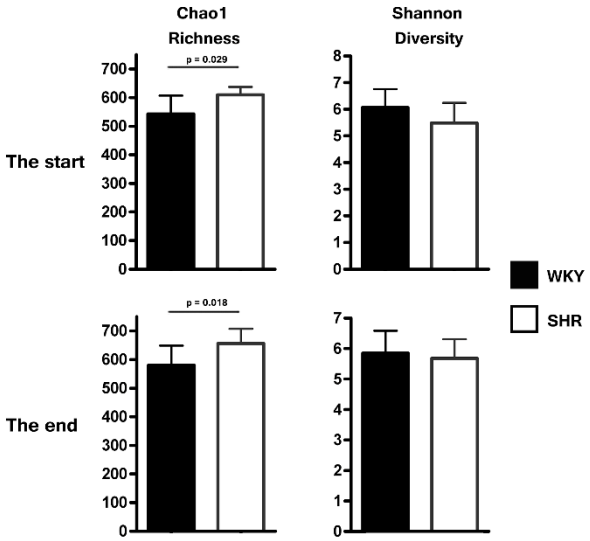

Figure S3. Comparison of alpha-diversity between all WKY [WKY(wky) and WKY(shr)](n=20) and SHRs [SHR(shr) and SHR(wky)](n=20) rats at the beginning and the end of the experiment. Means ± SD are presented

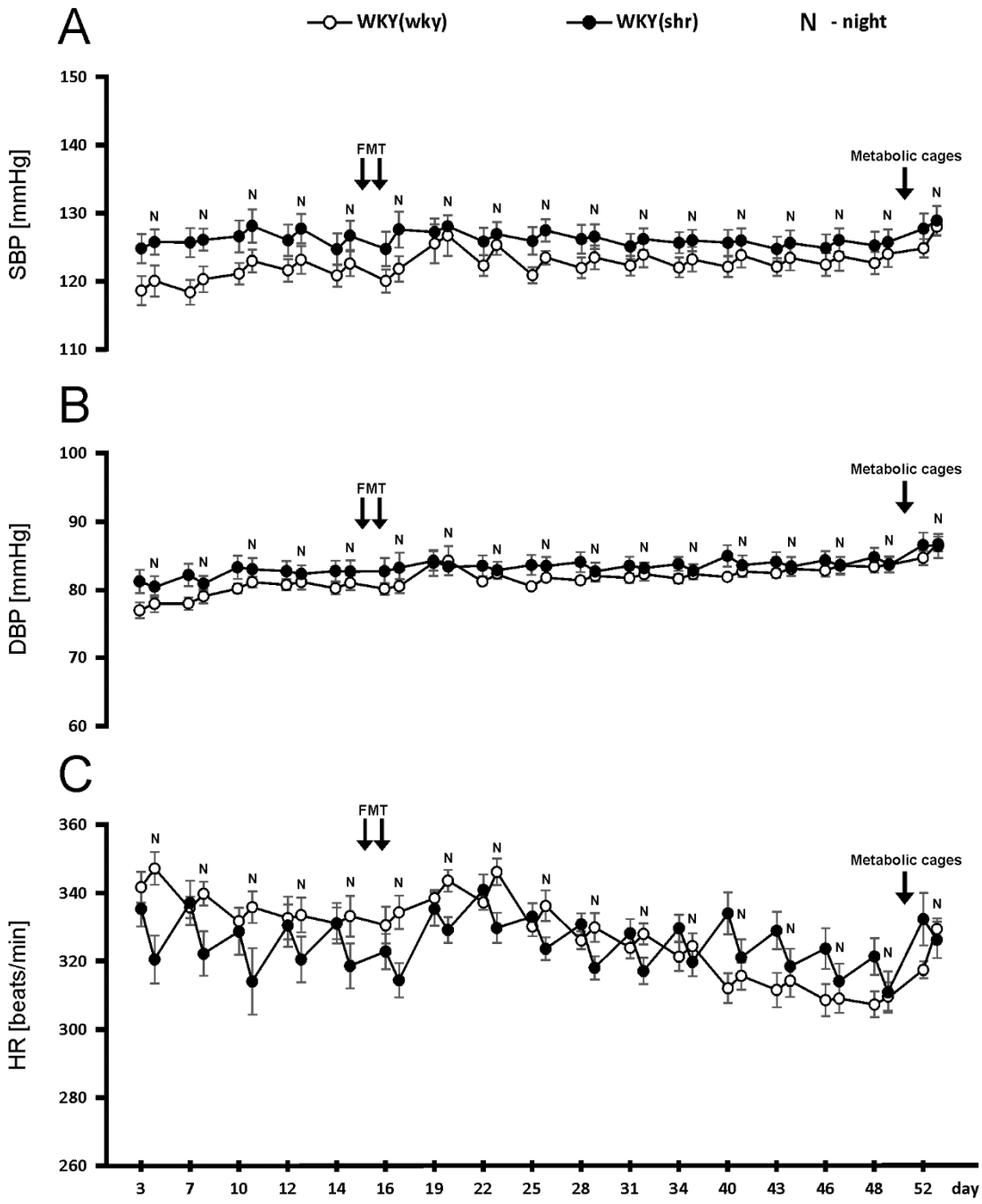

Figure S4. Changes in (A) systolic, (B) diastolic blood pressure and (C) heart rate in WKY rats receiving fecal transplantation from WKY [WKY(wky) series] (n=6) or SHR [WKY(shr) series] (n=7) separated by the day and night measurements. FMT – fecal microbiota transplantation, SBP – systolic blood pressure, DBP – diastolic blood pressure, HR – heart rate, N – night. Means ± SE are presented

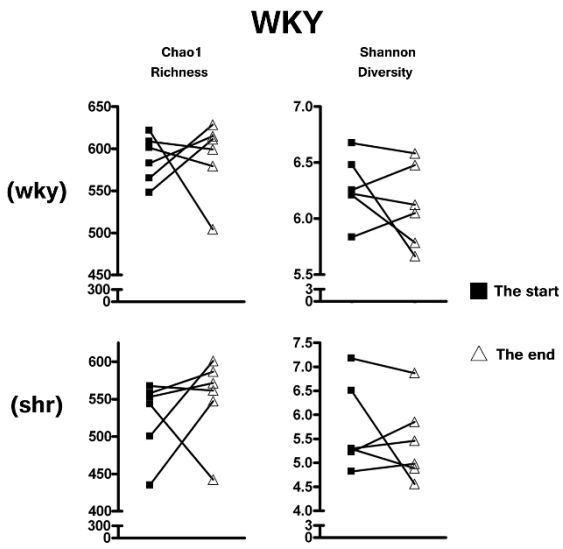

Figure S5. Comparison of alpha-diversity in WKY rats receiving fecal transplantation from WKY [WKY(wky) series](n=12) or SHR [WKY(shr) series] (n=12) rats at the beginning and the end of the experiment.

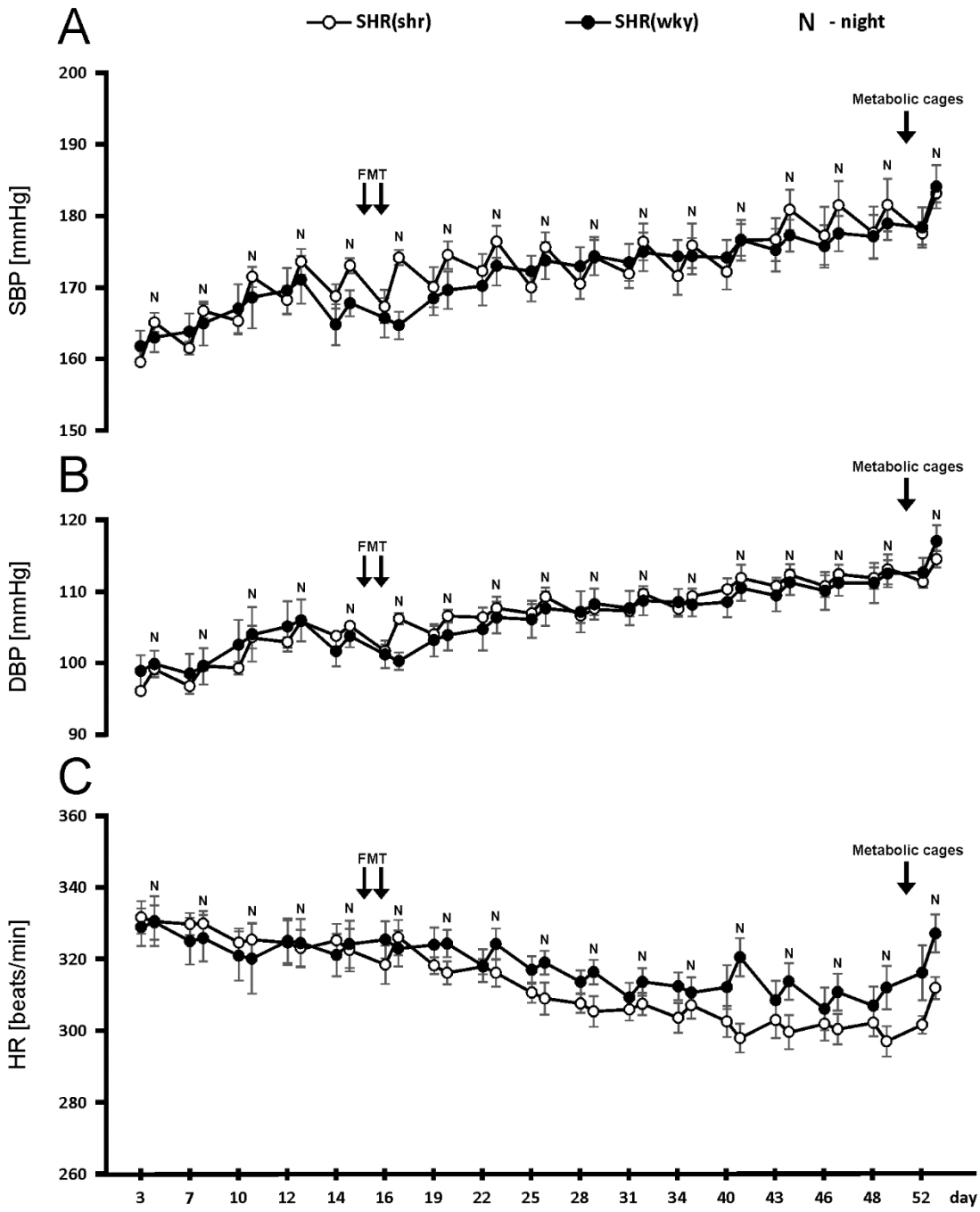

Figure S6. Changes in (A) systolic, (B) diastolic blood pressure and (C) heart rate in SHRs receiving fecal transplantation from SHR [SHR(shr) group] (n=4) or WKY [SHR(wky) group] (n=6) separated by the day and night measurements. FMT – fecal microbiota transplantation, SBP – systolic blood pressure, DBP – diastolic blood pressure, HR – heart rate, N – night. Means ± SE are presented

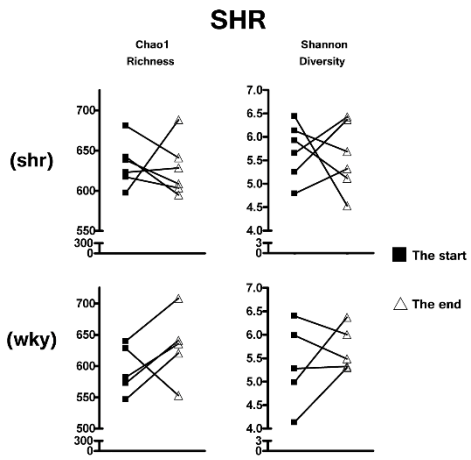

Figure S7. Comparison of alpha-diversity in SHR rats receiving fecal transplantation from SHR [SHR(shr) series] (n=12) or WKY [SHR(wky) series] (n=10) rats at the beginning and the end of the experiment.

Comparison of taxonomic abundance between WKY rats and SHRs

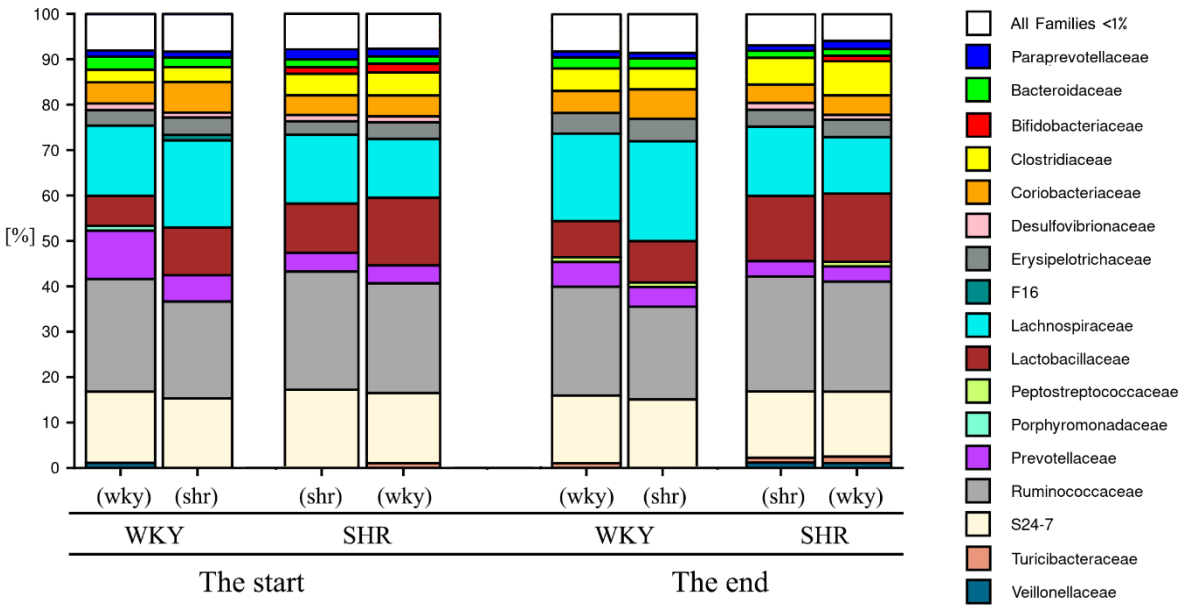

Figure S8. Fecal microbiota transplantation did not alter gut microbiota at the family level. Bar charts illustrate abundance at the beginning and the end of the experimental protocol.

Nutrients	Rat chow
Metabolizable Energy (MJ)	11.5
Crude protein (g/kg)	175
Crude fat (g/kg)	2.8
Crude fiber (g/kg)	70
Crude ash (g/kg)	57
Starch (g/kg)	330
Calcium (g/kg)	9.5
Phosphorus (g/kg)	6.5
Magnesium (g/kg)	3.0
Potassium (g/kg)	7.5
Sodium (g/kg)	1.9

Table S1. Nutritional composition of a rat chow

	all WKY (n=14)	all SHR (n=14)
	Metabolic parameters	
Body mass start (g)	244.52 ± 4.90	206.55 ± 5.34*
Body mass end (g)	365.62 ± 7.41	338.25 ± 4.32*
Weight gain (g)	121.09 ± 9.58	131.7 ± 5.60
Food intake (g/24h)	23.38± 0.69	23.86 ± 0.72
Water intake (mL/24h)	34.53± 1.75	36.80 ± 1.46
Urine output (mL/24h)	9.08 ± 0.87	11.54 ± 1.30
Stool output (g/24h)	13.76 ± 0.67	14.50 ± 0.57
	Plasma biochemistry	
Sodium (mmol/L)	135.21 ± 1.62	138.64 ± 1.27
Potassium (mmol/L)	5.31 ± 0.24	5.27 ± 0.07
Creatinine (mg/dL)	0.56 ± 0.04	0.56 ± 0.03

Values are Means ± SE. WKY, normotensive rats; SHR, Spontaneously Hypertensive Rats; * $p < 0.05$ WKY vs SHR comparison (Independent samples t-Test).

Table S2. Comparison of metabolic parameters and plasma biochemistry in all normotensive and hypertensive rats.

Experimental Group	Start of the experiment	End of the experiment
Microbiota-derived metabolites in stools		
	Butyric Acid (µM)	
WKY (n=14)	9440.54 ± 2472.03	11874.01 ± 1986.97
SHR (n=14)	16718.83 ± 2623.40 *	12746.02 ± 1392.39
	Valeric Acid (µM)	
WKY (n=14)	840.11 ± 162.02	944.59 ± 122.19
SHR (n=14)	1497.86 ± 159.34 *	918.71 ± 92.31
	Acetic Acid (µM)	
WKY (n=14)	73763.69 ± 6059.14	44987.69 ± 1939.74 †
SHR (n=14)	88126.13 ± 4553.55	46916.70 ± 2341.99 †

Values are Means ± SE. WKY, normotensive rats; SHR, Spontaneously Hypertensive Rats; * $p < 0.05$ – between groups (WKY vs SHR); † $p < 0.05$ – the start vs end of the experiment, (within a group);

Table S3. The concentrations of gut microbiota-derived metabolites in stools from all normotensive and hypertensive rats at the beginning and the end of the experiment

References

1. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; 30(15):2114-2120. doi: 10.1093/bioinformatics/btu170
2. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; 7(5):335-336. doi: 10.1038/nmeth.f.303
3. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, *et al.* Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* 2011; 21(3):494-504. doi: 10.1101/gr.112730.110
4. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* 2013; 10(12):1200-1202. doi: 10.1038/nmeth.2658
5. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013; 8(4):e61217. doi: 10.1371/journal.pone.0061217
6. Dabdoub SM, Fellows ML, Paropkari AD, Mason MR, Huja SS, Tsigarida AA, *et al.* PhyloToAST: Bioinformatics tools for species-level analysis and visualization of complex microbial datasets. *Sci Rep* 2016; 6:29123. doi: 10.1038/srep29123