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Probiotics prevent dysbiosis and the raise in blood pressure in genetic hypertension: role of short-chain fatty acids

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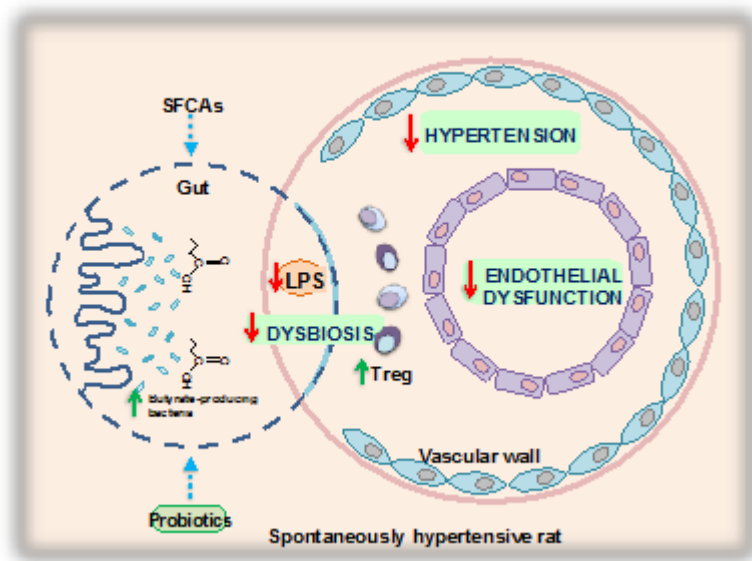
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The probiotics LC40 and BFM prevent dysbiosis and the development of endothelial dysfunction and high blood pressure in genetic hypertension. This effect seems to be related to endotoxemia reduction, vascular TLR4 pathway impairment, and to increase Treg accumulation in the vasculature.



Abbreviations:

BFM, *Bifidobacterium breve* CECT7263; CFU, colony-forming units; DOCA, deoxycorticosterone acetate; F/B, Firmicutes/Bacteroidetes; HR, heart rate; HW, heart weight; LC40, *Lactobacillus fermentum* CECT5716; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; LVW, left ventricular weight; MLNs, mesenteric lymph nodes; MUC, mucin; NMR, Nuclear Magnetic Resonance; NO, nitric oxide; OTU, operational taxonomic unit; RLU, relative luminescence units; ROS, reactive oxygen species; SBP, systolic blood pressure; SCFAs, short chain fatty acids; SHR, spontaneously hypertensive rats; TL, tibia length; TLR4, toll-like receptor 4; TSP, Trimethylsilyl propanoic acid; WKY, Wistar Kyoto rat; ZO-1, zonula occludens-1.

Keywords: Probiotic bacteria, gut microbiota, hypertension, endothelial dysfunction, immune system, SHR.

Abstract

Scope: The objective of this study was to determine the cardiovascular effects of the probiotics *Bifidobacterium breve* CECT7263 (BFM) and *Lactobacillus fermentum* CECT5716 (LC40), and the short chain fatty acids butyrate and acetate in spontaneously hypertensive rats (SHR).

Methods and results: Ten five-weeks old Wistar Kyoto rats (WKY) and fifty aged-matched SHR were randomly distributed into six groups: control WKY, control SHR, treated SHR-LC40, treated SHR-BMF, treated SHR-butyrate, and treated SHR-acetate. Chronic treatments with LC40 or BFM increased butyrate-producing bacteria and prevented the blood pressure increase in SHR. Oral treatment with butyrate or acetate also prevented the increase in both blood pressure and Firmicutes/Bacteroidetes ratio. All treatments restored the Th17/Treg balance in mesenteric lymph nodes, normalized endotoxemia, and prevented the impairment of endothelium-dependent relaxation to acetylcholine, as a result of reduced NADPH oxidase-driven reactive oxygen species production. These protective effects might be mediated by both the reduction in vascular lipopolysaccharide/toll-like receptor 4 pathway and the increase in Treg infiltration in the vasculature.

Conclusion: The probiotics LC40 and BFM prevent dysbiosis and the development of endothelial dysfunction and high blood pressure in genetic hypertension. These effects seem to be related to endotoxemia reduction and to increase Treg accumulation in the vasculature.

1. Introduction

Hypertension is among the most prevalent risk factors for cardiovascular events such as stroke and myocardial infarction. Up to present date, an ever-increasing number of studies have shown a link between gut microbial signatures and hypertension in both animal models and human patients [1-6]. In general, both manifest dysbiosis as a result of decreases in

evenness, microbial diversity, richness, and an increased Firmicutes/Bacteroidetes (F/B) ratio in the renin-dependent form of hypertension in both essential hypertensive patients and animals [1,3,4]. On the other hand, no significant changes in F/B were present in renin-independent hypertensive mice [5]. Angiotensin II-infused germ-free mice showed that changes in gut microbiota are involved in angiotensin II-induced vascular dysfunction and hypertension [7]. In addition, fecal transplantation from spontaneously hypertensive-stroke prone rats [8] or SHR [9,10] to Wistar Kyoto rats (WKY) or from hypertensive subjects [4] to germ-free mice, respectively, induces an increase in blood pressure. These observations suggest that gut microbiota regulates blood pressure. However, the exact roles of bacteria and gut health in hypertension have not been elucidated. Recently, we demonstrated the key role of T-cell activation in the gut immune system and vascular T-cells accumulation in the hypertensive response triggered by fecal microbiota transplantation from SHR to WKY rats [10].

The gut microbiota communicates with distal organs through the production of a high number of metabolites that can be absorbed into the systemic circulation and exert biological effects [11]. The signalling molecules are bacterial metabolic products, including short chain fatty acids (SCFAs) [12], and bacterial wall components such as lipopolysaccharide (LPS) [13]. In fact, gut dysbiosis in hypertension has been characterized by an increase in lactate-producing bacteria, and a decrease in acetate- and butyrate-producing bacterial populations [1, 10]. SCFAs can impact renin secretion and blood pressure regulation stimulating host G-protein-coupled receptor pathways [12]. In fact, butyrate has been shown to attenuate angiotensin II-induced hypertension in mice [14, 15], and both, acetate supplementation or a diet rich in fiber, which substantially increases the production of SCFAs such as acetate, prevented the development of hypertension in deoxycorticosterone acetate (DOCA)-salt [5]. Increased bacterial production of SCFAs is associated with reduced circulating CD4+

immune cells [15]. Bacterial LPS, through toll-like receptor (TLR)4 activation, contributes to the low-grade vascular inflammation and, ultimately, the increased blood pressure present in SHR [17].

Thus, it is highly probable that gut microbiota is functionally involved in blood pressure control. In fact, a meta-analysis demonstrated a significant reduction in blood pressure in probiotic-treated patients [18]. Furthermore, a beneficial role of *Lactobacillus* probiotics and kefir in blood pressure regulation and vascular protection have been described in SHR with established hypertension [19-21], as a result of restoring the imbalance in reactive oxygen species (ROS)/nitric oxide (NO) in the arterial wall. However, if probiotics consumption could prevent gut dysbiosis, reducing gut immune system T-cell activation and vascular T-cells infiltration in SHR, and if SCFAs play any role in this effect is unknown. We hypothesized that oral supplementation with probiotics would prevent the raise in blood pressure in SHR by changing SCFAs-producing bacteria populations, thus altering the gut communication with local secondary lymph organs and distal organs. Thus, the objective of this study was to evaluate the cardiovascular effects of probiotics *Bifidobacterium breve* CECT7263 (BFM), and *Lactobacillus fermentum* CECT5716 (LC40), and butyrate and acetate in genetic hypertension.

2. Materials and methods

This study was performed following requirements and regulations of the European Union on the protection of animals used for scientific purposes. All experimental protocols were officially sanctioned by the Ethic Committee of Laboratory Animals of the University of Granada (Spain; permit number 03-CEEA-OH-2013).

2.1. Probiotic preparation and administration

LC40 or BFM were obtained from Biosearch, S. A. (Granada, Spain) and were normally grown in de Man, Rogosa and Sharpe medium at 37°C in anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, Hants, UK). For the probiotics treatment, the lyophilized bacteria were aliquoted and stored at -20°C until usage, at that moment, they were suspended in tap water and administered.

2.2. Animals and experimental groups

Five weeks old male WKY and SHR were acquired from Harlan Laboratories (Barcelona, Spain). Animals were kept in a specific pathogen-free environment. Water and standard laboratory chow (SAFE A04, Augy, France) were provided *ad libitum*. Ten WKY and fifty SHR were randomly distributed into six groups of 10 rats each: a control WKY group and a control SHR group (both provided with tap water), a treated SHR-LC40 group and a treated SHR-BFM group (the probiotics were administered at the final concentration of 10^9 colony-forming units, CFU/day in 1 mL by oral gavage), a treated SHR-acetate (100 mM in the drinking water), and a treated SHR-butyrate ($0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ in the drinking water). The doses of probiotics selected were similar to that previously used to reduce BP in SHR [19]. We used a dose of acetate similar to that used to prevent hypertension in DOCA-salt mice [5], and a dose of butyrate that prevents the rise of blood pressure in angiotensin II-infused mice [14]. Oral gavage of probiotics were performed every day in the morning. Animals were maintained in individual ventilated cages. In the present experiment treatments started when SHR were normotensive (5 week-old) and during 13 weeks period. Water was changed daily. Both water and food consumption was documented every day. Every week, body weight was recorded.

In order to analyze the effects of experimental interventions in control WKY rats we tested the effects of probiotics and SCFAs in normotensive WKY rats, using 6 rats per group.

2.3. Blood pressure measurement

Systolic blood pressure (SBP) and heart rate (HR) measurements were performed weekly at room temperature by tail-cuff plethysmography as previously described [10]. At the end of the experimental period, animals were put under isoflurane anesthesia, a polyethylene catheter containing 100U heparin in isotonic, sterile NaCl solution was inserted in the left carotid artery to monitor intra-arterial BP in conscious, unrestrained conditions. Direct BP was recorded continuously (MacLab; AD Instruments, Hastings, UK) [9].

2.4. Cardiac and renal weight indices

When the experimental period was completed, 18 h fasting animals were anaesthetized with 2.5 mL/kg equitensin (i.p.). Finally, the animals were sacrificed by exsanguination, collecting blood from the abdominal aorta. The colon, kidneys and ventricles were then obtained and weighed. The heart was divided into right ventricle and left ventricle plus septum, and the cortex was excised of the rest of the kidney.

2.5. SCFAs in plasma and feces

At the end of the treatment, animals were sacrificed under isoflurane anesthesia. Blood samples from abdominal aorta were chilled on ice and centrifuged for 10 min at 3500 rpm at 4 °C. The resulting plasma was frozen at -80 °C. Plasma LPS levels were measured using the Limulus Amebocyte Lysate (LAL) chromogenic endotoxin quantitation Kit (Lonza, Valais, Switzerland), following the instructions of the manufacturer. Briefly, before the LPS determination, samples are treated with a 10 mM MgCl₂ solution (Lonza, Walkersville, USA) in order to remove all heparin used during blood extraction. Also, Pyrospense, a dispersing agent, was added to remove fat from the samples. After those two pre-treatments, samples

were incubated at 70 °C for 15 min. Sterile and pyrogen-free material was used at all moments to guarantee sample and test integrity. The chromogenic LAL test has a linear range for absorbance at 405-410 nm between 0.1 and 1.0 EU/mL endotoxin. The recovery rate of bacterial endotoxin obtained when a theoretical concentration (0.5 EU/mL) was added to the sample was 116 %, thereby meeting the criteria established by Agência Nacional de Vigilância Sanitária (ANVISA) for proof of accuracy of the bioanalytical method.

Plasma levels of SCFAs and other metabolites were measured by Nuclear Magnetic Resonance (NMR). Plasma samples (40 µL) were examined by 500 MHz High-Resolution Magic Angle Spinning NMR Bruker AMX500 spectrometer at CIC Biomagune (Donostia, Spain). Samples were placed into a 50-µl zirconium oxide rotor using a rinsed cylindrical insert, together with 15 µl 0.1 mM solution Trimethylsilyl propanoic acid (TSP) in deuterium water. Standard solvent-suppressed spectra were acquired using a sequence based on the first increment of the nuclear Overhauser effect spectroscopy pulse sequence. A number of bidimensional homonuclear and heteronuclear experiments such as standard gradient-enhanced correlation spectroscopy, ¹H-¹H total correlated spectroscopy, and gradient-selected heteronuclear single quantum correlation protocols were performed to carry out metabolites assignments. Spectral processing was performed using the “Metabonomic” R package [22]. ¹H-NMR spectra were referenced to the TSP signal at 0 ppm chemical shift and normalized to total sum of the spectral regions. Two-dimensional spectral processing and editing was performed using MestRenova v. 11.0.3 (Mestrelab Research S.L., Santiago de Compostela, Spain). To determine plasma butyrate concentrations the samples (200 µL) were acidified with 20 µL of 5% *o*-phosphoric acid (final concentration 0.5%) and after vortexing were extracted with 200 µL of methyl tert butyl ether. Samples were homogenized with a vortex and centrifuged for 10 min at 17,000 g at 4°C [23]. Organic phase was collected, and 50 µL was transferred to and insert in a vial for the injection into a gas chromatograph (PerkinElmer

Autosystem GC-FID, Waltham, MA, USA) equipped with a capillary column (CPWAX 52CB, 60 m × 0.25 mm, 0.25 μm, Varian, Middelburg, The Netherlands) and connected to a Star Chromatography WorkStation program (version 6, Varian) to quantify the samples.

SCFAs concentrations in the feces were quantified by gas chromatography, as previously described [24]. Briefly, the samples were homogenized with 150 mM NaHCO₃ (pH 7.8) (1:5 w/v) in an argon atmosphere. Samples were incubated for 24 h at 37 °C and stored at -80 °C until the extraction. To extract the SCFAs, 50 μL of the internal standard 2-methylvaleric acid (100 mM), 10 μL of sulfuric acid, and 0.3 mL of chloroform were added to 1 mL of the homogenate and, then, centrifuged at 10000g for 5 min at 4 °C. The organic layer was dehydrated with sodium anhydrous sulfate and centrifuged at 10000g for 5 min at 4 °C. One microliter of the supernatant was injected into a gas chromatograph.

2.6. Vascular reactivity studies

Thoracic aortic rings were incubated in organ baths filled with Krebs solution (composition in mmol/L: CaCl₂ 2, glucose 11, KCl 4.75, KH₂PO₄ 1.2, MgSO₄ 1.2, NaCl 118 and NaHCO₃ 25) at 37 °C and infused with carbogen (95% O₂ and 5% CO₂) and were subjected to a resting tension of 2 g. Isometric tension was recorded using an isometric force-displacement transducer (Letigraph 2000) connected to an acquisition system, as previously described [10]. The concentration-relaxation response curves to acetylcholine (10⁻⁹ mol/L-10⁻⁵ mol/L) were performed in rings pre-contracted by 10⁻⁶ mol/L phenylephrine, in the absence or in the presence of the NADPH oxidase inhibitor VAS2879 (5 μmol/L), or the endothelial NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) for 30 min. In some rings, concentration- relaxation response curves to nitroprusside (10⁻⁹-10⁻⁶ mol/L) were performed in

the dark in aortic segments denuded of endothelium precontracted by 1 $\mu\text{mol/L}$ phenylephrine. Relaxant responses were expressed as a percentage of precontraction.

2.7. NADPH oxidase activity, arginase activity and nitric oxide synthase activity in aorta

In order to determine NADPH oxidase activity, the lucigenin-enhanced chemiluminescence assay was performed in intact aortic rings, as previously described [10]. We used a colorimetric Nitric Oxide Synthase (NOS) Activity Assay Kit (abcam, Abingdon, UK) to determine NO production in aortic segments from all groups. Sample processing and analysis were performed following the manufacturer's instructions. For the determination of arginase activity in aorta, we used an Arginase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions.

2.8. Flow Cytometry

Mesenteric lymph nodes (MLNs) and spleen were obtained from all experimental groups. To decrease friction, all samples were mashed with wet slides and the resulting solutions were filtered through a 70 μM cell strainer. 1×10^6 cells were counted and incubated with a protein transport inhibitor (BD GolgiPlugTM) and a 50 ng/mL phorbol 12-myristate 13-acetate plus 1 $\mu\text{g/mL}$ ionomycin stimulus for an optimum detection of intracellular cytokines by flow cytometry. After 4.5 hours, aliquot cells, of each sample, were blocked with anti-CD32 (clone D34-485) at 4°C for 30 minutes to avoid non-specific binding to mouse Fc-gamma receptors. Next, cells were transferred to polystyrene tubes for the surface staining with viability dye (LIVE/DIED[®] Fixable Aqua Dead cell Stain Kit, Molecular Probes, Oregon, USA) and mAbs anti-CD45 (APC, clone RA3-6B2 BD PharmigenTM, New Jersey, USA), anti-CD4 (PerCP-Vio700, clone REA482, Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C in the dark. The lymphocytes were then fixed, permeabilized with the Fix/Perm Fixation/Permeabilization kit (eBioscience, San Diego, USA) and intracellular staining was

achieved with mAbs anti-IL-17A (PE-Cy7, clone eBio17B7, eBioscience, San Diego, USA) and anti-FoxP3 (PE, clone FJK-16s, eBioscience, San Diego, USA) for 30 min at 4°C in the dark. All samples were analyzed using a flow cytometer CANTO II (BD Biosciences) with BD FACSDIVA™ software.

For vascular T-cell infiltration, we analyzed leukocytes in aorta. Following dissection, aortae were digested using a solution of collagenase type I-S (450 U/mL), collagenase type XI (125 U/mL), DNase I (60 U/mL) and hyaluronidase I-S (60 U/mL) in heparinized PBS (20U/mL) at 37 °C for 60 min [25]. The digested tissue was then homogenized and filtered as described above. Cells from aortae were isolated followed by lysis of red blood cells with Gey's solution. The cells were then stained for 20 minutes at 4°C with fluorescently-labeled antibodies described below, were then fixed and resuspended in PBS and analyzed using multi color flow cytometry as previously described.

Antibodies for staining were from Miltenyi Biotec, Bergisch Gladbach, Germany and were used in different multi-color combinations: CD45-VioBright FICT (clone REA504); CD3- PerCPVio700 (clone REA223); CD4-APC (clone REA489); CD8-PE (clone REA437); CD44-PE-Vio770 (clone REA505); and viability dye (LIVE/DIED® Fixable Aqua Dead cell Stain Kit, Molecular Probes, Oregon, USA).

2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For RT-PCR analysis, total RNA was extracted from the colon, renal cortex, aortae, and mesenteric lymph nodes by homogenization and retrotranscribed to cDNA using standard methods. The polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to analyze mRNA expression [10]. The sense and antisense primer sequences used for amplification are described in **Table S1**.

2.10. 16S rDNA V4-V5 region sequencing

Fecal DNA was extracted from the samples collected from all experimental groups by using quick-DNA fecal/soil microbe kit (ZymoResearch, Irvine, CA). Primers compatible with illumina Miseq v2 2x250bp kit (Illumina, San Diego, CA) were used to amplify bacterial 16S V4-V5 variable regions [1]. The PCR amplicons were purified by QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified by Qubit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of purified PCR product from each sample were pooled together as one library. The library was quantified by real time PCR (Kapa Biosystems, Wilmington, MA) prior to Miseq sequencing (Illumina, San Diego, CA). The sequencing data had a Q30 score $\geq 93.5\%$ and $97.17 \pm 0.34\%$ of total cluster passes the filter.

2.11. Bioinformatics analysis

The raw paired-reads from Miseq were processed using QIIME 1.9.1. Briefly, reads were trimmed to remove bases with Phred score lower than 30 and quality-filtered with parameters set as previously optimized [26]. Open reference operational taxonomic unit (OTU)-picking was performed and taxonomical assignment to the generated OTUs was performed with 97% identity against Greengenes database 13.8. Alpha diversity and unweighted principal coordinate analyses plots using the phylogenetic tree-based unifrac distance metric were generated using scripts from QIIME package.

2.12. Chemicals

All chemicals used were obtained from Sigma (Alcobendas, Madrid, Spain). Distilled deionized water was used as solvent when necessary.

2.13. Statistical analysis

Chao, observed species, Pielou and Shannon whole indexes were calculated using QIIME (PAST 3x). Reads in each OTU were normalized to total reads in each sample. For the analysis, only taxa with a percentage of reads $> 0.001\%$ were used. Additionally, Partial Least Square analysis was applied to these data to identify significant differences between groups. Linear Discriminant Analyze scores greater than 2 were displayed. Taxonomy was summarized at the genus level within QIIME-1.9.0 and uploaded to the Galaxy platform [27]. The nested design was used to compare the evolution of tail SBP in time, with treatment and days as fixed factors and the rat as random factor. This method also carried out with groups and concentrations to compare the concentration-response curves to acetylcholine. Results for all measurements are expressed as the mean \pm SEM. Comparisons were made using Bonferroni's test with an appropriate error if the overall difference was significant. The remaining variables were tested on normal distribution using Shapiro-Wilk normality test and compared using an one-way ANOVA and Tukey post hoc test in case of normal distribution, or Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparison test in case of abnormal distribution. Statistical significance was considered as $P < 0.05$.

3. Results

3.1 Probiotics and SCFA prevented the raise on blood pressure, and cardiac hypertrophy in SHR

As expected, a significant time-dependent increase in SBP (≈ 62 mm Hg) was observed in SHR from 5 to 18 weeks old (**Fig. 1A, 1B**). Long-term administration of both probiotics prevented the raise in SBP ($28.4 \pm 7.8\%$, and $23.6 \pm 7.0\%$ by LC40 and BFM, $P < 0.01$ and $P < 0.5$ vs untreated SHR, respectively) (**Fig. 1A**). Similarly, both acetate and butyrate consumption also inhibited the development of high BP ($17.2 \pm 5.4\%$, and $21.4 \pm$

7.0 %, respectively, $P < 0.05$ vs untreated SHR) (**Fig. 1B**). The antihypertensive effect of these treatments was confirmed at the end of the experiment by a direct pressure recording in the carotid (**Fig. 1C**). However, no significant changes in heart rate were found among all experimental groups (**Fig. 1D**). Interestingly, none of the treatments was able to change SBP in normotensive WKY rats (**Fig. S1**).

Both WKY and SHR control groups experienced an increase in body weight between 5-18 weeks old (311.7 ± 11.6 % and 283.8 ± 12.0 %, respectively). The treatments did not change weight gain and we were not able to find a statistical difference among all experimental groups in final body weight (**Table S2**). Absolute heart weight (HW) and left ventricle weight (LVW) and their relative values expressed as a ratio to tibia length (TL) were higher (13, 24, 18, and 26%, respectively) in SHR control group as compared with WKY control group. The probiotics LC40 and BFM and butyrate and acetate significantly reduced LVW/TL index, by 7, 8, 7, and 8% respectively (**Table S2**).

3.2. Probiotics and SCFA reduced gut dysbiosis in SHR

The bacterial communities composition was evaluated calculating major ecological parameters, including Chao richness, Pielou evenness, and the number of observed species. Significant differences among experimental groups were not found (**Fig. 2A**). The analysis of the phyla composition (**Fig. 2B, Table S3**) showed that Firmicutes and Bacteroidetes were the most abundant phylum in rat feces. The proportion of bacteria from the Firmicutes phylum was significantly higher ($\approx 18\%$, $P < 0.05$) in SHR than in WKY and both butyrate and acetate consumption normalized the proportion of bacteria belonging to this phylum. Moreover, bacteria from Bacteroidetes phylum were decreased ($\approx -37\%$, $P < 0.01$) in SHR and BFM, butyrate and acetate significantly increased this proportion similar to WKY rats. LC40 tended to reduce Firmicutes and increase Bacteroidetes but these changes were not

statistically significant. The F/B ratio, a signature of gut dysbiosis in hypertension [1], was \approx 2-fold higher in SHR than in WKY, and this ratio returned to normal values by the action of BFM, butyrate and acetate, whereas LC40 tended to reduce it but not significantly ($P=0.352$) ((**Fig. 2C**). In addition, significant lower percentages of acetate- and butyrate-producing bacteria (\approx -50 and -42%, respectively, $P<0.05$), and a \approx 5-fold, $P<0.05$, higher percentage of lactate-producing bacteria were found in SHR compared to WKY (**Fig. 2D**). Both LC40 and BFM increased \approx 2-, and 3-fold, respectively, $P<0.05$, butyrate-producing bacteria in SHR but had no effect on acetate- and lactate-producing bacteria. Acetate increased \approx 2-fold, $P<0.05$ acetate-producing bacteria while butyrate halved lactate-producing bacteria (**Fig. 2D**). Acetate content in feces was reduced 2.7 fold in SHR as compared to WKY, which was unchanged by BFM, LC40 and butyrate treatment. However, acetate treatment increased 6.3 fold the acetate concentration in feces. Butyrate content in feces was similar between WKY and SHR, but both BFM and butyrate treatment in SHR increased by 3.6 and 2.4 fold, respectively, butyrate concentration (**Fig. S2**).

At the family level, a significant increase of \approx 3-fold in *Lactobacillaceae*, \approx 2-fold *Turicibacteriaceae*, >100 -fold *Peptostreptococcaceae*, and \approx 4-fold *Anaeroplasmataceae*, and a \approx 40% reduction of *S24-7* were found in SHR feces as compared to WKY (**Fig. 3A**). Both probiotics and acetate treatments prevented ($P<0.05$) the increase in *Peptostreptococcaceae* in SHR. At the genus level, *Lactobacillus* and *Turicibacter* were increased (\approx 3-, and 2-fold, respectively, $P<0.05$) and *S24-7g* were reduced \approx 40%, $P<0.05$ in SHR as compared to WKY (**Fig. 2B**) as previously described [10]. Both probiotics and SCFAs treatments tended to reduce *Lactobacillus* and increase *S24-7g*, but only acetate reduced significantly ($P<0.05$) by \approx 2.5-fold the abundance of *Lactobacillus* (**Fig. 3C**). The treatments did not alter the *Turicibacter* proportion in SHR (**Fig. 3C**). The ratio

Lactobacillus/ S24-7g positively correlated with SBP ($r^2=0.1375$, $P<0.05$), and was normalized by all treatments (**Fig. 3D**).

3.3. Probiotics reduced endotoxemia, increased butyrate but did not change circulating acetate and lactate levels in SHR

An approximately two-third reduction in mRNA levels of barrier-forming junction proteins (zonula occludens-1 (ZO-1) and occludin) in the colon of SHR compared to WKY were found (**Fig. 4A**). Both probiotic treatments restored ZO-1 and occludin mRNA levels, suggesting a possible preserved barrier function. As expected, both acetate and butyrate treatments affected mRNA levels encoding tight junction proteins in SHR, normalizing ZO-1, and increasing 2- and 5-fold, respectively, occludin. We have also found downregulation of mucin (MUC)-2 and MUC-3 transcripts by ≈ 90 , and 75%, respectively, in SHR, which were significantly increased by both SCFAs (≈ 10 - and 30-fold MUC-2, and ≈ 15 - and 12-fold MUC-3, butyrate and acetate, respectively) but unaffected by LC40 and BFM (**Fig. 4B**). We measured endotoxin levels in plasma, and found them to be $\approx 70\%$ significantly ($P<0.01$) higher in SHR compared with the WKY group (**Fig. 4C**). Interestingly, the long-term treatment with both probiotics and SCFAs significantly prevented endotoxemia in SHR. These results suggest that intestinal permeability is increased in SHR and allow bacterial components (e.g., LPS) to enter the blood stream. We also found that colonic expression of IL-18 (**Fig. 4D**) was $\approx 80\%$ lower ($P<0.05$) in SHR as compared to WKY. All treatments increased mRNA levels of IL18 in colon (**Fig. 4D**).

As we found significant changes in SCFAs-producing bacteria in feces, we next analyzed the colonic expression of the transporters of SCFAs, MCT1 and MCT4 (**Fig. 4E**), and the plasma levels of SCFA from the NMR spectra (**Fig. 4F**). Both MCT1 and MCT4 transcripts levels were reduced in SHR by $\approx 60\%$, $P<0.05$ and 65%, $P<0.01$, respectively, as compared to

WKY and were unchanged by all treatments, except for BFM that increased \approx 4-fold MCT-1 and butyrate that increased \approx 7-fold MCT-4. Lactate was significantly decreased in plasma (\approx -22%, $P < 0.05$), without changes in acetate levels in plasma from SHR as compared to WKY. Probiotic treatments did not significantly change the plasma levels of these SCFAs. In addition, neither acetate nor butyrate chronic consumption altered the levels of acetate and lactate in SHR. We were not able to detect butyrate levels in the NMR spectra. However, plasma butyrate concentrations, measured by gas chromatography, were reduced in SHR as compared WKY, and increased by LC40, BFM and butyrate treatment (**Fig. 4G**).

Unsupervised classification studies with PCA were carried out to analyze the differences between spectra from SHR and WKY rats. The plasma spectra provided nearly perfect discrimination between the two groups (**Fig. S3A**). Metabolic differences between WKY and SHR are highlighted in representative plasma spectra (**Fig. S3B**). The chemical shifts for the identified metabolites are listed in **Table S4**. SHR showed higher concentration of leucine, phenylalanine, creatinine and glucose (\approx 23, 64, 23 and 43%, respectively), and \approx 20% lower concentration of aliphatic chains, as compared to WKY. Both probiotic LC40 and BFM normalized plasma phenylalanine, without affecting other metabolites. Plasma creatinine concentration was increased \approx 25% by butyrate treatment.

3.4. Probiotics restored T cell populations changes in lymphoid organs in SHR

We found that the number of total T cells in MLNs was similar in SHR compared to WKY (**Fig. S4A**). However, a \approx 30% lower T cell percentage was detected in spleen from SHR as compared to WKY (**Fig. S4B**). No change was observed by treatments in total T cells from both secondary lymph organs. The percentage of Treg ($CD4^+/FoxP3^+$) was reduced \approx 65%, whereas Th17 ($CD4^+/IL-17^+$) lymphocytes were \approx 2-fold significantly increased in both MLNs (**Fig. 5A**) and spleen (**Fig. 5B**) in SHR compared to WKY. All treatments increased

Treg and reduced Th17 to levels similar to that found in WKY in both secondary lymph organs, with the exception of chronic acetate in spleen.

3.5. Probiotics treatment improves endothelial function, oxidative stress and T cells infiltration in SHR

A reduced endothelium-dependent vasodilator response to acetylcholine when stimulating with phenylephrine in aortae from control SHR was shown as compared with aortae from control WKY ($E_{max} = 59 \pm 4\%$ vs $84 \pm 3\%$, respectively). Both LC40 and BFM treatments increased the relaxation induced by acetylcholine in SHR rats ($E_{max} = 70 \pm 5\%$, and $69 \pm 2\%$, respectively, $P < 0.05$ vs SHR control) (**Fig. 6A**). Similarly, chronic butyrate and acetate also improved the relaxation to acetylcholine ($E_{max} = 74 \pm 5\%$, and $63 \pm 3\%$, respectively, $P < 0.05$ vs SHR control) (**Fig. 6A**). This relaxation was unaltered by chronic interventions with both probiotics and SCFAs in WKY rats (**Fig. S5**). In all experimental groups, the acetylcholine-induced relaxation was fully inhibited by L-NAME (data not shown), which shows that in this vessel relaxation induced by acetylcholine in both WKY and SHR was completely dependent on NO derived from endothelium. The endothelium-independent vasodilator responses to nitroprusside, which directly activates soluble guanylyl cyclase in vascular smooth muscle, were not different among groups (data not shown), showing no change in the signaling of NO in vascular smooth muscle. No significant changes in NOS activity and arginase activity in aorta from all experimental groups were observed (**Fig. S6**).

ROS production from the NADPH oxidase is a crucial element in endothelial dysfunction in SHR. In fact, we found that the presence of the selective NADPH oxidase inhibitor VAS2870 in the organ chamber increased the relaxant response to acetylcholine in untreated SHR ($E_{max} = 80 \pm 4\%$), reaching similar relaxation percentages to those found in

WKY ($E_{max} = 85 \pm 3 \%$). In the presence of this agent we were not able to find any differences between groups, as compared to SHR group (**Fig. 6B**). In agreement with this, NADPH oxidase ROS production was increased $\approx 60\%$, $P < 0.01$, in aortic rings from SHR as compared with WKY rats (**Fig. 6C**). Both probiotics prevented this increase in NADPH oxidase activity in SHR. In aortic tissue from SHR, a significant increase in mRNA levels of NADPH oxidase subunits, NOX-1, NOX-4, p47^{phox} and p22^{phox} (≈ 2 -, 8.5-, 8.8- and 3.5-fold, respectively) was observed as compared with WKY rats (**Fig. 6D**). Again, both probiotics normalized the gene expression of NADPH oxidase subunits in SHR. TLR4 mRNA levels in aortic homogenates were ≈ 8 -fold higher in SHR as compared with WKY (**Fig. 6E**). In SHR, probiotics and SCFAs restored the TLR4 mRNA levels to similar values to those of WKY.

The infiltration of total T cells was similar in aorta from SHR than from their normotensive counterparts, and was unchanged by all treatments (**Fig. 7A**). However, we found reduced by $\approx 75\%$ the Treg (FoxP3⁺/CD4⁺) populations without significant change in the Th17 (IL-17⁺/CD4⁺) populations in aortas from the SHR group as compared to WKY rats (**Fig. 7B**). LC40, BFM and butyrate, but not acetate, treatments restored the aortic accumulation of Treg with no significant changes in Th17 infiltration (**Fig. 7B**).

4. Discussion

The main new findings of this study are the following: 1) chronic treatments with the probiotics LC40 or BFM prevented both gut dysbiosis (reduced F/B ratio, increased butyrate-producing bacteria) and the blood pressure increase in SHR; 2) Oral treatment with the SCFAs, butyrate or acetate, also prevented the raise in both blood pressure and F/B ratio; 3) SBP is directly correlated to the *Lactobacillus/S24-7g* ratio, and the SBP reduction induced by all treatments was linked to a decrease in this ratio; 4) All treatments restored the Th17/Treg balance in MLNs, and normalized endotoxemia; 5) All treatments prevented the

impairment of endothelium-dependent relaxation to acetylcholine, as a result of reduced NADPH oxidase-driven ROS production; 6) All treatments were unable to change SBP and endothelial function in normotensive WKY rats; 7) The protective effects induced by treatments in vascular oxidative stress and endothelial function seem to be independent of acetate and lactate plasma levels and might be mediated by the reduction in vascular LPS/TLR4 pathway, the increase in Treg infiltration in the vasculature in rats with genetic hypertension, and for LC40, BFM and butyrate by the increase in plasma butyrate concentration.

Multiple studies have demonstrated the association between gut dysbiosis and hypertension [1, 2, 4, 6, 10]. Our results are consistent with the main features of dysbiotic microbiota described in SHR [1, 10, 28]: a) an increased F/B ratio, and b) a reduction in acetate- and butyrate-producing bacteria, with higher proportion of lactate-producing bacteria. We have previously demonstrated that when SHR with established hypertension were treated for 5 weeks with the probiotics, LC40 or *Lactobacillus coryniformis* CECT5711 plus *Lactobacillus gasseri* CECT5714 (1:1) an improvement of endothelial dysfunction and a decrease in blood pressure were found [19]. These cardiovascular protective effects induced by *Lactobacillus* strains were associated with changes in some bacterial genera (increased *Lactobacillus* spp and reduced *Bacteroides* and *Clostridium* spp) measured by qRT-PCR. Data of the microbiota composition from the present study is difficult to compare with this previous work because we now performed 16s ribosomal DNA sequencing. Recently, we found a positive and negative correlation of SBP with *Turicibacter* and *S24-7g* bacterial abundance, respectively [10]. In agreement with this study we showed that *Turicibacter* was increased and *S24-7g* was reduced in SHR as compared to WKY. However, the pathological value of the lactate-producing genus *Turicibacter* to induce a hypertensive phenotype has not been established. In the present study, we found a significant direct correlation between

Lactobacillus/S24-7g ratio and SBP. The unclassified genus belonging to the S24-7 family, the unidentified taxon from order *Bacteroidales*, was found in higher numbers in non-diabetic mice also correlated positively with splenic FoxP3⁺CD4⁺ Treg cells and the delayed diabetes onset age [29]. The probiotics LC40, a strain of *Lactobacillus fermentum*, and BFM, a strain of *Bifidobacterium breve*, did not change the relative abundance of its own genera in the gut, *Lactobacillus* or *Bifidobacterium*, respectively, which indicates that their effect is not just due to a replacement of other bacteria but rather to a positive effect on the whole bacterial community. The main change at genera level induced by chronic consumption of LC40 and BFM, or SCFAs was a reduced *Lactobacillus/S24-7g* ratio. However, we did not perform any experiment addressed to clarify if this reduction is mechanistically involved on the antihypertensive effects of these treatments.

Dysbiotic microbiota from SHR increased blood pressure, at least in part, as a consequence of its effects on T-cell activation in the gut immune system and in vascular T-cells accumulation [9]. Interestingly, both LC40 and BFM restored the proportion of butyrate-producing bacteria, which were found reduced in SHR. SCFAs, such as butyrate, acetate and propionate, can influence immune function in this part of the intestine. In particular, the number and function of peripheral Treg cells in the colon are enhanced by SCFAs [30-32]. In fact, we found increased Treg cells populations in MLNs from SHR treated with probiotics that increased butyrate-producing bacteria and butyrate content in feces or with oral acetate or butyrate. Moreover, significant changes in the proportion of Treg cells were found in spleen from SHR-treated groups as compared to SHR, with the exception of acetate-treated SHR. This lack of effects could be related to the surprising absence of significant change in the acetate plasma levels, despite oral consumption of acetate. This could be partially explained by: a) the low plasma half-life of acetate [33]; b) reduced expression of MCT1 for active transport of SCFAs; and c) reduced passive diffusion of

acetate because the expression of genes associated with tight junction such as occluding and ZO-1 were upregulated by treatment with acetate. The most important discrepancy between SCFAs-producing bacteria in the gut and plasma SCFAs levels is lactate, which showed an increased proportion of lactate-producing bacteria and reduced plasma lactate levels in SHR as compared to WKY. One possible explanation might be related to the reduced expression of MCT1 in SHR, the major SCFA transporter expressed in the gut for the uptake of lactate [34]. Interestingly, a decreased membrane transporter specific for butyrate (MCT4) was observed in colonic samples from SHR, which was significantly up-regulated by butyrate, similar to that previously described [15]. Interestingly, LC40 and BFM, that increased butyrate-producing bacteria, and butyrate consumption increase plasma levels of butyrate in SHR. This SCFA inhibits the differentiation of Th17 cells [35, 36]. In fact, only these treatments change T cells polarization in spleen, being without effects acetate consumption. In addition, systemic butyrate might act directly at the vascular wall reducing oxidative stress and improving endothelial dysfunction, as previously described in ApoE^{-/-} mice [37].

Hypertension is associated with the altered expression of gut tight junction proteins, increased permeability and gut pathology [38]. SCFAs have been demonstrated to exert many beneficial effects on intestinal epithelium, including inhibition of inflammation [39], and modulation of oxidative stress [40]. Furthermore, an improved barrier function by SCFAs has been reported *in vitro* [41-44], *ex vivo* [45], and in animal studies [15, 46]. In agreement with these data, we also found that probiotics, and acetate and butyrate consumption increased mRNA levels of tight junction protein occludin and ZO-1 in the colon. However, no direct measures of gut permeability were performed in the present study. Increased intestinal permeability in adult hypertensive SHR has been related to reduced goblet cells [38]. These cells produce mucins, which protect the gut from pathogen invasion, thereby regulating the gut immune response [47]. However, MUC-2, the main structural component of the mucus

layer, and MUC-3 transcripts in SHR, were only increased by both SCFAs. In addition, IL-18, a cytokine important for tissue repair [41], was also increased in colon from SHR-treated groups as compared to untreated SHR. Overall, our results suggest that probiotics might improve gut integrity with the subsequent reduced translocation of bacterial endotoxin into the circulation.

In the presence of altered gut mucosa, bacteria can translocate across the intestinal epithelium resulting in the activation and migration of CX3CR1⁺ cells, including dendritic cells and macrophages, to draining lymph nodes of the lower intestinal tract [48]. Additionally, they present soluble antigens to naïve CD4⁺ T cells, leading to T cell activation. Moreover, intestinal epithelial cells constitutively secrete IL-18 that acts directly on IL-18R1-expressing CD4⁺ T cells to limit colonic Th17 cell differentiation [49]. Our results showing reduced levels in colonic IL-18 expression in SHR are consistent with increased levels of Th17 in MLNs from SHR, and treatments increasing colonic IL-18 significantly reduced Th17 in secondary lymph organs in the gut.

The activation of TLR4 contributes to high blood pressure and low-grade vascular inflammation present in SHR [17]. Actually, TLR4^{-/-} mice demonstrated a full blood pressure protection against L-NAME-induced hypertension [50]. Thus, enhanced TLR4 expression might be linked to the development and maintenance of hypertension. Bacterial LPS stimulates and increase the expression of TLR4 in the vasculature, which resulted in increased NADPH oxidase-dependent O₂⁻ production and inflammation [51, 52]. We found that TLR4 expression was higher in aorta from SHR than in age-matched Wistar controls, linked to increased LPS plasma levels. We also found that the impaired aortic endothelium-dependent relaxation to acetylcholine in SHR is mediated by increased NADPH oxidase O₂⁻ production, which swiftly reacts with NO, decreasing its bioavailability, since it was restored

by the selective NADPH oxidase inhibitor VAS2870. This endothelial dysfunction seems to be induced, at least in part, by the gut bacterial product endotoxin. In fact, all treatments reduced endotoxemia resulting in impaired LPS/TLR4 pathway in the vasculature and the subsequent improvement of the endothelium- and NO-dependent relaxation. This protective effect seems to be independent of changes in NOS or arginase activity in aorta, since both activities were similar among all experimental groups.

Various hypertensive stimuli cause T-cell activation and infiltration into target organs such as the vessels, which promotes vascular dysfunction and a raise in blood pressure [53]. Gut microbiota is a highly relevant factor in blood pressure regulation, due to its effects on T-cell activation in the gut immune system and in vascular T-cells accumulation [10]. We found increased Th17 cells in aortas with impaired relaxation to acetylcholine from SHR as compared to WKY. The proinflammatory cytokine IL-17, produced by Th17, causes Rho-kinase-mediated endothelial dysfunction by increasing phosphorylation of the inhibitory eNOS residue Thr⁴⁹⁵ [54]. By contrast, in agreement with Katsuki *et al.* [55], Treg accumulation was decreased in SHR group. IL-10, the main cytokine released by Tregs, attenuates NADPH oxidase activity, which is a critical process in the improvement of vascular endothelial function in hypertension [56]. Improvement of endothelial function by LC40, BFM, and butyrate could be also related to increased Treg infiltration in the vasculature, possibly as a result of increased differentiation of Tregs in secondary lymph nodes. In addition, reduced phenylalanine plasma levels induced by both probiotic treatments might contribute to improve endothelial dysfunction, since vessels from hyperphenylalaninemic mice showed reduced levels of the essential cofactor of eNOS tetrahydrobiopterin, improving eNOS uncoupling [57].

In conclusion, we have found for the first time that probiotics LC40 and BFM increased butyrate-producing bacteria in SHR and prevented the development of endothelial dysfunction and hypertension. This effect seems to be related to its capacity to reduce endotoxemia. The effects were reproduced in rats consuming acetate or butyrate. Furthermore, the increased Tregs accumulation in the vasculature and the increase in plasma butyrate concentration induced by both probiotic might also contribute to protect the vasculature and reduce BP. Taken into account that these results were obtained in a renin-dependent model of hypertension, the effects of probiotics in renin-independent models, such as deoxycorticosterone acetate (DOCA)-salt induced hypertension, should be explored. Thus, our results are a new proof of concept showing that gut microbiota might be intertwined functionally with chronic probiotics consumption to prevent genetic hypertension. However, caution is advised when extrapolating these findings to humans because of the possible differences between the animal and human gut microbiota.

5. References

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Authors contribution

IRV, MT and JD participated in the research design. IR-V, MT, N.d.I.V, MS, MG-G, MR, TY, JLI-G, EG-H, and RJ performed the experiments. IR-V, MT, MR, RJ, JR-C, MKR, FP-V, and JD contributed to data analysis. FP-V, and JD wrote or contributed to the writing of the manuscript. All authors approved the final version to be published.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Figure 1. Probiotic treatments prevent elevated blood pressure in spontaneously hypertensive rats (SHR). Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography in all experimental groups (**A, B**). Mean arterial blood pressure (MABP), measured by intra-arterial recording into left carotid artery, at the end of the experimental period (**C**) and heart rate (HR) (**D**). Results are shown as mean \pm SEM (n = 7-10). **P < 0.01 compared with Wistar Kyoto (WKY) group. #P < 0.05 and ###P < 0.01 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

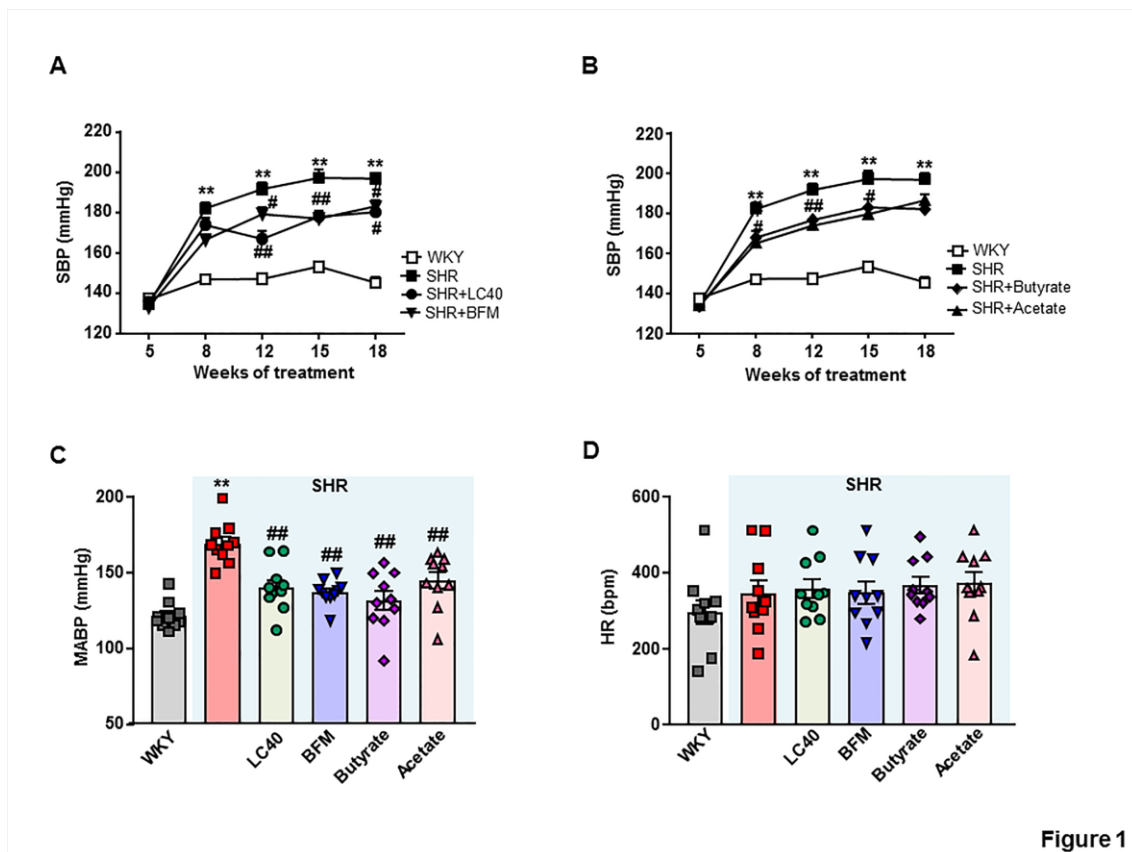


Figure 2. Probiotic treatments prevent gut dysbiosis in spontaneously hypertensive rats (SHR).

Bacterial 16S ribosomal DNA were amplified and sequenced to evaluate three major ecological parameters, Chao richness, Pielou evenness, and the number of observed species

(A). Phylum breakdown of the 7 most abundant bacterial communities (B). The

Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker for gut dysbiosis

(C). Relative proportions of acetate-, butyrate-, and lactate-producing bacteria in the gut

microbiota from Wistar Kyoto (WKY) and SHR groups (D). Results are shown as mean \pm

SEM (n = 5-6). *P < 0.05 and **P < 0.01 compared with WKY group. #P < 0.05 compared

with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM,

Bifidobacterium breve CECT7263.

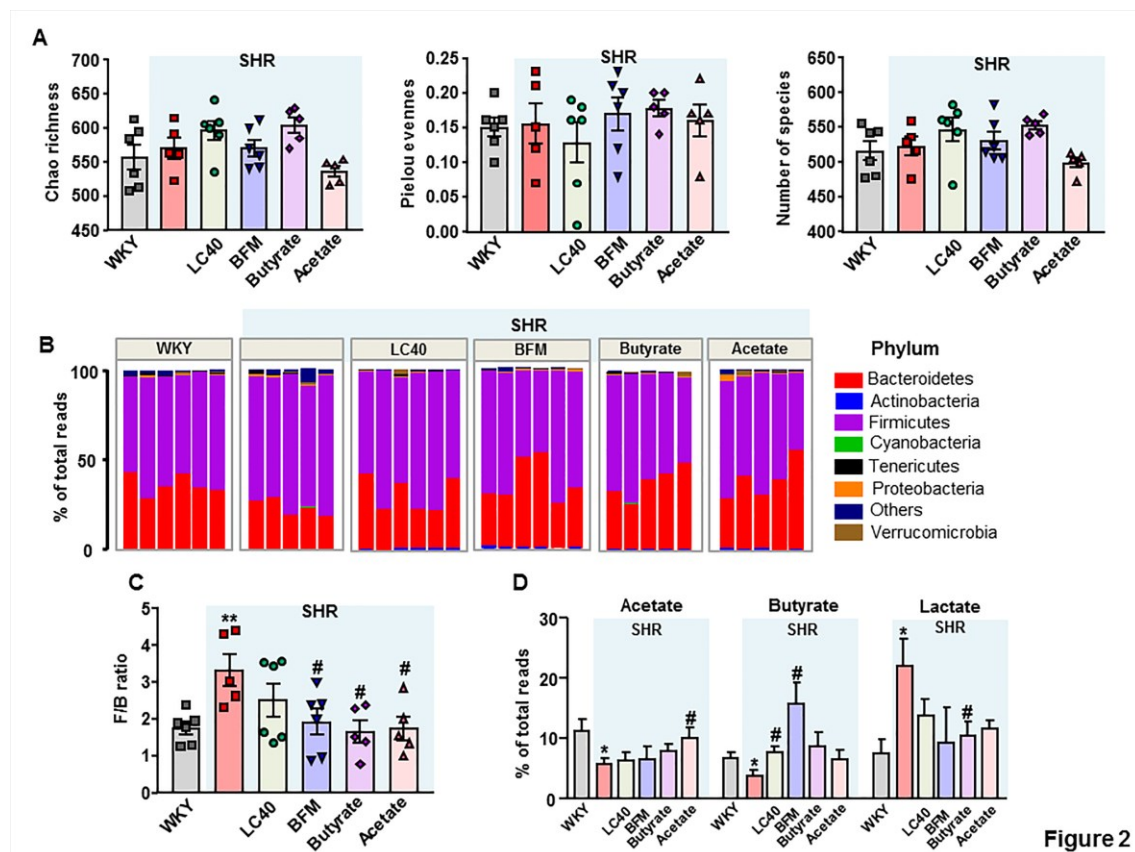


Figure 2

Figure 3. Probiotics treatments contribute gut microbiota homeostasis in spontaneously hypertensive rats (SHR). Heat map showing the bacterial families and genera most differing in abundance between the experimental groups. Samples clustered by treatment group showed that the treatments resulted in distinct populations of bacteria at the family and genus level. The heat map colors represent the relative percentage of microbial family assigned within each sample as compared to SHR (A, B). Main significantly modified bacterial genera (C) in the gut microbiota in Wistar Kyoto (WKY) and SHR groups. Ratio *Lactobacillus*/ *S24-7g* and correlated with systolic blood pressure (SBP) (D). Results are shown as mean \pm SEM (n = 5-6). *P < 0.05 and **P < 0.01 compared with WKY group. #P < 0.05 and ###P < 0.01 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

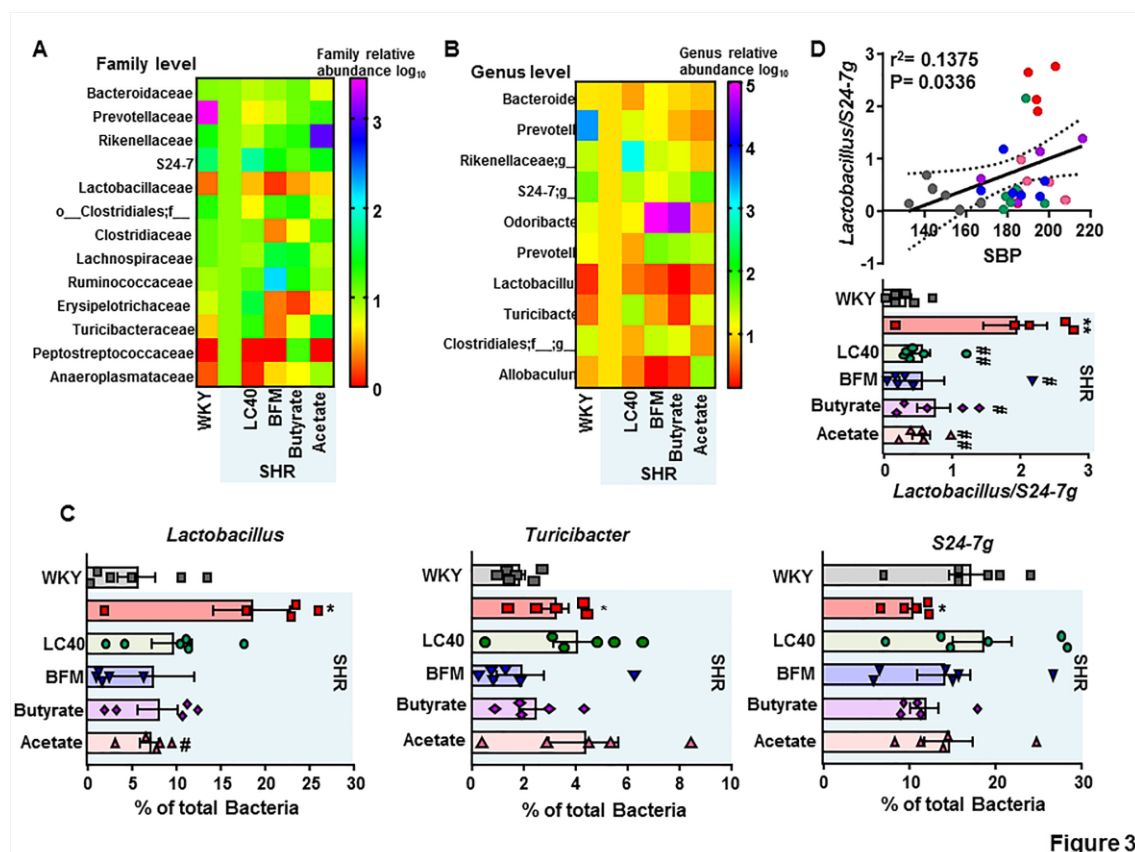


Figure 3

Figure 4. Probiotic treatments improve altered gut integrity in spontaneously hypertensive rats (SHR). Colonic mRNA levels of occludin, and zonula occludens-1 (ZO-1) (A). Mucin (MUC)-2, and MUC-3 (B). Plasma endotoxin concentrations (EU/mL, endotoxin units/mL (C). Tissue repair cytokine IL-18 (D). Expression of the short-chain fatty acids (SCFAs) transporters, MCT-1 and MCT-4 (E), the plasma levels of SCFAs from the Nuclear Magnetic Resonance (NMR) spectra (F), and plasma butyrate levels by GC (G) in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean \pm SEM (n = 7-10). *P < 0.05 and **P < 0.01 compared with WKY group. #P < 0.05 and ##P < 0.01 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

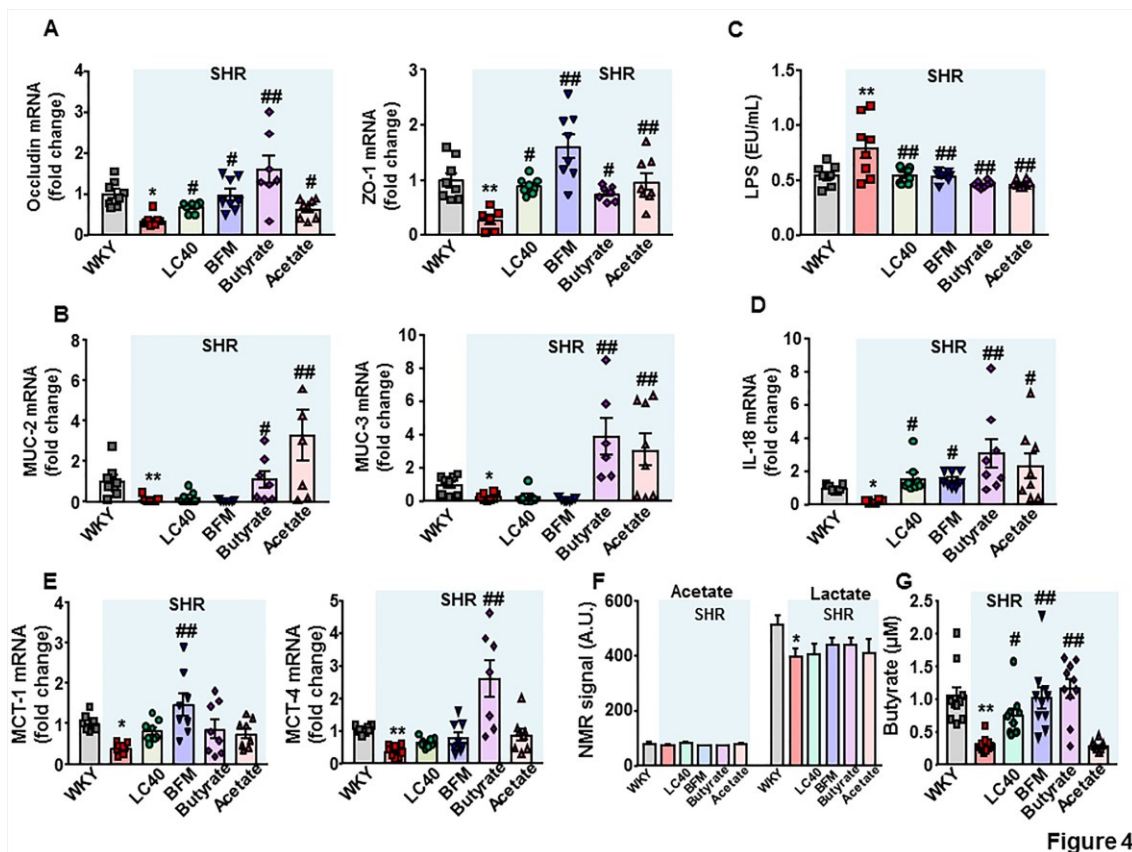


Figure 4

Figure 5. Probiotic treatments restore T cell imbalance in spontaneously hypertensive rats (SHR). Regulatory T (Treg) cells, and T-helper (Th) 17 cells measured in mesenteric lymphoid nodes (A) and spleen (B) in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean \pm SEM (n = 7-10). *P < 0.05 and **P < 0.01 compared with WKY group. #P < 0.05 and ##P < 0.01 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

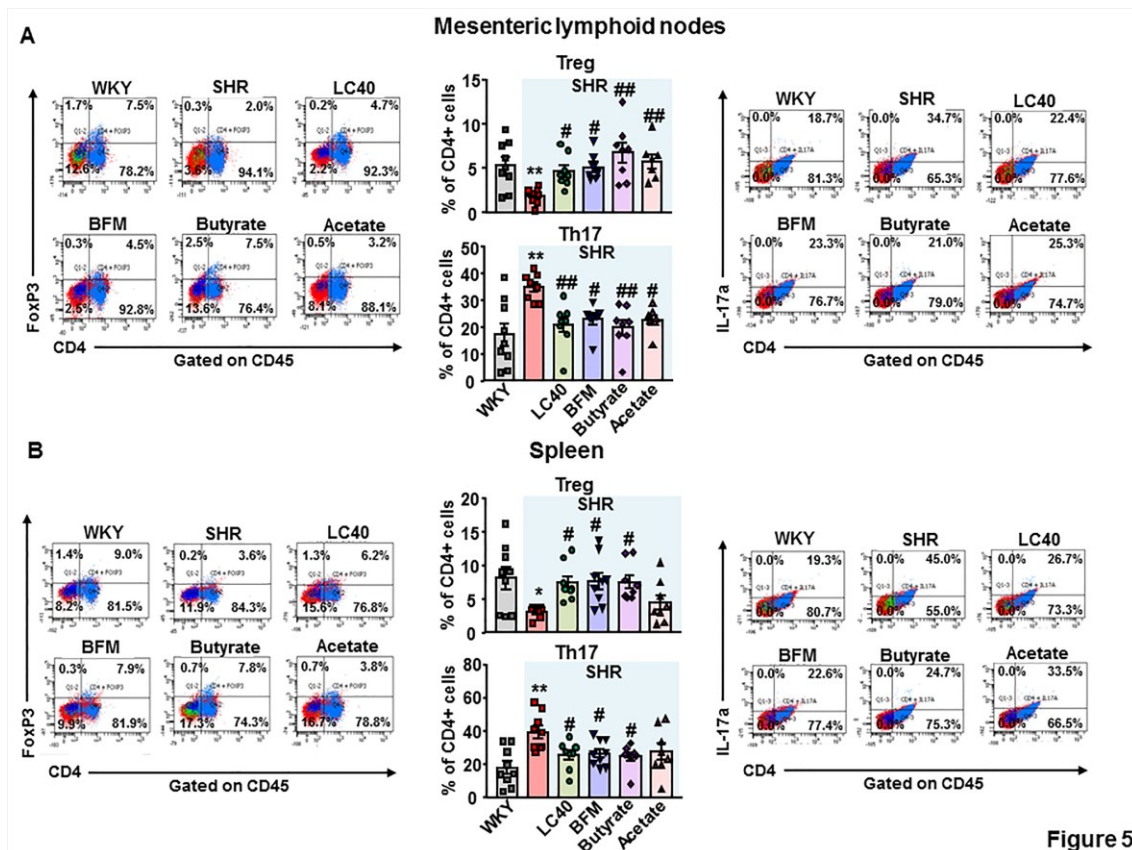


Figure 5

Figure 6. Protective effects of probiotic treatments in endothelial function and oxidative stress in spontaneously hypertensive rats (SHR). Vascular relaxant responses induced by acetylcholine (Ach), in endothelium-intact aortae pre-contracted by phenylephrine (Phe) in the absence (**A**) and in the presence of the NADPH oxidase inhibitor VAS2870 (5 μ M; **B**) in Wistar Kyoto (WKY) and SHR groups. NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (**C**), and aortic mRNA levels expression of NADPH oxidase subunits NOX-4, p47^{phox}, NOX-1, and p22^{phox} (**D**), and toll-like receptor (TLR)-4 (**E**) in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean \pm SEM (n = 7-10). *P < 0.05 and **P < 0.01 compared with WKY group. #P < 0.05 and ##P < 0.01 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

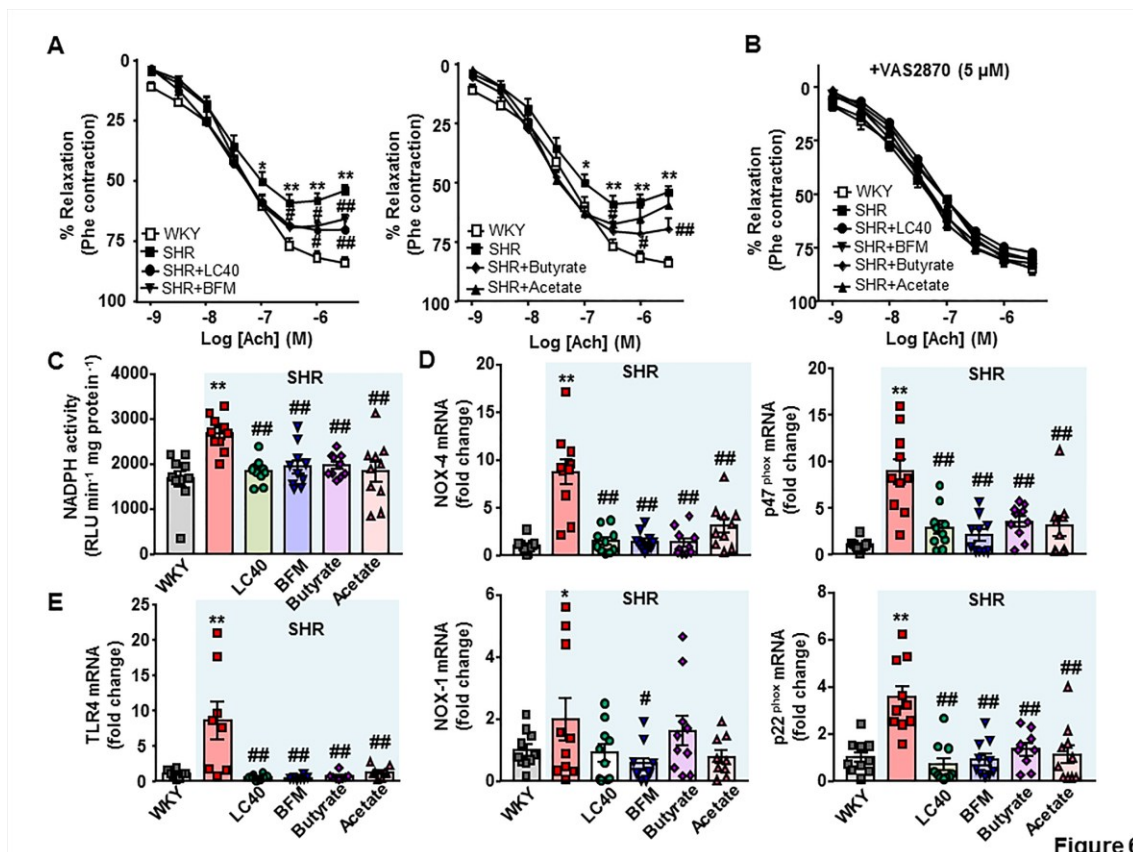


Figure 6

Figure 7. Probiotic treatments improve T cell infiltration in spontaneously hypertensive rats (SHR). Total T cells (A), regulatory T (Treg) cells, and T-helper (Th) 17 cells (B) measured in aortae of Wistar Kyoto (WKY) and SHR groups. Results are shown as mean \pm SEM (n = 7-10). *P < 0.05 compared with WKY group. #P < 0.05 compared with the non-treated SHR group. LC40, *Lactobacillus fermentum* CECT5716; BFM, *Bifidobacterium breve* CECT7263.

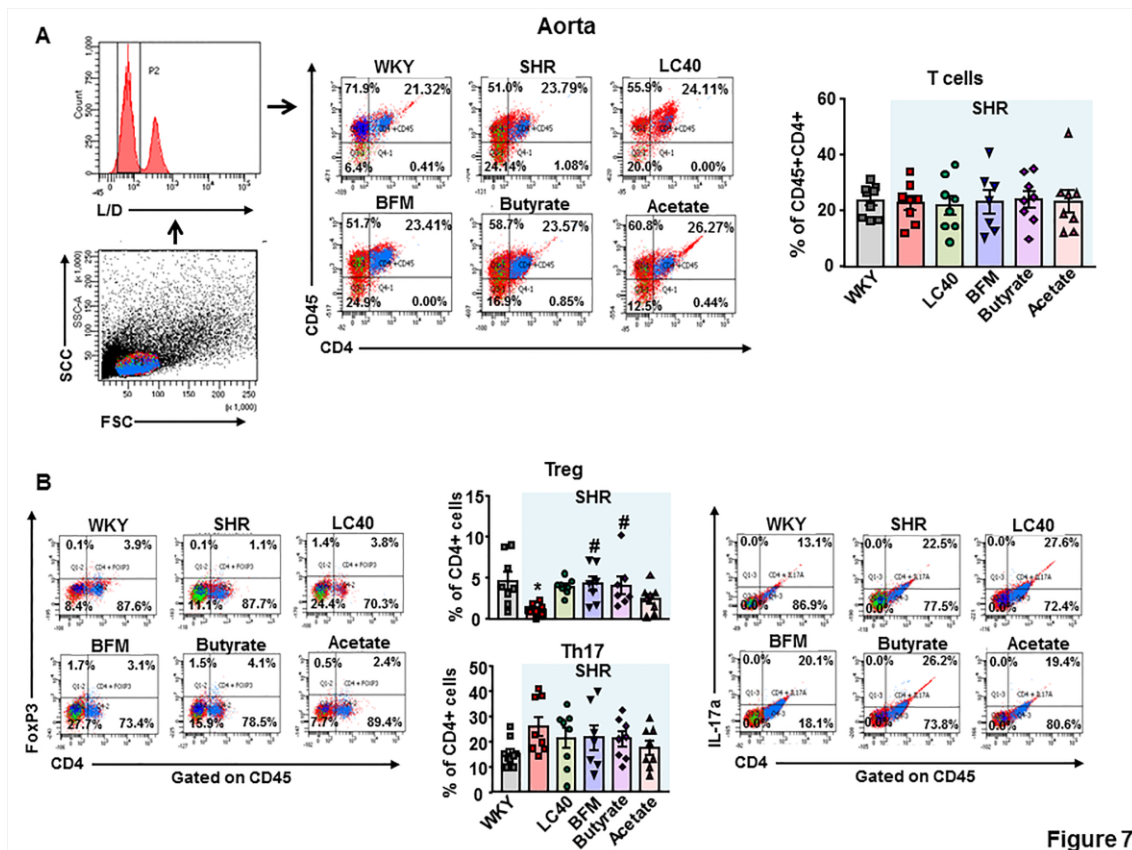


Figure 7