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Microbiota intestinal en hipertensión. Papel del sistema inmunológico.

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ABBREVIATIONS

ABBREVIATIONS

3MST
3-mercaptopyruvate sulfurtransferase

A23187
Calcium ionophore calimycin

ACE
Angiotensin-converting enzyme

ACh
Acetylcholine

AGT
Angiotensin

cAMP
Cyclic adenosine monophosphate

AngI
Angiotensin I

AngII
Angiotensin II

AT1
Angiotensin II receptor type 1

AT2
Angiotensin II receptor type 2

AU
Arbitrary units

AUC
Area under the curve

AP1
Targeting activator protein 1

APC
Antigen presenting cell

BFM
Bifidobacterium breve

BM
Bone marrow

BP
Blood pressure

BSA
Bovine serum albumin

BW
Body weight

Ca^{2+}
Calcium

CECT
Spanish type culture collection

CFU
colony-forming unit

CCR9
C-C chemokine receptor type 9

CM-H2DCFDA
5-(and-6) chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate

COX
Cyclooxygenase

CSE
Cystathionine-lyase

CTLA-4
Cytotoxic T-lymphocyte-associated protein 4

DAF-2
Diaminofluorescein-2

DAG
Diacylglycerol

DAPI
4,6-diamidino-2-phenylindole dichlorohydrate

DAMPs
Damage-associated molecular patterns

DBP
Diastolic Blood Pressure

DCs
Dendritic cells

DHE
Dihydroethidium

DMSO
Dimethylsulfoxide

DNA
Deoxyribonucleic acid

DOCA
Deoxicorticosterone acetate

DTPA
Diethylenetriaminepenta-acetic acid

EDCF
Endothelium-derived contracting factors

EDHF
Endothelium-derived hyperpolarizing factor

EDTA
Ethylenediaminetetraacetic acid

EDRFs
Endothelium-derived relaxing factors

eNOS
Endothelial nitric oxide synthase

ET-1
Endothelin-1

F/B
Firmicutes/Bacteroidetes

FBS
Fetal bovine serum

FFAR
Free fatty acids receptor

FMO
Fluorescence minus one

FMT
Fecal microbiota transplantation

FSC
Forward-scattered light

FoxP3
Forkhead box P3

GADPH
Glyceraldehyde-3-phosphate dehydrogenase

GATA-3
GATA Binding Protein 3

GPR
G-protein-coupled receptor

H_2S
Hydrogen sulfide

HDZ
Hydralazine

HEPES
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTN
Hypertension

HDAC
Histone deacetylase

HFD
High-fat diet

HR
Heart range

HW
Heart weight

I3P
Inositol trisphosphate

IECs
Intestinal epithelial cells

IL
Interleukin

INFγ
Interferon gamma

ip
Intraperitoneally

IR
Insulin resistance

ISAPP
International Scientific Association for Probiotics and Prebiotics

KMO
Kaiser-Meyer-Olkin

KW
Kidney weight

LAL
Limulus amebocyte lysate

LC40
Lactobacillus fermentum

LDA
Linear discriminant analysis

L-NAME
N_G-nitro-L-arginine methyl ester

Lefse
Linear discriminant analysis Effect Size

LOS
Losartan

LPS
Lipopolysaccharides

LVW
Left ventricle weight

MABP
Mean arterial blood pressure

MLNs
Mesenteric lymph nodes

mRNA
Messenger RNA

MCT
Monocarboxylate transporters

MHC
Major histocompatibility complex

MUC
Mucin

NA
Noradrenalin

NADPH
Nicotinamide adenine dinucleotide phosphate

NF-κB
Nuclear factor-κB

NIACR1
Niacin receptor 1

NK
Natural killer

NLRP3
NLR Family Pyrin Domain Containing 3

NMR
Nuclear magnetic resonance

NO
Nitric oxide

O_2^-
Superoxide

Olfr
Olfactory receptor

OTU
Operational taxonomic unit

PAMPs
Pathogen-associated molecular pattern molecules

PAST
Paleontological statistics

4-PBA
4-phenyl butyric acid

PBS
Phosphate-buffered saline

PCA
Principal component analysis

PCoA
Principal Coordinates Analysis

PFA
Paraformaldehyde

PKC
Protein kinase C

PGA1
Prostaglandin A1

PGD2
Prostaglandin D2

PGI2
Prostaglandin 2

PKA
Protein kinase A

PKC
Phosphorylation of protein kinase C

Phe
Phenylephrine

PRR
Pattern recognition receptors

PVR
Peripheral vascular resistances

PVN
Paraventricular nucleus

QIIME
Quantitative Insights Into Microbial Ecology

RAS
Renin-angiotensin system

RAECs
Rat endothelial cells

RDP
Ribosome Database project

RLU
Relative luminescence unit

ROS
Reactive oxygen species

RORγ
RAR-related orphan receptor gamma

RNP
Rat neutrophil-derived alpha-defensins

rpm
Revolutions per minute

RT-PCR
Reverse transcriptase-PCR

SBP
Systolic blood pressure

SCID mice
Severe combined immunodeficient mice

SCC
Side-scattered light

SCFA
Short Chain fatty acid

SDS
Sodium dodecyl sulphate

SEM
Standard error of the mean

SHR
Spontaneously hypertensive rats

SNP
Nitroprusside sodium

SNS
Sympathetic nervous system

SOD
Super oxide dismutase

T-BET
T-box transcription factor

TCR
T-cell receptor

TLR
Toll-like receptor

TNF-α
Tumor necrosis factor-α

Th
T-helper

TH
Tyrosine hydroxylase

Treg
Regulatory T cells

TMAO
Trimethylamine N-oxide

TMA
Trimethylamine

VSMC
Vascular smooth muscle cells

WKY
Wistar-Kyoto

ZO-1
Zonula occludens-1

ABSTRACT

RESUMEN

El intestino alberga billones de bacterias que modulan la homeostasis del huésped dentro y fuera del tracto intestinal. Existen numerosas evidencias que asocian la existencia de disbiosis intestinal y la enfermedades neurogénicas, como la hipertensión. Se conoce que tanto el sistema inmunológico como el sistema nervioso simpático, controlado desde el sistema nervioso central, contribuyen al desarrollo de la hipertensión. También, es conocida la interacción entre intestino y cerebro y que la microbiota intestinal se adapta a las condiciones de salud o enfermedad del individuo. Además se sabe que el uso de probióticos puede reducir la presión arterial (PA) en animales y en humanos. **La hipótesis** de nuestro estudio es la siguiente: La disbiosis intestinal detectada en hipertensión genética altera el sistema inmunitario intestinal y la descarga simpática desde el sistema nervioso central, contribuyendo al mantenimiento de la PA elevada. La microbiota intestinal está alterada en la hipertensión por el excesivo tono simpático en el intestino y los probióticos pueden reducir la PA cambiando la modulación del sistema inmunitario intestinal y la descarga simpática en el intestino. Para constatar si esta hipótesis es cierta hemos realizado varios estudios secuenciales que se resumen a continuación. Las respuestas inmunes innatas y adaptativas participan en la generación de especies reactivas de oxígeno (ROS) y en los cambios inflamatorios en los riñones, los vasos sanguíneos y el cerebro en la hipertensión. La co-estimulación de células T a través de ligandos B7 es esencial para el desarrollo de la hipertensión experimental, y la inhibición de este proceso podría tener un beneficio terapéutico en el tratamiento de esta enfermedad. El aumento de la infiltración de macrófagos en el espacio peri-adventicia de los vasos y un equilibrio entre la reactividad de las células T pro-inflamatorias y las células T reguladoras (Tregs) con carácter anti- inflamatorio determinan el desarrollo de hipertensión. Sin embargo, se desconoce si los cambios en la activación de las células T en los órganos linfáticos intestinales, posiblemente inducidos por la microbiota intestinal, contribuyen a controlar la función endotelial y la PA en la hipertensión genética. El objetivo de nuestro **primer estudio** (*Toral M, Robles-Vera I, de la Visitación N, Romero M, Sánchez M, Gómez-Guzmán M, Rodríguez-Nogales A, Yang T, Jiménez R, Algieri F, Gálvez J, Raizada MK,* ***Duarte J****. Role of the immune system in vascular function and blood pressure control induced by faecal microbiota transplantation in rats. Acta Physiol (Oxf). 2019 Sep;227(1):e13285. doi: 10.1111/apha.13285. Epub 2019 Jun 2. PubMed PMID: 31004464*), *índice de impacto: 5,868 (2018 JCR Science Edition), Physiology 8/81 Q1*

(Decil 1)) fue investigar si los cambios en la microbiota intestinal inducidos por el intercambio de la microbiota entre ratas espontáneamente hipertensas (SHR) y el normotensiva Wistar Kyoto (WKY) alteran la interacción del sistema inmunitario intestinal, induciendo cambios en la función vascular y la PA. **Métodos:** Las ratas receptoras WKY y SHR de veinte semanas de edad fueron tratadas oralmente con contenido fecal de ratas donantes WKY o SHR. En experimentos adicionales, utilizamos un diseño para determinar si el bloqueo de la co-estimulación dependiente de B7 con CTLA4-Ig o el bloqueo de IL-17 con anticuerpo neutralizante de IL-17 podría prevenir la hipertensión causada por el trasplante de microbiota fecal (FMT) de SHR a WKY. **Resultados:** Los análisis de correlación identificaron la abundancia bacteriana de *Turicibacter y S24-7_g* que, respectivamente, se correlacionaron positiva y negativamente con la PA sistólica. El FMT de ratas WKY a ratas SHR redujo la PA sistólica basal, restableció el desequilibrio entre Th17/Treg en los ganglios linfáticos mesentéricos (MLN) y en la aorta, y mejoró la disfunción endotelial y el estado oxidativo vascular en SHR trasplantado con heces SHR. El FMT de SHR a WKY aumentó los niveles de ARNm de CD80 y CD86 y la activación de células T en MLN, las células T circulantes y la infiltración de células T aórticas, deterioró la función endotelial y aumentó PA sistólica basal. Estos efectos fueron abolidos por el bloqueo de la co-estimulación dependiente de B7 con CTLA4-Ig. El anticuerpo neutralizador IL-17a redujo la PA sistólica y mejoró la disfunción endotelial inducida por FMT de SHR a WKY. **Conclusión:** La microbiota intestinal es un factor importante involucrado en el control de la PA, como consecuencia de su efecto en la activación de las células T en el sistema inmunitario intestinal y la acumulación de células T vasculares.

Se ha descrito una señalización bidireccional entre el cerebro y la microbiota intestinal que podría regular la PA a través de la modulación de la interacción entre el sistema nervioso simpático y el sistema inmunitario. El objetivo de nuestro **segundo estudio** (*Toral M, Robles-Vera I, de la Visitación N, Romero M, Yang T, Sánchez M, Gómez-Guzmán M, Jiménez R, Raizada MK,* ***Duarte J.*** *Critical Role of the Interaction Gut Microbiota - Sympathetic Nervous System in the Regulation of Blood Pressure. Front Physiol. 2019 Mar 8;10:231. doi: 10.3389/fphys.2019.00231. eCollection 2019. PubMed PMID: 30930793; PubMed Central PMCID: PMC6423906, índice de impacto: 3,201 (2018 JCR Science Edition), Physiology 25/81 Q2*) fue investigar si los cambios en la microbiota intestinal alteran las interacciones intestino-cerebro que inducen cambios en

la PA. Las ratas receptoras WKY y SHR de veinte semanas de edad fueron tratadas oralmente con contenido fecal de ratas donantes WKY o SHR. Dividimos a los animales en cuatro grupos: WKY trasplantado con WKY microbiota (W-W), SHR con SHR (S-S), WKY con SHR (W-S) y SHR con WKY (S-W). La PA sistólica basal (PAS) y la PA diastólica (PAD) se redujeron, sin cambios en la frecuencia cardíaca, como resultado del FMT de ratas WKY a SHR. Del mismo modo, el FMT de SHR a WKY aumentó PAS basal y PAD. Se encontraron aumentos tanto en la producción de especies de oxígeno reactivo mediada por la NADPH oxidasa como en las citocinas proinflamatorias en el núcleo paraventricular cerebral vinculadas a una mayor caída de la PA inducida por pentolinio y noradrenalina plasmática (NA) en el grupo S-S en comparación con el grupo W-W. Estos parámetros fueron reducidos por FMT de WKY a SHR. Se encontraron niveles aumentados de citocinas proinflamatorias, niveles de ARNm de tirosina hidroxilasa y contenido de NA en el colon proximal, mientras que se encontraron niveles reducidos de ARNm de proteínas de unión en el grupo S-S en comparación con el grupo W-W. Estos cambios fueron inhibidos por FMT de WKY a SHR. De acuerdo con nuestros análisis de correlación, la abundancia de *Blautia* y *Odoribacter* mostró una correlación negativa con una PAS alta. **En conclusión**, en SHR, la microbiota intestinal es un factor importante involucrado en el control de la PA, al menos en parte, como consecuencia de su efecto sobre la neuroinflamación y la actividad del sistema nervioso simpático.

La microbiota intestinal se conoce comúnmente que es un órgano esencial adquirido y que su composición y riqueza se adaptan constantemente a los desafíos que ocurren en el medio ambiente o en el huésped, como la edad, la dieta y las modificaciones en el estilo de vida. Además, se ha observado habitualmente que un cambio en el estado de salud del huésped se acompaña por un cambio en la microbiota intestinal. En los estudios anteriores hemos descrito un papel crítico de la interacción entre la microbiota intestinal y el sistema nervioso simpático en la regulación de la PA. Pensamos que la reducción de la PA en la hipertensión establecida podría contribuir a corregir la disbiosis intestinal en SHR, y también podría contribuir a mantener la PA baja a través de cambios en la infiltración de células T en la pared vascular. El objetivo de nuestro **tercer estudio** (*Robles-Vera I, Toral M, de la Visitación N, Sánchez M, Gómez-Guzmán M, Muñoz R, Algieri F, Vezza T, Jiménez R, Gálvez J, Romero M, Redondo JM, **Duarte J**. Changes to the gut microbiota induced by losartan contributes to its antihypertensive effects. Br J Pharmacol. 2020 May;177(9):2006-2023. doi: 10.1111/bph.14965. Epub 2020 Feb 3. PubMed PMID:*

31883108, índice de impacto: 6,583 (2018 JCR Science Edition), Pharmacology & Pharmacy, 14/267 Q1 (Decil 1)) fue evaluar los efectos de losartán, un fármaco del grupo de los antagonistas del receptos AT1 de la angiotensinaII, sobre la microbiota intestinal en SHR y el papel de la microbiota intestinal en el efecto antihipertensivo de losartán. **Diseño Experimental:** Se utilizaron ratas Wistar Kyoto (WKY) y SHR de veinte semanas de edad divididas en tres grupos: WKY (WKY) no tratado, SHR (SHR) no tratado y SHR tratado con losartán durante 5 semanas (SHR-losartán). También se realizó un experimento de trasplante de microbiota fecal (FMT) mediante administración mediante sonda gastroesofágica del contenido fecal del grupo SHR-losartán como donante al grupo SHR como receptor. **Resultados clave:** Las heces de SHR mostraron disbiosis intestinal, caracterizada por una mayor relación *Firmicutes/Bacteroidetes*, una menor proporción de bacterias productoras de acetato y un aumento en bacterias productoras de lactato, así como una reducción en bacterias anaerobias estrictas, parámetros que fueron restaurados mediante el tratamiento con losartán. La mejora de la disbiosis intestinal se relacionó con una mayor integridad del colon y un menor activación simpática intestinal. En contraste, la hidralazina redujo la presión arterial pero no restableció la disbiosis intestinal ni la integridad del colon. Curiosamente, el FMT de SHR-losartán a SHR redujo la PA, mejoró la relajación vascular dependiente del endotelio a la acetilcolina y redujo la actividad de la NADPH oxidasa. Estos cambios vasculares estaban relacionados tanto con el aumento de Treg como con la disminución de la población de células Th17 en la pared vascular. **Conclusiones e implicaciones:** El tratamiento con losartán mejoro la disbiosis intestinal en SHR. Este efecto parece estar relacionado con su capacidad para reducir el impulso simpático intestinal, mejorando la integridad intestinal. Los cambios inducidos por losartán en la microbiota intestinal contribuyeron, al menos en parte, a proteger la vasculatura y reducir la PA, posiblemente mediante la modulación el sistema inmunitario intestinal.

La microbiota intestinal se comunica con los órganos distales mediante la producción de una gran cantidad de metabolitos que pueden ser absorbidos por la circulación sistémica y ejercen efectos biológicos. Las moléculas de señalización son tanto productos metabólicos bacterianos, incluidos los ácidos grasos de cadena corta (SCFAs), como componentes de la pared bacteriana como el lipopolisacárido (LPS). Se ha demostrado una reducción significativa de la PA en pacientes tratados con probióticos. Además, se

ha descrito un papel beneficioso de los probióticos de *Lactobacillus* y el kéfir en la regulación de la presión arterial y la protección vascular en SHR con hipertensión estabilizada, como resultado de restaurar el desequilibrio en (ROS)/óxido nítrico (NO) en la pared arterial. Sin embargo, si el consumo de probióticos puede prevenir la disbiosis intestinal, reducir la activación de las células T del sistema inmunitario intestinal y la infiltración vascular de las células T en SHR, y si los SCFA juegan algún papel en este efecto, se desconoce. Pensamos que la suplementación oral con probióticos evitaría el aumento de la presión arterial en SHR al cambiar las poblaciones de bacterias productoras de SCFA, alterando así la comunicación intestinal con los órganos linfáticos secundarios locales y los órganos distales. El objetivo del **cuarto estudio** *(Robles- Vera I, Toral M, de la Visitación N, Sánchez M, Gómez-Guzmán M, Romero M, Yang T, Izquierdo-Garcia JL, Jiménez R, Ruiz-Cabello J, Guerra-Hernández E, Raizada MK, Pérez-Vizcaíno F,* ***Duarte J****. Probiotics Prevent Dysbiosis and the Rise in Blood Pressure in Genetic Hypertension: Role of Short-Chain Fatty Acids. Mol Nutr Food Res. 2020 Mar;64(6):e1900616. doi: 10.1002/mnfr.201900616. Epub 2020 Feb 6. PubMed PMID: 31953983, índice de impacto: 4,653 (2018 JCR Science Edition), Food Science & Technology 9/135 Q1 (Decil 1)*) fue evaluar los efectos cardiovasculares de los probióticos *Bifidobacterium breve* CECT7263 (BFM) y *Lactobacillus fermentum* CECT5716 (LC40), y butirato y acetato en la hipertensión genética. **Métodos y resultados**: Diez ratas WKY de cinco semanas de edad y cincuenta SHR de edad similar se distribuyen aleatoriamente en seis grupos: control WKY, control SHR, SHR-LC40 tratado, SHR-BMF tratado, SHR-butirato tratado y de SHR-acetato tratado. Los tratamientos crónicos con LC40 o BFM aumentan las bacterias productoras de butirato y previenen el aumento de la PA en SHR. El tratamiento oral con butirato o acetato también evita el aumento de la presión arterial y la relación F/B. Todos los tratamientos restablecen el equilibrio Th17/Treg en los ganglios linfáticos mesentéricos, normalizan la endotoxemia y evitan el deterioro de la relajación dependiente del endotelio a la acetilcolina, como resultado de la reducción de la producción de especies reactivas de oxígeno reactivas mediada por NADPH oxidasa. Estos efectos protectores podrían estar mediados tanto por la reducción de la vía del LPS/receptor tipo toll-4 (TLR-4) vascular como por el aumento de la infiltración de Treg en la vasculatura. **Conclusión:** Los probióticos LC40 y BFM previenen la disbiosis y el desarrollo de disfunción endotelial y PA alta en la hipertensión genética. Estos efectos parecen estar relacionados

con la reducción de la endotoxemia y con el aumento de la acumulación de Treg en la vasculatura.

El endotelio tiene una gran importancia en el control de la PA mediante la liberación de una gran variedad de factores moduladores del tono vascular. El NO derivado del endotelio es un regulador clave de la PA. El NO, sintetizado por eNOS, es el principal factor vasodilatador del endotelio, y tiene un papel principal en el control de la HTN y la disfunción endotelial. Como se describió anteriormente, los probióticos ejercen efectos antihipertensivos en pacientes hipertensos humanos y también en animales con hipertensión genética. De hecho, se ha descrito cómo el consumo crónico de cepas de Lactobacillus fue capaz de reducir la PA y el daño en los órganos diana (corazón, riñón) y mejorar la disfunción endotelial. El efecto antihipertensivo parece estar relacionado con el aumento de la biodisponibilidad de NO, como resultado de la reducción del estado proinflamatorio y prooxidativo vascular. Sin embargo, no está claro si los probióticos pueden inducir niveles más altos de NO, porque no hay información sobre los efectos antihipertensivos de los probióticos en otras formas no genéticas de hipertensión, por lo que el objetivo del **quinto estudio** ***(****Lactobacillus fermentum Prevents Dysbiosis and Vascular Oxidative Stress in Rats with Hypertension Induced by Chronic Nitric Oxide Blockade. Mol Nutr Food Res. 2018 Oct;62(19):e1800298. doi: 10.1002/mnfr.201800298. Epub 2018 Aug 7. PMID: 30028078.)* Conocer si el efecto antihipertensivo podría estar relacionado con la capacidad de los probióticos para aumentar la biodisponibilidad de NO, debido a una activación de eNOS o tal vez solo como resultado de la reducción del estado proinflamatorio y prooxidativo vascular.

Métodos y resultados: las ratas se dividieron aleatoriamente en cuatro grupos diferentes y se trataron durante 4 semanas: a) vehículo (control), b) vehículo más L-NAME (50 mg 100 mL^{-1} en agua potable), c) LC40 (10^9 colonias -formando unidades / día por sonda), y d) LC40 más L-NAME. L-NAME indujo disbiosis intestinal, caracterizada principalmente por un aumento de la relación Fimicutes/Bacteroidetes (F/B) y un contenido reducido de Bifidobacterium, aumento de células Th17 y Treg reducido en ganglios linfáticos mesentéricos (MLN), aumento de infiltración aórtica Th17 y especies reactivas de oxígeno, reducción respuesta relajante aórtica dependiente del endotelio a la acetilcolina y la hipertensión. LC40 previno la disbiosis intestinal, alteró el equilibrio Th17/Treg en MLN, el estrés oxidativo vascular y la inflamación, mejoró ligeramente la disfunción endotelial pero no inhibió el desarrollo de hipertensión inducida por L-NAME.

Conclusión: el tratamiento crónico con LC40, en este modelo de inhibición crónica de la síntesis de NO, redujo los eventos tempranos involucrados en el desarrollo de la aterosclerosis, como el estrés oxidativo vascular y el estado proinflamatorio, como resultado de la prevención de la disbiosis intestinal y los cambios inmunes en MLN, pero no hipertensión, confirmando el papel crítico del NO en los efectos antihipertensivos de LC40 en la hipertensión genética.

INTRODUCTION

Introduction

1. Hypertension

Hypertension (HTN) is one of the most powerful risk factors for cardiovascular events, including myocardial infarction and stroke. HTN is present in about one quarter of the world's population and is responsible for about 41% of the cardiovascular disease related deaths (Wong et al., 2012). Treatment with any commonly used antihypertensive regimen reduces the risk of total major cardiovascular events, the larger the reductions in blood pressure (BP) the larger the reductions in risk (Turnbull et al., 2003). Currently, the BP levels considered as hypertensive are higher than 90 mmHg for diastolic blood pressure (DBP) and 140 mmHg for systolic blood pressure (SBP) (Chopra et al., 2019). Initially, the HTN may present no symptoms, or mild symptoms such as dizziness, headaches or palpitations.

HTN is associated with several risk factors which are potentially controllable (overweight, smoke, salt intake, drugs, stress, exercise) and not controllable (age, sex or genetics factors). Traditionally, HTN has been classified according to several criteria, among which is worth noting the etiological classification (Aoki., 1986). According to it, HTN could be considered as essential or idiopathic and not essential or secondary. In essential HTN, which appears in the 90% of the hypertensive population, the cause is unknow. Usually, essential hypertension can be controlled by treatments with two or three anti-hypertensive drugs. Secondary hypertension, which is more usual in young patients is derived from renal pathology, pheochromocytoma, thyrotoxicosis among others and frequently displays high plasmatic levels of aldosterone, vasopressin and endothelin and low plasmatic renin levels (Akpunonu et al., 1997). Although more than 60 distinct drugs (Diuretics, α- or β-blocking, renin–angiotensin–aldosterone system blocking and calcium channel blockers) are available to lower BP, less than half of the treated hypertensives reach the currently recommended BP targets (James et al., 2014). Furthermore, an estimated 10-15 % of the general hypertensive population presents resistant HTN defined as high BP not adequately controlled by three antihypertensive agents from different classes, one of them a diuretic (Mancia et al., 2014).

2. Physiopathology of Hypertension

BP is a multifactorial pathology determined by a great number of factors, both genetical as well as environmental. Alterations in these factors could induce changes in the cardiovascular system inducing hypertensive stimuli and tissue damage. The alterations usually are mediated by changes in the sympathetic nervous system (SNS) and renin angiotensin system inducing damages mainly in kidney, heart and vascular vessels. The main factors able to regulate BP are: the vascular smooth muscle cell (VSMC) tone, cardiac function, renal Na^+ excretion and plasma and whole-body volume, which control peripheral vascular resistances (PVR), cardiac output and intravascular volume, respectively. When unnormal working of any of these mechanisms occurs, and/or some of these factors are found elevated, the result will be an elevation in BP. Under normal conditions, the hemodynamic factors, which are involved in the maintenance of BP, are regulated by hormones induced by the SNS, which are able to have direct effects on the alpha- and beta-adrenergic receptors at blood vessel and heart. Besides, other not neural or humoral factors, such as mineralocorticoid hormones, prostaglandins and the renin-angiotensin system, are also involved in the regulation of BP.

Typically, HTN has been linked to endothelial dysfunction, which is characterized by a reduction in nitric oxide (NO) bioavailability and a higher level of reactive oxidative species (ROS) at vascular and endothelial level that would induce a reduction in the endothelium-mediated vasorelaxation capacity. In recent years, progress in basic research has led to the identification of new VSMC signaling pathways and possible therapeutic targets for the treatment of HTN, and several promising drugs have subsequently been developed (Yang T et al., 2015).

There is also strong evidence for the central nervous system playing a critical role in HTN (Oparil et al., 2003). Increased sympathetic outflow is a uniform finding in essential HTN, and contributes to vasoconstriction, vascular remodeling, and VSMC proliferation (Mancia et al., 1999). In addition, the immune system has also been linked to BP control due to small increases in prohypertensive factors, such as angiotensin II (Ang II), salt retention, or aldosterone, being able to activate the innate immune system by activation and release of damage-associated molecular patterns (DAMPs) that activate Toll-like receptors (TLRs). These mechanisms could damage the kidney directly, which

is one of the main tissues regulating the BP. Besides, HTN would induce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–dependent ROS production in dendritic cells (DCs), which leads to the formation of neoantigens that can activate adaptive immune cells. Activation of innate-like lymphocytes, such as $\gamma\delta$ T cells and natural killers (NK) cells, as well as the adaptive immune system, consequently aggravates renal injury **(Figure 1).**

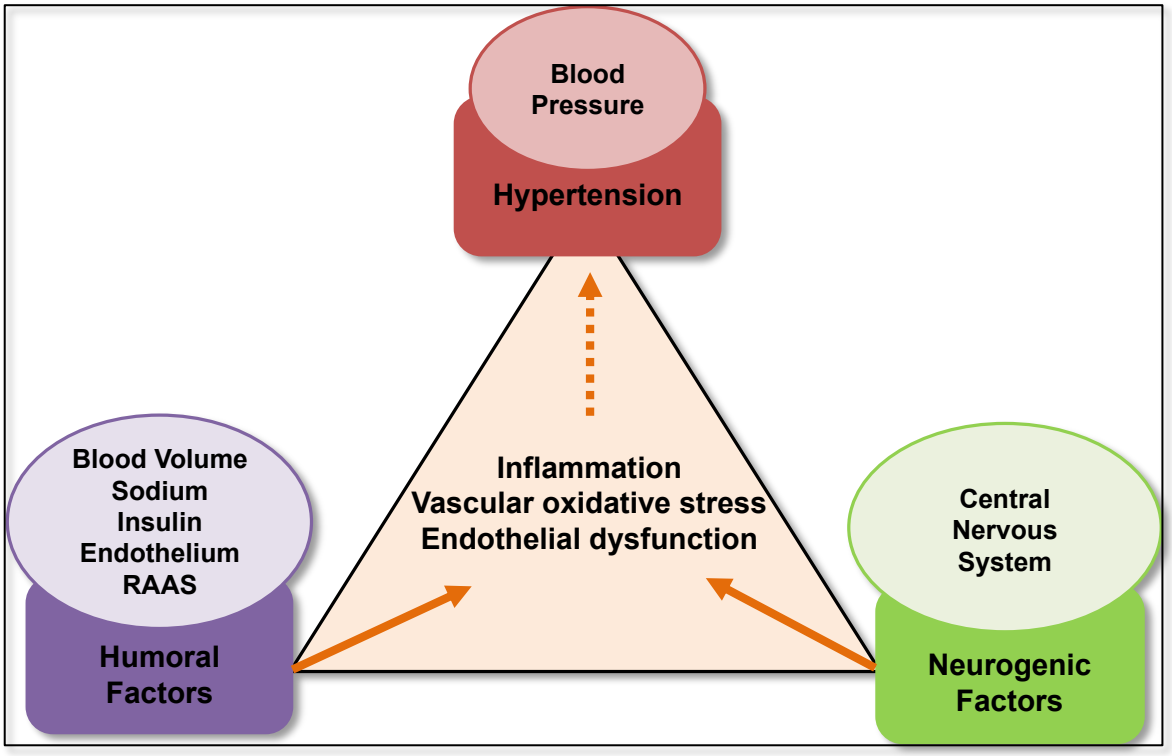

Figure 1. *Summary of traditional factors involved in the development of hypertension. RAAS, renin-angiotensin-aldosterone system*

2.1 Humoral Factors

As was describe above, there are a great variety of factors with certain role in the regulation of BP, some of which do not have a neural origin. These factors are traditionally known as humoral factors, highlighting the blood volume, sodium concentration, insulin, renin-angiotensin system and endothelium-derived factors among others (**Figure 1).**

2.1.1 Blood Volume

The circulating blood volume has a critical role in the alteration of BP, a reduction in blood volume induces a reduction in arterial pressure and, subsequently, in renal perfusion. Low renal perfusion stimulates of renin release from juxtaglomerular cells, which induces angiotensinogen proteolysis to rend angiotensin I (Ang I). The angiotensin converting enzyme (ACE) mediated the transition of Ang I to Ang II. Ang II is able to induce an important vasocontraction and also stimulates the secretion of aldosterone by the suprarenal gland. Ang II and aldosterone have a synergistic effect inducing sodium and water retention. Besides, the changes in the blood volume induce the secretion of renin in the juxtaglomerular apparatus, which induces higher secretion levels of Ang II and an elevation in BP. (Santos et al., 2019).

2.1.2 Sodium

High level of sodium in the blood vessel wall induces an elevation in the effects of catecholamines on itself, inducing and elevation in vasoconstriction and periphery resistance (Monte et al., 2013). Besides, it has been recently described by Nicola et al., 2017 how even a moderate salt intake may affect the persistence of intestinal *Lactobacilli* and other bacteria, along with an increase in pro-inflammatory T-helper (Th)17 cells and induce salt-sensitive BP changes. Moreover, they described how salt intake also has profound actions on hormonal systems, such as the renin-angiotensin-aldosterone axis, inducing also an increase in BP mediated by the elevation in the activity of RAAS (Nicola et al., 2017).

2.1.3 Insulin

Insulin has an important protective effect on the vascular function, mainly due to his capacity to induce the activation of eNOS and increase NO production. Under insulin resistant conditions, reduced endothelium derived NO was detected associated with an increase in BP. In this situation, a trophic effect on the vascular smooth muscle and endothelial cells could be found mediated by insulin receptor (IR), inducing vascular hypertrophy, and so, HTN. Also, the modulation in the insulin concentration could induce HTN by other two mechanisms mediated by the modulation in the catecholamines secretion and sodium reabsorption. Both of those mechanisms can justify hyperinsulinemia, obesity, intolerance to carbohydrates, hypertriglyceridemia, decreased concentration of high-density lipoproteins, and high BP.

2.1.4 Renin-Angiotensin-Aldosterone System

The RAAS is a hormone system that is able to regulate BP. The main mediator of the pressor actions of RAAS is Ang II. This peptide is a potent vasoconstrictor agent, inducing an increased BP. Ang II effects not only derivate from its vasoconstrictor action on the arterial wall, it has others important crucial effects on several tissues. In the kidney, is able to constrict glomerular arterioles; concretely, Ang II has an important effect on the efferent arterioles inducing an increase in arteriolar resistance, so Ang II forces blood to build up in the glomerulus, increasing glomerular pressure. In general, the actions of Ang II include the contraction of smooth muscle cells, inducing the synthesis and secretion of aldosterone from glomerular cells and the release of noradrenaline in the adrenergic fibers, and modulating the sodium transport at the renal tubular level. Besides what was described above, Ang II controls the sodium reabsorption by stimulation of hypertrophy of renal tubule cells and water reabsorption derived of its capacity to stimulate the release of vasopressin from the hypothalamus and its release from the pituitary gland. In addition, Ang II is able to induce the activity of NADH and NADPH oxidases, which are the main source of ROS at the vascular level.

The great numbers of effects induced by Ang II are the result of several receptors with different isoforms of on which Ang II or its fragments could work. Several authors have found at least four different isoforms, AT1, AT2, AT3 and AT4. The effects derived from the activation of AT1 and AT2 receptors are opposite. The AT1 receptor is expressed mainly in the adrenals, vascular smooth muscle, kidneys, and heart and also in

the brain. The activation of AT1 induces its effects mediating the activation of phospholipase C, stimulating voltage-dependent calcium channels, activating phospholipase A2, phospholipase D and stimulating adenyl cyclase. In addition, inducing the delayed activation of the formation of superoxide (O_2^-) anions and hydrogen peroxide causes endothelial dysfunction and nitric oxide degradation (Esther, 1998; Yamata, 1998; Nakajima, 1998; Allen, 1999; Ardaillou, 1999; Sharma, 2000). For these reasons, the AT1 receptor is the main receptor to mediate BP increase. On the other hand, the AT2 receptor regulates growth and cell apoptosis. It is abundant in various fetal tissues, where it is expressed temporarily. The AT2/AT1 proportion in human depends on the tissue. Importantly, the AT2 receptor appears after vascular damage, myocardial infarction, heart failure, and peripheral nerve damage, reflecting the reactivation of a fetal genetic program (Suzanne, et al., 2000). The role of the AT3/AT4 receptors in hypertension is not completely known, even currently is unknown if they are present in humans (Singh et al., 2016).

2.1.5 Endothelium-derived factors

The endothelial cells form the inner layer of the blood vessel, which is able to produce various metabolites, classically called endothelium-derived relaxing factors (EDRFs) and endothelium-derived constricting factors (EDCFs), based on their ability to modulate the arterial wall. Besides, it has been described how the endothelium could be able to induce vasorelaxation by endothelium-derived hyperpolarizing factors (EDHFs) (Kang et al., 2014).

2.1.5.1 Prostacyclin

Prostacyclin 2 (PGI_2) was the first endothelium derived relaxing factor discovered. Prostaglandin production is derived from the arachidonic acid metabolism (Patrono et al., 2006). Its synthesis pathway is began by the release of arachidonic acid from membrane phospholipids due to the action of phospholipase A_2, after that, the cyclooxygenases (COX 1, 2, or 3) catalyze the reaction to generate PGH_2 and finally, the effect of prostaglandin synthases such as prostacyclin synthase, leading to the formation of PGI_2; or thromboxane synthase, which generates thromboxane A_2 (TXA_2). The phospholipase A_2 could be activated by the action of different stimuli as Ang II, epinephrine and bradykinin in vascular tissues. The prostacyclin action is induced by activation of

prostacyclin receptors (IP) which leads to G-protein coupled increase in the second messenger cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activation, resulting in decreased $[Ca^{2+}]_i$ and vasorelaxation.

2.1.5.2 Endothelin

Other main metabolite produced by the endothelium is Endothelin (ET), one of the most powerful endogenous vasoconstrictors produced by the endothelium (Yanagisawa, et al., 1988). Endothelin is obtained from pro-endothelin, which is converted into a big endothelin and then to the active endothelin through the endothelin-converting enzyme (ECE). There are three isoforms of endothelin (ET-1, ET-2, and ET-3), being ET-1 the most relevant. Several stimuli could induce endothelin release by endothelial cells, such as thrombin, catecholamines, shear stress, Ang II, vasopressin, and hypoxia (Luscher et al., 1993). Endothelin acts through the activation of ET-A or ET-B receptors. The ET-A is the principal receptor located on VSMC and cardiomyocytes. The activation of this receptor leads to the activation of phospholipase C joined to an increase in intracellular calcium and contraction. However, ET-B receptor can be found on both vascular smooth muscle and endothelial cells. In the endothelium, ET-B activation induces the release of vasodilating substances such as NO, PGs, and adrenomedullin, but in muscle cells induces vasoconstriction. Specially, the release of NO by ET-B receptors on endothelial cells promotes the maintenance of balance between the vasodilatory effect of NO and the vasoconstrictor effect of ET-1 (Nishiyama et al., 2017).

2.1.5.3 Hydrogen sulfide

Endothelial cells are also able to produce hydrogen sulfide (H_2S) due to the activation of cystathionine-lyase and 3-mercaptopyruvate sulfuresterase (CSE). Different experimental genetic models have shown that the deletion of CSE reduces H_2S levels in the aorta and other tissues leading to a reduction in endothelium-dependent vasorelaxation after muscarinic cholinergic stimulation of vascular endothelial cells and HTN (DiBona et al., 2010).

2.1.5.4 Nitric Oxide

The main product synthesized by the endothelium is NO, which is closely involved in the regulation of circulation and vascular wall homeostasis. NO is formed by the enzyme nitric oxide synthase (NOS) from the amino acid L-arginine. NO diffuses to the underlying VSMC and activates soluble guanylyl cyclase-induced vasorelaxation (Vane et al., 1990). Three isoforms of NOS have been described: neuronal NOS (nNOS) present in neural cells, inducible NOS (iNOS), and an endothelial NOS (eNOS) present mainly in the endothelium. NO is released from endothelial cells in response to physical stimuli and by stimulation of receptors, such as bradykinin and muscarinic receptors, which induce the liberation of Ca^{2+} from the endoplasmic reticulum by a I_3P/DAG-dependent pathway. The calcium-calmodulin complex stimulates eNOS to release NO.

A great number of studies have demonstrated the key role of the endothelium in the regulation of cardiovascular homeostasis leading to the concept of endothelial dysfunction (Konishi et al., 1983; Luscher et al., 1987; Panza et al., 1990). Several mechanisms have been suggested to explain endothelial dysfunction, such as an impaired NO synthesis and release or a reduction in bioavailability due to an increase in ROS production in tissues. The oxidative stress is mainly due by an elevation of O_2^- anions, which are potent scavengers of NO. The main source of ROS at the vascular level is the NADPH oxidase, which is composed by several catalytic (NOX1, 2, 4 or 5) and regulatory ($p22_{phox}$, $p47_{phox}$, $p67_{phox}$ and Rac-1) subunits. Under physiological conditions, there is a balance between the production and elimination of ROS. Nonetheless, under endothelial dysfunction conditions, an alteration in this balance is present, and then, an oxidative stress carries out a reduction in NO levels. Currently, it is unknown whether endothelial dysfunction is a primary or secondary event in HTN. However, several experiments have shown that endothelial dysfunction could precede the development of HTN and play a primary role in the genesis of the disease (Taddei et al., 1995).

2.2 Neurogenic factor

The SNS plays an important role in the pathogenesis of primary HTN. In particular, its activity increases progressively parallelly with HTN. Classically, HTN has been described as a disease with multifactorial etiology. Among the factors involved, the role of neuroadrenergic factors is well established. The role of SNS has been confirmed indirectly, by measuring circulating blood levels of the adrenergic neurotransmitters

epinephrine and norepinephrine or by evaluating, via the power spectral approach, vagal and sympathetic frequency components and directly by quantifying efferent postganglionic muscle sympathetic nerve traffic in peripheral nerves as well as regional norepinephrine release and reuptake by adrenergic nerves via the norepinephrine radio-labelled technique (Esler et al., 2011, DiBona et al., 2013). The main factors that are regulated by SNS are the cardiac output and systemic vascular resistance.

The arteriolar vasomotor tone is determined by SNS by several neurotransmitters (norepinephrine, epinephrine, and dopamine). The main receptor through which these neurotransmitters exert their vasomotor action are adrenergic and dopaminergic receptors. Those receptors can be found in a great variety of tissues with different levels of density. Activation of the receptors leads to vasoconstriction and increases cardiac output linked to an elevation of heart rate. The physiological activity of the dopaminergic receptors in BP regulation is not completely understood.

The role of adrenergic receptors, which are a class of G protein-coupled receptors that are targets of many catecholamines, like norepinephrine and epinephrine, has been tested in several hypertensive animal models (Lohmeir et al., 2007). Authors have described how cardiovascular hypertrophy is mediated by adrenergic receptors. In contrast to the dopaminergic receptors and α-adrenoreceptors, the stimulation of β-adrenergic receptors leads to vasodilation. β-adrenergic receptors also have an influence on the heart rate and cardiac output, not so much on vascular resistance. Deficiency of adrenergic receptors in animal models blunts the cardiac effects of adrenergic stimulation (Chruscinski et al., 1999; Rokosh et al., 2002). Besides, a great body of evidence has shown the role of the SNS on the control of the renin-angiotensin system and renal sodium excretion. Indeed, the electrical stimulation of renal sympathetic nerves increases renin release from juxtaglomerular cells by indirect and direct mechanisms, through changes in renal blood flow or through the stimulation of beta-adrenergic receptors located in the juxtaglomerular cells, respectively (Kopp et al., 1993; Johns et al., 2011; Iliescu et al., 2012). The SNS is also able, through the stimulation on renal sympathetic nerves, to keep the sodium homeostasis, exerting anti-natriuretic effects by a direct action on tubular renal sodium reabsorption (Kopp et al., 1993).

The increase in SNS activity is characterized by an elevation of norepinephrine plasma and tissue level. Moreover a marked increase in sympathetic nerve traffic has also a key role in promoting HTN-related target organ damage, such as left ventricular

hypertrophy, congestive heart failure, and deterioration in renal function (Greenwood et al., 2001;Schlaich et al., 2003; Grassi et al., 2009).

The effects of SNS on BP have been described in young and elderly hypertensive patients. Julius et al. (1971) showed that in young subjects intravenous administrated of atropine, which blocks the effects of the parasympathetic neurotransmitter acetylcholine on muscarinic receptors, induced the increase of heart rate and cardiac output of lesser magnitude. The role of SNS was also showed, analyzing the level of norepinephrine, in young subjects with borderline BP elevation. In elderly hypertensives, when sympathetic nerve traffic was recorded, a clear sympathetic activation was observed when the values were compared to those found in elderly normotensive controls (Grassi et al., 2000).

Finally, the effects of different antihypertensive drugs on sympathetic function have been studied, with certain evidence that the various classes of drugs are able to lower elevated BP values due to heterogeneous effects on the adrenergic function. Diuretics and some calcium channel blockers type-dihydropyridine increase the SNS activity, while others, namely ACE-inhibitors and Ang II receptor blockers, reduce the adrenergic drive. The different effects of antihypertensive drugs on the SNS give us information about how the values of BP can be reduced even by drugs which are able to activate the SNS, that situation has allowed to generate the so called “neurogenic hypothesis” of HTN, which cannot explain alone the pathogenesis of all the essential hypertensive forms (**Figure 1).**

2.3 Immune system in Hypertension

The participation of the immune system is a consequence of a complex interaction between activated immune cells, oxidative stress, and Ang II activity that drives a low-grade inflammation in the kidney, arteries, and central nervous system (**Figure 2).**

The idea of inflammation and immune system as important elements in the pathology of HTN is an old concept. White and Grollman (1964) described that immunosuppression induces a reduction in BP in rats that had partial renal infarction. In 1967, Okuda and Grollman showed that the transfer of lymphocytes from rats with unilateral renal infarction to non-hypertensive rats was able to cause HTN in recipient rats. Besides, Olsen (1972) described a periadventitial accumulation of T cells and monocytic cells in hypertensive humans. In the 1970s, Svendsen discovered that mice that were thymectomized or athymic nude mice do not maintain HTN after renal infarction. In

1982, Ba et al. discovered that transplant of a thymus from a Wistar-Kyoto rat (WKY) reduced BP in a recipient spontaneously hypertensive rat (SHR). They also noted that transplanting a compatible thymus into neonatal SHRs resulted in a significant suppression of BP if full immunological restoration was achieved (Ba et al., 1982).

Innate immunity is a system that could be activated by several stimulus under hypertensive situations. These signals correspond to molecular patterns in pathogenic microorganisms (PAMPs) or endogenously generated cellular stress signals, which activate DAMPs. These signals are recognized by pattern recognition receptors (PRR). Among the PRR, the TLRs are, one of the most important group that has been shown to be involved in the inflammation associated with HTN. TLRs are expressed by T and B lymphocytes, monocytes, DCs, and other somatic cells, such as endothelial and vascular smooth muscle cells. The TLRs engage several pathways, which drives to the upregulation of NF-kB, AP-1, and interferon-regulatory factors. The activation of these transcription factors result in the upregulation of genes that control inflammasome, such as the sensor molecule NLRP3, procaspase, pro-IL-1b, and pro-IL-18.

In addition to the activation of TLR in models of HTN, it was demonstrated that TLR4 expression is increased in the kidneys of SHR and inflammatory cytokine production by splenocytes from SHR rats is increased following stimulation of TLR 7/8 or 9. Furthermore, splenocytes of the SHR treated with TLR ligands showed an enhanced cytokine production in the presence of nicotine, in contrast to the reduction observed in the splenocytes from control WKY rats (Harwani et al., 2012). Direct evidence of the role of TLR4 in HTN was shown by the ability of the anti-TLR4 antibody treatment to ameliorate HTN in the SHR (Bomfim et al., 2012) and by the failure of TLR4. mice to develop NG-nitro-L-arginine methyl ester (L-NAME)-induced HTN (Sollinger et al., 2014). The importance of TLRs in specific areas of the brain is suggested by the demonstration that TLR4 is upregulated in the paraventricular nucleus (PVN) of the hypothalamus in the SHR, but not in the normotensive WKY rats. Furthermore, injection of a specific TLR4 blocker to this brain area lowered BP, reduced mRNA and protein abundance of TNF-α, IL-1β, and inducible NOS, and suppressed NFκB activity in SHR.

The adaptive immune system is characterized by a specific immune response directed to exogenous or endogenous antigens. The most important effector cells of the adaptive immune system are T and B lymphocytes. Activation of T cells requires that antigens are presented in the context of the major histocompatibility complex (MHC) by antigen presenting cells (APCs). For the T cells to be activated, they need two signals:

first, an antigen in the MHC of the APC that is recognized by a specific T-cell receptor (TCR) and second, independent co-stimulation by B7 ligands (CD80 or CD86) that are linked to CD28 in the T cell. In addition, the clonal expansion of activated T cells requires a third signal to proliferate that is provided by cytokines (Curtsinger et al., 2010). The generation of memory T cells requires the interaction of the CD27 molecule present in the T lymphocyte with the co-stimulatory molecule CD70 in antigen presenting cells and is also a central feature of adaptive immunity. Memory T cells are responsible for the accelerated protective response to subsequent antigen exposure.

In 2007, Guzik et al., showed that RAG1$^{-/-}$ mice, which lack both T and B cells, develop blunted HTN and have preserved vascular endothelial function when infused with Ang II or after being challenged with deoxycorticosterone acetate (DOCA) and salt. Adoptive transfer of T cells, but not B cells, restored the hypertensive response and vascular dysfunction observed in WT mice. Subsequently, Mattson et al. (2013) deleted the RAG1 gene in Dahl salt-sensitive rats and showed that this blunted the BP increase that occurs upon high-salt feeding. A striking finding was that the RAG1$^{-/-}$ rats seem protected against glomerular damage, albuminuria, and renal damage. In a following study, this group deleted the chain (CD247) from CD3+ cells in Dahl salt-sensitive rats and found a very similar phenotype to that observed in the rats lacking RAG1 (Rudemiller et al., 2014).

In keeping with these findings in RAG1-deficient animals, Crowley et al. (2010) showed that SCID mice, which are deficient in the production of B and T cell, develop blunted HTN and cardiac hypertrophy during Ang II infusion. CD8+ T cells were protected from HTN, whereas mice lacking CD4+ T cells or MHC class II were not. In this study, deep sequencing revealed an increase in V chain clonality of CD8+ T cells in the kidney, but not in blood vessels or the spleen of hypertensive mice. The naive CD4 T cell, depending on the cytokine environment, polarizes to Th1, Th2, Th17, or regulatory T cell (Treg) phenotypes. The Th1 phenotype is generated in environments rich in IL-12 and interferon (IFN) and predominantly secretes IL-2, TNF-α, and IFN-γ. The Th2 phenotype is generated in IL-4 environments and predominantly secretes IL-4 and IL-10. The Th17 phenotype requires IL-6, IL-21, IL-23, transforming growth factor (TGF)-β, and IL-1β. It is activated by aldosterone and secretes IL-17A, IL-17F, IL-21, and IL-22 (Gaffen, 2011). The Treg phenotype is generated in TGF-β environments with low concentrations of IL-6, and its anti-inflammatory activity is exerted by secretion of immuno-suppressive immune factors such as IL-9, IL-10, TGF-β, and cytotoxic T-

lymphocyte antigen 4 (CTLA-4) and by direct cell-to-cell contact (Zhang and Crowley, 2015; Toral et al., 2018).

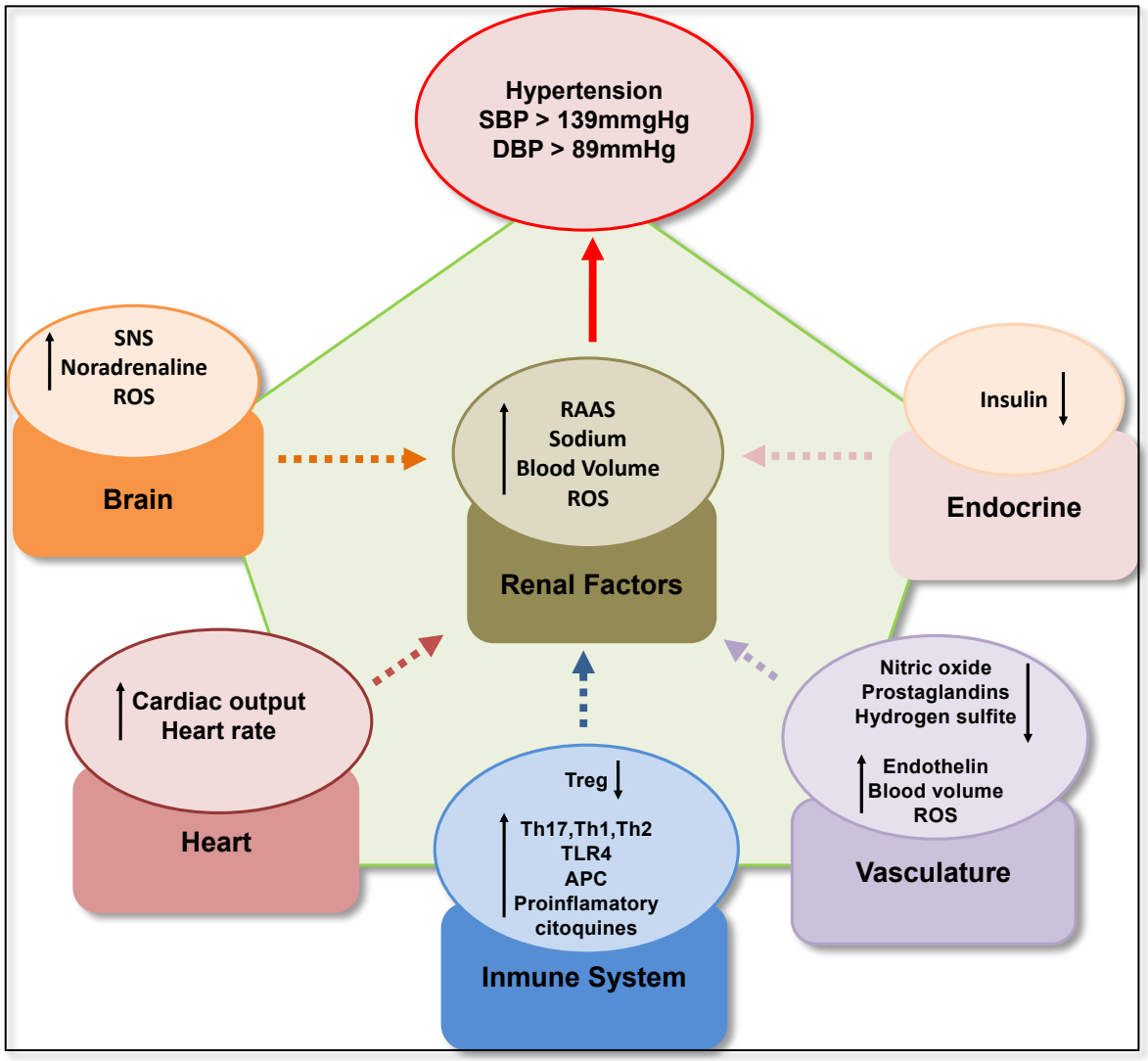

Figure 2. *Summary of alterations in the different systems and main tissues during the development of hypertension. Th, T-helper, TLR, Toll like receptor, APC, antigen presenting cells, SNS, sympathetic nervous system, RAAS, Renin-Angiotensin-Aldosterone System, ROS, reactive oxygen species.*

3. Gut microbiota in Hypertension

The mammalian microbiome consists of unique assemblages of microorganisms (i.e., bacteria, archaea, fungi, and viruses) associated with various niches in and on the body. The gut microbiota is mainly dominated by bacteria belonging to phyla *Firmicutes* and *Bacteroidetes*, and to a lesser extent by *Actinobacteria*, *Tenericutes, Proteobacteria and Verrumicrobia* (Qin et al., 2010). The gut microbiota is constantly adapting itself to lifestyle modifications, such as diet, exercise and even has been commonly observed that a change in the host health status has been accompanied by a shift in the gut microbiota.

There are several studies indicating a direct association between HTN and gut microbiota in both animal models and humans. Recently, it has been demonstrated that the healthy gut microbiota would be able to influence BP. Karbach et al. (2016) did not find alterations in the gut microbiota of germ-free compared with conventionally raised mice, which is supported by previous observations describing no effect in BP after drastic reduction in fecal microbial biomass induced by antibiotic treatment (Pluznick et al., 2013). Similarly, it has been described that both, the *Firmicutes*/*Bacteroidetes* (F/B) ratio, and the abundance and diversity levels of selected genera in pre-hypertensive SHR were not significantly different from the age-matched WKY (Santisteban et al., 2016). Li et al., (2017) also analyzed the gut microbiota of pre-hypertensive and hypertensive patients. They described significant differences between the gut microbiota of hypertensives and normotensives, but there was no distinguishable difference between pre- and hypertensive patients. However, the absence of gut microbiota protects mice from Ang II-induced HTN, vascular dysfunction, and HTN-induced end-organ damage, showing for the first time that commensal microbiota, an ecosystem that is acquired directly after birth, could represent an environmental factor promoting Ang II-induced high BP (Karbach et al., 2016). Similarly, oral administration of antibiotics improved BP in Ang II-induced HTN and in SHR (Yang et al., 2015). It has also been reported that a treatment based on the combination of antibiotics was able to reduce the BP of resistant hypertensive patients from 160/90 mmHg to 130/60 mm Hg (Qi et al., 2015).

Recently, both in animal models and humans HTN, alterations have been demonstrated to occur in the gut microbiota. A delicate balance in the gut microbiota composition is key in maintaining intestinal immunity and whole-body homeostasis. An imbalance in gut microbiota composition relative to the healthy state or homeostasis is

commonly known as dysbiosis. In hypertensive animal models, gut dysbiosis was characterized by: i) Reduction of diversity, less richness (Chao Richness, Shannon, Simpsons and Pielou) and a higher F/B ratio, due to a contraction of *Bacteroidetes* and an expansion of *Firmicutes*; ii) Increase in lactate-producing bacteria and a reduction in acetate- and butyrate-producing bacteria. However, the clinical evidences of a role for dysbiosis in HTN are limited (Pluznick et al., 2013; Yang et al., 2015; Santisteban et al., 2016; Durgan et al., 2016). In addition, the BP has been significantly negatively associated with abundance of butyrate-producing bacteria and butyrate production in the gut microbiota. Concretely, *Odoribacter*, a butyrate-producing genus was inversely correlated with systolic BP.

A significant change in gut microbiota populations between adult SHR and age-matched WKY has also been shown (Gómez-Guzmán et al., 2015), characterized by increased numbers of Gram-negative *Bacteroides* spp. (phylum *Bacteroidetes*) and Gram-positive *Clostridium* spp. (phylum *Firmicutes*) and reduced numbers of *Bifidobacteria* (phylum *Actinobacteria*). Considering the different methodology used to explore gut microbiota in this study (qRT-PCR for some bacteria genera) in comparison with that used by Yang et al., 2015 (16s ribosomal DNA sequencing), it is difficult to compare the data from both analyses. However, a significant depletion of *Bifidobacterium sp*. in SHR was found in both studies. *Bifidobacterium* is commonly considered a beneficial bacterial genus that plays a critical role in the maturation and regulation of the immune system (Grangette, 2012), and, in consequence, *Bifidobacterium* depletion might contribute to the dysregulation of the immune system found in SHR (Bomfim et al., 2012). These findings support the suggestion that the BP increase in the SHR is closely associated with the development of gut dysbiosis. However, in other studies, no significant changes in gut microbiota after L-NAME treatment in rats (Xu et al., 2013) or between WKY and SHR (Petriz et al., 2014) were observed, which could be attributed to differences in the technologies used.

The gut microbiota has also been analyzed in another experimental model, the renin-angiotensin-system (RAS)-dependent HTN induced by Ang II-infusion. The gut microbiota of Ang II models is characterized by an expansion of *Firmicutes* and reduction of *Bacteroidetes* joined to a reduction of Chao Richness and Shannon (Yang et al., 2015). Specifically, the studies have described some features of that microbiota such as a larger amount of *Parabacteroides* and *Blautia* linked to a *Ruminococcus* and *Oscillospira* reduction (Yang et al., 2015). The short-chain fatty acids (SCFAs)-producing bacteria

profile in faeces is similar to SHR. The RAS-independent model of HTN induced by DOCA-salt has been described as not presenting change in the F/B ratio neither in richness or diversity. At the phylum level, the authors found elevated *Cyanobacteria* and *Proteobacteria* and a reduction in the phyla *Firmicutes* and *Bacteroidetes*. At genus level, only the non-cultured bacteria *S24-7g* was found reduced (Marques et al., 2017). The induction of HTN could also be carried out using some immunosuppressive drugs as tacrolimus (inhibited the T cell proliferation). In the microbiota of the tacrolimus model have been found an expansion of *Firmicutes* and a contraction of *Bacteroidetes* and *Actinobacteria* without a reduction in the Shannon parameters. Concretely, the analysis of microbiota showed a major proportion of *Clostridiales* and less *Butyricimonas* and *Bifidobacterium* (Toral et al., 2018).

These associations between gut microbial dysbiosis and HTN does not necessarily indicate a cause-effect relationship. The demonstration of the involvement of these microbial populations in pathophysiology requires a more complex experimental design. Adnan et al., (2017) using fecal microbiota transplantation (FMT) between WKY and SHR conclude that gut dysbiosis can directly affect BP. Interestingly, FMT from hypertensive patient donors into germ-free mice also elevated BP (Li et al., 2017). Collectively, these studies indicate that gut dysbiosis contributes to the development of HTN in various animal models and patients. Despite these strong evidences, the mechanisms involved in the control of BP are unknown.

3.1 Physiological systems involved in Hypertension are controlled by microbiome

Few years ago, a triangular connection involving a dysfunctional sympathetic-gut-bone marrow communication has been involved in the pathophysiology of HTN (Santisteban et al., 2016). The authors have described a bidirectional communication between the microbiome and the host via the nervous system. Neural pathways from the gut to brain regions, such as the PVN, involved in BP control were shown to be altered in animal models of HTN. Increased sympathetic activity to the gut drives to elevated gut permeability, and modulates the inflammatory status and microbial dysbiosis (Santisteban et al., 2016; 2017). Moreover, the gut epithelial barrier serves as a site of constant interaction between the microbiome and the host's immune system. It is critical to analyze the link between the two and the role they play in the pathogenesis of HTN. Here,

we review the role of both sympathetic and immune system in the BP regulation induced by gut microbiome.

3.1.1 Role of sympathetic nervous system (SNS)

Noradrenergic fibers innervate the stomach, and the small and large intestines. These sympathetic fibers affect gastrointestinal functions such as secretion, absorption, fluid, and electrolyte transport across the epithelium, as well as vessel smooth muscle contraction and hence the blood flow to the gastrointestinal tract (Furness and Costa, 1974). Early investigations highlighted the importance of the splanchnic vascular bed and sympathetic nerve activity in BP regulation in humans and animals (Rowell et al.. 1972). Recently, Raizada's group has linked hypothalamic neuroinflammation and increased sympathetic drive with changes in gut physiology and microbiota associated with AngII-induced HTN (Santisteban et al., 2016).

Changes in gut microbiota composition have been associated with gut integrity (Kim et al., 2018). The mammalian digestive tract epithelial cells create a tight barrier in the gut, contributing to the hypoxic environment of the lumen. Damage to this barrier makes the environment less hypoxic, conducive to aerobic bacterial growth (Konig et al., 2016; Earley et al, 2015). In fact, intestines of Ang II-hypertensive mice were less hypoxic and with increased aerobic bacteria in faeces (Kim et al., 2018). Interestingly, the elevated splanchnic sympathetic nerve activity and mild gut pathology in juvenile pre-hypertensive rodents precede HTN-related gut dysbiosis, suggesting that elevated gut sympathetic nerve activity modulates the gastrointestinal environment before the development of HTN (Santisteban et al., 2017).

More effort is needed to understand the cellular and molecular mechanisms involved in gut-brain interconnexion to control BP (**Figure 3).**

3.1.2 Role of the immune system

A growing body of evidence supports the role of the immune system and exaggerated inflammatory responses in the development of HTN (Caillon et al., 2017). In particular, preclinical models indicate that subsets of T lymphocytes such as Th1, Th2, Th17, and Treg cells are involved in the regulation of BP and end-organ damage. In fact, Th1 and Th17 cells play a key role in the development and maintenance of HTN, while Treg cells offer protection against increased BP (Madhur et al., 2010). A fragile balance

in the composition of gut microbiota is basic in maintaining integral homeostasis and intestinal immunity. The modulation of the immune system by gut microbiota is not, however, limited to the gut itself. Strikingly, gut microbes shape systemic immune responses through direct interaction with immune cells or the production of metabolites that travel to distal sites and activate or inhibit the inflammatory cascade. It has been proposed that the gut microbiota may be, at least partially, responsible for the development of HTN and the activation of the immune system and inflammatory response observed in HTN. In fact, the absence of gut microbiota protects mice from Ang II-induced HTN and vascular dysfunction, at least in part, inhibiting the accumulation of inflammatory cells in the vasculature (Karbach et al., 2016) (**Figure 3).**

3.2 Bacterial products and mechanisms involved in Hypertension

The gut microbiota produces a variety of metabolites that are able to reach the circulating blood and act as signaling molecules in the host. Gut-derived metabolites include both potentially beneficial (such as spermidine) and potentially harmful molecules (uremic toxins such as 4-ethylphenyl sulfate, p-cresol sulfate, and p-cresol glucuronide), which were upregulated with Ang II. However, Ang II down-regulated a compound N,N,N-trimethyl-5-aminovalerate associated with renal damage, and a compound reported to relax vessels and lower BP, ferulic acid 4-sulfate (Rymenant et al., 2017). Overall, the effects of the gut microbiota on host health may be pleiotropic, being in some cases protective and in other cases detrimental in the HTN scenario. There is abundant evidence that gut microbiota has important influences on host cell physiology through bacterial metabolic products such as SCFAs (Pluznick et al., 2013) or Trimethylamine N-oxide (TMAO) (Bennett et al., 2013), or bacterial wall components such as lipopolysaccharides **(**LPS) (Cani et al., 2012; Toral et al., 2014) (**Figure 3).**

3.2.1 Short chain fatty acids

HTN has been linked with SCFAs-producing bacteria and an increase in lactic acid producing bacteria (Pluznick et al., 2017). SFCAs are metabolites resulting from the fermentation of undigested carbohydrates in gut microbiota with known beneficial effects. SCFAs are fatty acids with fewer than six carbon atoms, including the most abundant, acetate (C2), propionate (C3), and butyrate (C4), and in lower proportion valeric acid and caproic acid. SCFAs can be absorbed via the gut epithelium, reaching the

circulation in different concentration levels (acetate (70-250 μM), propionate (88 μM) and butyrate (29 μM)) to participate in various physiological processes or excreted in feces (Stumpff., 2018).

The SCFA are able to carry out several interactions with the host. Butyrate is usually the principal source of energy for colonocytes but also is able to reduce the pro-inflammatory status. The SCFAs fulfill his effect by different ways. Recently, it has been shown how acetate and butyrate are able to have anti-inflammatory effects on myeloid cells as well as intestinal epithelial cells (IECs) by histone deacetylase (HDAC) inhibition (Chang et al., 2014). Besides, SCFAs are able to bind to GPR 41, 43, 109a, (also known as FFAR3, FFAR2, NIACR1 respectively) and olfactory receptor (Olfr)78 in mice (Olfr59 in rats) to trigger intracellular signaling in various cell types (Amedei and Morbidelli., 2019). The affinity by the receptor is different among the SCFAs, GPR41/FFAR3 (Gi) C3>C4>>C2, GPR43/FFAR2 (Gi, Gq) C2>C3, GPR109A (Gi, Gbg) butyrate (Koh et al., 2016).

SCFAs could exert certain actions on the intestinal cell and also at local immune system level. The function of SCFAs in the intestinal homeostasis is keeping a balance between tolerance to commensals and immunity to pathogenic bacteria. SCFAs can selectively support the development of Th1 and Th17 effector cells and Treg cells depending on the cytokine milieu and immunological context (Park et al., 2015). This effect of SCFAs on T cells is independent of GPR41 or GPR43, but dependent on direct HDAC inhibitor activity. The different SCFAs have a great variety of function, in fact, butyrate is able to achieve this balance by generation of Treg and IL-10-producing by activation of IL-18 secretion by IECs derived from the activation of GPR109A (Singh et al., 2014). Acetate is able to activate GPR43 in neutrophils localized in the lamina propia, and carry out the HDAC on Treg. Currently, it is unclear if GPR43 is expressed in colonic Treg and myeloid cells. Smith el al., (2013) showed the expression of GPR43 in colonic Treg and myeloid cells, but, Park et al., (2015) reported that T cells do not significantly express GPR43 also they described that GPR43 is not functional in regulating cytokine expression in T cells. Acetate supplementation or a diet rich in fiber, which substantially increases the production of acetate by gut bacteria, prevented the development of HTN in DOCA-salt mice (Marques et al., 2017).

In the distal gut, SCFAs are able to penetrate in colonocytes by diffusion or mediated by the solute carrier family 5 member 8 (SLC5A8) transport. SCFAs (mostly acetate and possibly propionate and in lesser extent butyrate) could reach the circulation

and induce certain effects on the brain, adipose tissue, liver and vascular cells. The systemic effects of SCFA on BP are antagonistic among GPR41, GPR43, GPR109 and Olfr78. Olfr78 has been found expressed in blood vessels and kidney being activated by acetate and propionate, but the sensitivity of Olfr78 to SCFAs compared with GPR41/43 is lower (Miyamoto et al., 2016). The activation of Olfr78 is able to increase BP, whereas stimulation of GRP41, GPR43 or GPR109a reduces BP. In addition, pro-hypertensive Ang II also induces similar qualitative expressional changes in rat aortic endothelial cells (RAECs). GPR41 induces vasodilatation in response to SCFAs, and subsequently decreases BP (Pluznick et al., 2013). SCFA receptors in aorta may play a role in the elevated BP of SHR. In contrast, other receptors for SCFAs do not appear to play any role. The role of SCFA receptors seems to be different in other hypertensive animal models, in hypertensive DOCA-salt mice both receptor GPR43 and Olfr78 were not expressed in the kidney or heart (Marques et al., 2016), although the expression of GPR43 mRNA was found reduced in the gut of this experimental model. Collectively, all data suggest that microbiota-derived SCFAs, by modulating T cells polarization in local secondary lymph organs, and distal effects in brain, kidney and vascular cells might regulate BP.

3.2.2 Lipopolysaccharides

LPS is a structural component of cellular wall from gram-negative bacteria, such as *Prevotella* and *Klebsiella*, which acts as a TLR4 agonist. The role of LPS in BP regulation is not completely known. Several authors have suggested that the up-regulation of TLR4 is able to contribute to the pathogenesis of HTN in animal models. TLR4 protein expression was found higher in vascular tissues from 15-week-old SHR than in age-matched WKY or in 5-week-old SHR (Bomfim et al., 2012). Similarly, in faecal samples of hypertensive subjects an increase in LPS or LPS-producing bacteria was also found (Li et al., 2017, Yan et al., 2017). The *in vivo* blockage of TLR4 has been proved able to prevent some of the organ damage induced by LPS. In addition, the systemic long-term treatment with a TLR4 antibody was able to reduce BP, remodeling and cardiac hypercontractility in SHRs (Bomfim et al., 2012; Bomfim et al., 2015). Besides, it was shown that TLR4 inhibition in the PVN of the hypothalamus decreases BP in SHRs (Dange et al., 2015). The chronic L-NAME treatment also augmented TLR4 expression and plasmatic LPS levels (Eissler et al., 2011). In fact, in TLR4$^{-/-}$ mice the L-NAME-

induced HTN was completely abolished (Sollinger, et al., 2014). Thus, TLR4 expression might be linked to the development and maintenance of HTN. LPS is able to stimulate and increase the expression of TLR4 in the vasculature joined to an augmentation of NADPH oxidase-dependent O_2^- production and inflammation (Liang et al., 2013). Besides, systemic TLR4 blockade in the Ang II-infusion model of HTN results in less pronounced BP changes as compared with SHR. Ang II-infused mice with a TLR4 deficiency, showed significant inhibition of vascular remodeling by reducing levels of ROS. However, this reduction did not impact Ang II's effects on BP (Nakashima et al., 2015). In agreement with these results, Ang II-infused mice treated with a TLR4 antibody restored, at least in part, the vascular function but could not induce any effects in BP compared with untreated animals (Nunes et al., 2017). Based on these results, it could be concluded in response to higher levels of Ang II TLR4 has a key role in vascular dysfunction and oxidative stress but not, at least directly, in BP. The partial knowledge the role of LPS in HTN has increased the interest of TLR4 as a possible target for novel pharmacological strategies to downstream the pathway overactivation.

3.2.3 Trimethylamine N-oxide

Both host and gut microbial metabolisms of trimethylammonium-containing nutrients (e.g., choline, phosphatidylcholine, and l-carnitine) result in the formation of Trimethylamine (TMA). In liver, flavin-containing monooxygenases (FMOs) convert TMA into TMAO. By far, the microbial-derived metabolite TMAO has garnered the most traction as a predictor of poor cardiovascular health and a potential target for therapy. In experimental HTN, SHR were found to have significantly elevated plasma TMA levels when compared to normotensive WKY rats (Jaworska et al., 2017). However, the pathophysiological role of TMA and TMAO in the development of high BP is unclear. TMAO infusion to normotensive rats did not increase BP, but prolongs the hypertensive effect of low concentrations of Ang II (Ufnal et al., 2014). In addition, TMAO exacerbated inflammatory reactions in the vascular wall, and increased ROS production (Sun et al., 2016). By contrast, a chronic low-dose TMAO treatment reduces diastolic dysfunction and heart fibrosis in hypertensive rats, providing new evidence for a potentially beneficial effect of a moderate increase in plasma TMAO on the pressure-overloaded heart (Huc et al., 2018). The effects of non-lethal inhibition of microbial production of TMA using 3,3-dimethyl-1-butanol, which reduced also the production of

TMAO (Wang et al., 2015), has not been studied in the context of HTN. Overall, the increased cardiovascular risk in patients with increased plasma TMAO levels may depend on increased plasma levels of its precursor (i.e. TMA).

Collectively, all data suggest an association between gut microbial dysbiosis and HTN, possibly by altering bacterial metabolism and/or structural products, with subsequent changes in T-lymphocytes populations, inflammation, vascular oxidative stress and endothelial dysfunction, impacting in the central nervous system, renal and vascular regulation of BP. In that context the microbiota could be considered a new possible pharmacological target to regulate BP. To modulate it, different therapeutic strategies, such as probiotics, could be used.

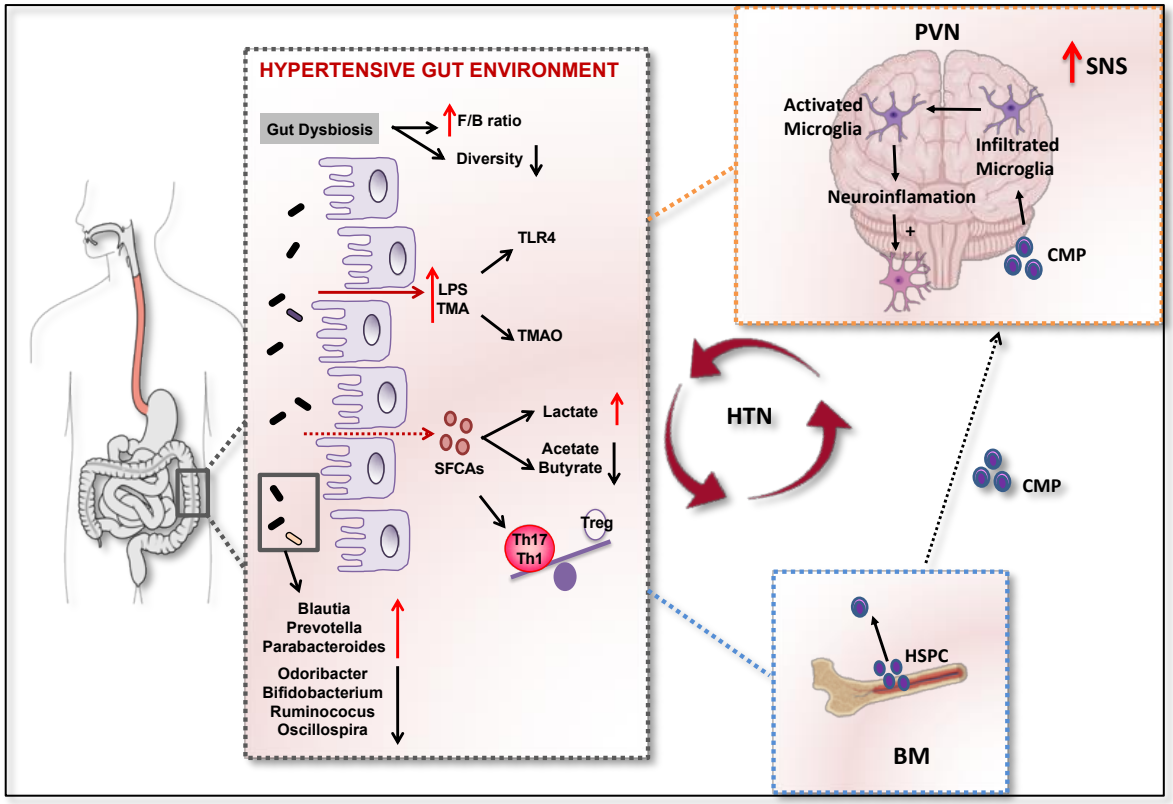

Figure 3. *Summary of the situation of the intestinal microbiota and its effects on the immune system and the brain in a hypertensive situation. F/B, Firmicutes/Bacteroidetes ratio; TLR4, Toll Like Receptor-4; LPS, lipopolysaccharide; TMAO, Trimethylamine N-oxide; TMA, Trimethylamine; SCFA, short chain fatty acids; Th17, T-helper 17; Th1, T-helper 1; Treg, T-regulatory; BM, Bone marrow; HSPC, Hematopoietic stem progenitor cell; CMP, common myeloid progenitor; SNS, sympathetic nervous system; PVN, paraventricular nucleus.*

4. Probiotics in Hypertension

Current guidelines for the management of arterial HTN propose lifestyle measures, including dietary approaches in all patients, including those who require drug treatment. The purpose of these lifestyle measures is to lower BP, to control other risk factors and to reduce the number or the dose of antihypertensive drugs (Mancia et al., 2014). These preventive strategies have had limited success. Therefore, there is an urgent need for alternative strategies. In this context several authors have included the consumption of probiotics as new therapeutic approach to prevent or treat HTN.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Santisteban et al., 2016). There are increasing numbers of probiotic products available to consumers, which include yogurt, other fermented milk and food products as well as various forms of dietary supplements. These products are usually prepared using lactic acid bacteria of four general species; *Lactobacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp., and *Streptococcus* sp., although the probiotic bacteria type and composition varies from product to product. Common probiotic yogurts often contain one or two bacterial strains, such as *Bifidobacterium lactis* and/or *Lactobacillus acidophilus*, whereas kefir, another fermented milk product, contains many more strains. Probiotics can also affect the composition and the diversity of the gut microbiota and their potential role in different diseases is currently a hot topic for research and discussion (Hill et al., 2014; Cani and Delzenne, 2009). Although the use of probiotics has been primarily associated with the improvement of gastrointestinal health, recent evidence has also shown that probiotics play an important role in other diseases, including HTN.

Few studies are currently available on the use of probiotic microorganisms at reducing cardiovascular disease. For example, some probiotics display cholesterol-lowering properties (Singh et al., 2013, Rerksuppaphol and Rerksuppaphol., 2015), improve atherosclerosis (Ishimwe et al., 2015; Chan et al., 2016a), or attenuate myocardial hypertrophy and heart failure after myocardial infarction (Chan et al., 2016b).

The beneficial effects of probiotic strains on BP have been reviewed recently (Gan et al., 2014; Thushara et al., 2016; Upadrasta et al., 2016; Daliri et al., 2016) The ability of probiotics to reduce BP has been attributed to the release of bioactive peptides during the fermentation of food products, such as the angiotensin-converting enzyme inhibitory

peptides. However, the bioavalability of such peptides is unclear. In addition, it has been described that some lactic acid bacteria, such as, *Lactobacillus johnsonii* La1 (LJLa1), a probiotic strain adhesive onto intestinal epithelial cells, has hypotensive action in urethane-anesthetized rats (de Brito Alves et al., 2016). Intraduodenal injection of LJLa1 reduced renal sympathetic nerve activity (RSNA) and BP and enhanced gastric vagal nerve activity (GVNA). Pre-treatment with thioperamide, a histaminergic H3-receptor antagonist, eliminated the effects of LJLa1 on RSNA, GVNA, and BP. Furthermore, bilateral lesions of the hypothalamic suprachiasmatic nucleus, the master circadian oscillator, abolished the suppression of RSNA and BP and the elevation of GVNA caused by LJLa1. These findings suggest that LJLa1 or its metabolites might lower BP by changing autonomic neurotransmission via the central histaminergic nerves and the suprachiasmatic nucleus in rats. By contrast, the intragastric *Lactobacillus casei* Shirota injection reduced efferent sympathetic nerve outflow to the adrenal gland and liver, but did not alter GVNA, RSNA, as well as mean BP in anesthetized rats ((Tanida et al., 2005). These results suggest that BP regulation induced by probiotics is strain-specific. Furthermore, the molecular mechanisms involved in the antihypertensive effects are unknown.

Only few animal studies analyzed the mechanisms involved on the antihypertensive effects of chronic probiotics consumption. It is well established that consumption of probiotics inhibited the production of proinflammatory cytokines (Matsuzaki et al., 2007; Arribas et al., 2009; Tanida et al., 2014). A few years ago, Toral et al. (2014) evaluated for the first time the effects of a probiotic with immunomodulatory properties, *Lactobacillus coryniformis* CECT5711, originally isolated from a traditional goat cheese, in BP and vascular function in obese mice fed on a high-fat diet (HFD). The probiotic treatment was given for 12 weeks, and it did not affect the weight evolution, although it reduced high BP, basal glycaemia and insulin resistance. *L. coryniformis* administration to HFD-induced obese mice induced marked changes in microbiota composition and reduced the metabolic endotoxaemia, as it decreased the LPS plasma levels, which was associated with a significant improvement in gut barrier disruption. Furthermore, it lowered tumor necrosis factor α (TNFα) expression in liver, improving the inflammatory status, and thus the glucose metabolism. Additionally, the probiotic reversed the endothelial dysfunction observed in obese mice. It also restored the increased vessel O_2^- levels observed in obese mice, by reducing NADPH oxidase activity and increasing antioxidant enzymes. Moreover, chronic probiotic administration for 2 weeks

also improved endothelial dysfunction and vascular oxidative stress induced by *in vivo* administration of LPS in control mice fed on a standard chow diet. These results are in agreement with the following hypothesis: HFD induces changes in gut microbiota that disrupt the gut barrier leading to a higher LPS plasma levels. Thus, LPS impacts with target metabolic organs (such as the liver), altering glucose metabolism, and the vascular wall, leading to oxidative stress. Both metabolic and direct vascular effects are involved on endothelial dysfunction and HTN. *L. coryniformis* administration induced changes in the gut microbiota that improved gut barrier dysfunction reducing plasma LPS and the subsequent alterations in glucose metabolism and vascular oxidative stress. In addition, by unknown mechanisms, the probiotic inhibited LPS signaling contributing to improve endothelial dysfunction (Toral et al., 2014)

The effects of oral supplementation with probiotics in non-obese SHR, an established model of genetic HTN characterized by elevated BP, arterial remodeling, endothelial dysfunction, and vascular inflammation were also studied (Gómez-Guzmán et al., 2015). Two groups of treatments with probiotics with inmunomodulatory properties were used: a) *Lactobacillus fermentum* CECT5716, or b) *L. coryniformis* plus *L. gasseri*. Both probiotic treatments induced a change in the cecum microbiota of SHR, with higher counts of the *Lactobacillus* spp. cluster, and lower counts of *Bacteriodes* spp. and *Clostridium* spp. These changes were associated with the improvement of vascular oxidative and inflammatory status resulting in increased NO bioavailability. Similarly, antihypertensive and vascular protective effects in SHR have been described after 8 weeks of treatment with *Lactobacillus casei* 10_{11} CFU/day (Yap et al., 2016). In addition, kefir treatment for 60 days was able to improve the endothelial function in SHR by partially restoring the ROS/NO imbalance and the endothelial architecture due to endothelial progenitor cells recruitment (Friques et al., 2015). By contrast, adding probiotics to a blueberry-enriched diet does not enhance and actually may impair the anti-hypertensive effect of blueberry consumption. However, probiotic bacteria are not interfering with blueberry polyphenol metabolism into hippuric acid (Blanton et al., 2015).

In human, several clinical trials have been performed with disparate conclusions. Furushiro et al. (1990) demonstrated the ability of *Lactobacillus casei* to reduce the BP and heart rate in hypertensive patients. In 2002, Naruszewicz et al., showed that food supplementation with *Lactobacillus plantarum* was able to induce a significant decrease in systolic BP in heavy smokers. Moreover in 2011 a Norwegian study lead by

Brantsaeter showed that the incidence of preeclampsia, which is associated with hypertension and inflammation, is decreased by chronic intake of probiotics (Brantsaeter et al., 2011). In addition, in a randomized double-blind clinical trial with type II diabetes mellitus, probiotic soy milk containing *Lactobacillus plantarum* induced a significantly reduction in the systolic/diastolic BP (Hariri et al., 2015). In a meta-analysis of randomized, controlled trials suggests that consuming probiotics may improve BP (Khalesi et al., 2014). In the nine trials included, probiotic consumption significantly lowered systolic BP by 3.56 mm Hg and diastolic BP by 2.38 mm Hg compared with the control groups. This meta-analysis suggests that consuming probiotics may improve BP by a modest degree, with a potentially greater effect when baseline BP is elevated, multiple species of probiotics are consumed, the duration of intervention is ≥8 weeks, or daily consumption dose is $\geq 10^{11}$ CFU.

However, several studies have questioned the role of some probiotics in producing low BP in humans. In a clinical trial, probiotics strains of *Bifidobacterium animalis, Lactobacillus acidophilus*, were unable to improve cardiovascular risk factors since they did not modify BP or concentrations of total cholesterol LDL-C, HDL-C, or triglycerides in overweight or obese individuals (Ivey et al., 2015). Additionally, a study of postmenopausal women with metabolic syndrome showed that administration of milk supplemented with *Lactobacillus plantarum* produced several beneficial effects, but it did not provide a significant decrease in BP. This is not the only one negative result to probiotics to reduce the BP (Barreto et al., 2014).

Due to these controversial results, it will be necessary to perform more studies to analysis the concrete role of probiotics in hypertension. Overall, the results suggest that maybe the different effect of the probiotic in HTN could be due to the complicate mechanisms involved in the development of high BP, the different bacterial strains, and the own idiosyncrasy of the gut microbiota of the patients.

JUSTIFICATION AND AIMS

Justification and Aims

Numerous studies have discussed a potential role for gut microbiota in the development of cardiovascular diseases. However, there are a limited number of studies pointing to a direct link between hypertension and gut microbiota in both humans and animal models. As was described in the introduction, there are strong evidences showing that gut microbiota has a key role in the development of hypertension. However, the precise mechanisms involved in the control of BP are still unknown. To try to clarify the underlying mechanisms several experiments will be performed during the development of the current Doctoral Thesis. So, the *main aim of this Thesis* is to try to know how microbiota could regulate BP, involving immune system and SNS, and how probiotic intervention could exert antihypertensive effects.

5.1 Analyze the role of immune system in the changes in BP induced by gut microbiota.

In the introduction section was described how both innate and adaptive immune responses participate in the generation of ROS and inflammatory changes in the kidneys, blood vessels and brain in hypertension (Wenzel et al., 2016). T-cell co-stimulation via B7 ligands is essential in the development of experimental hypertension, and the inhibition of this process could have a therapeutic benefit in the treatment of this disease. (Vinh et al., 2010) Increased macrophage infiltration into the peri-adventitial space of vessels and a balance between pro-inflammatory T cells reactivity and inflammatory suppression induced by Treg determine the development of hypertension (Didion et al., 2009; Kassan et al., 2011; Kasal et al., 2012). Intestinal microbiota has a marked effect on host immune functions (Gensollen et al., 2016), mainly through metabolic products, such as SCFAs, and structural microbial products, such as LPS (Furusawa et al., 2013; Asarat et al., 2016). In fact, Adnan et al., using FMT between WKY and SHR conclude that gut dysbiosis can directly affect SBP. In addition, FMT from tacrolimus hypertensive mice increased SBP and induced endothelial dysfunction in control recipient mice (Toral et al., 2018). In fact, microbiota manipulation with the probiotic *Lactobacillus fermentum* restored the imbalance Th17/Treg in mesenteric lymph nodes (MLNs) and inhibited the raise in BP induced by tacrolimus (Toral et al.,

2018). However, if changes in T cell activation possibly induced by gut microbiota contribute to control the endothelial function and BP in genetic hypertension, is unknown. Thus, the first aim of this doctoral thesis was:

Article 1: Investigate whether changes in gut microbiota induced by exchanging the gut microbiota between spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) alter the gut-immune system interaction inducing changes in vascular function and BP.

5.2 Analyze the role of the interaction gut microbiota-SNS in the regulation of BP

The association between gut dysbiosis and neurogenic-related diseases, such as hypertension has been previously discussed (Mell et al., 2015; Yang et al., 2015). A common characteristic of resistant hypertension is chronically elevated SNS activity accompanied by a high release of noradrenaline (NA) (Tsioufis et al., 2011), which indicates a neurogenic component that contributes to the initiation, maintenance and progression of hypertension (Yang and Zubcevic., 2017). The link between hypertension and gut dysbiosis has recently been suspected (Mell et al., 2015; Yang et al., 2015), several groups are investigating this interaction. In this way, different studies have shown that FMT from hypertensive human and rat donors elevate the BP of the host, normotensive mice and rats, respectively (Adnan et al., 2017; Li et al., 2017; Toral et al., 2018), pointing out that gut dysbiosis plays a possible contributing or even causal role in hypertension. A newly identified interaction between the brain, gut and bone has been identified as a possible mechanism in the pathogenesis of hypertension (Santisteban et al., 2016). For instance, an increase in sympathetic drive to BM and the gut may also trigger a sequence of signalling events that can, ultimately, contribute to an overall increase in BP and the establishment of HTN, by affecting the structure and function of BP target organs, such as vasculature, kidney, heart, and brain. The immune and sympathetic systems are recognized to contribute to the development of hypertension (Yang et al, 2017), while there exists a bidirectional signaling between the brain and gut microbiota, which can regulate BP through the modulation of the interaction between SNS and the immune system (Yang and Zubcevic., 2017).

However, the communication between gut microbiota and the SNS in hypertension is not completely understood. To address these questions, the second aim of this Doctoral Thesis was:

Article 2: Study whether changes in gut microbiota induced by exchanging the gut microbiota between spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) alter the gut-brain interaction inducing changes in vascular function and BP.

5.3 Analyze the effect of AT1 receptor blockade on gut microbiota composition in genetic hypertension

Gut microbiota is commonly referred to as an essential acquired organ because its composition and richness are constantly adapting to the challenges occurring in the environment or in the host, such as age, diet, and lifestyle modifications. In addition, it has been commonly observed that a change in the host health status has been accompanied by a shift in the gut microbiota (Marques et al., 2017). The BP reduction by minocycline was able to rebalance the hypertension-related dysbiotic gut microbiota by reducing the F/B ratio in Ang II-induced hypertension (Yang et al., 2015). Similarly, the consumption of probiotic *Lactobacillus* strains reduced BP in SHR (Gómez-Guzmán et al., 2015), in tacrolimus-induced hypertension in mice (Toral et al., 2018) and in high salt diet-induced hypertension in mice (Wilck et al., 2017), showing that restoring the microbiota composition to a similar composition as that found in normotensive animals led to the improvement of BP. In addition, increased permeability of gut epithelial barrier, intestinal inflammatory status and dysbiosis are associated with BP elevation in Ang II-infused mice (Kim et al., 2018). In fact, the inhibition of the renin-angiotensin system (RAS) by the ACE inhibitor captopril reduces BP, reverses gut pathology (Santisteban et al., 2017). However, whether BP reduction by other RAS inhibitors, such as the angiotensin receptor blocker losartan, shifts the gut microbiota composition is unclear. Then, the third aim was:

Article 3: Evaluate the effects of losartan on gut microbiota in SHR and the role of gut microbiota in the antihypertensive effect of losartan.

5.4 Analyze the effect of the modulation of gut microbiota by probiotics in genetic hypertension

In the introduction section was describe how currently there is a trend on looking for alternative/complementary treatments to control BP, since some patients under treatment showed resistant HTN. Among the different proposals, the modulation of the gut microbiota by probiotics could be interesting.

The gut microbiota is able to communicate with distal organs by producing numerous metabolites that may be absorbed into the systemic circulation and exert biological effects (Marques et al., 2018). The signalling molecules are bacterial metabolic products, such as SCFAs (Pluznick et al., 2013) and TMAO (Bennett et al., 2015), or bacterial wall components such as LPS (Toral et al., 2014). In fact, gut dysbiosis in hypertension was characterized by a decrease in acetate- and butyrate-producing bacteria and increase in lactate-producing bacterial populations (Yang et al., 2015). SCFAs can function to stimulate host GPR pathways that impact renin secretion and blood pressure regulation (Pluznick et al., 2013). In fact, butyrate attenuates Ang II-induced hypertension in mice (Kim et al., 2018), and both, a diet rich in fiber, which substantially increases the production of SCFAs such as acetate, or acetate supplementation, prevented the development of hypertension in the DOCA-salt model (Marques et al., 2017). Increased bacterial production of SCFAs is associated with reduced circulating CD4+ immune cells (Bartley et al., 2018). Bacterial LPS, through TLR4 activation, contributes to increase BP and low-grade vascular inflammation displayed by SHR (Bomfim et al., 2012).

Thus, gut microbiota is potentially intertwined functionally to control BP. In fact, a meta-analysis demonstrated a significant decrease in BP in patients treated with probiotics (Khalesi et al., 2014). Furthermore, a beneficial role for *Lactobacillus* probiotics and kefir in blood pressure regulation and vascular protection has been described in SHR with stablished hypertension (Gómez-Guzmán et al., 2015; Friques et al, 2015; Yap et al., 2016), as a result of restoring ROS/NO imbalance in arterial wall. However, if probiotics consumption could prevent the raise in BP in SHR and if SCFAs play any role in this effect is unknown. Then, the fourth aim of this Doctoral Thesis was:

Article 4: Evaluate the cardiovascular effects of probiotics *Bifidobacterium breve* CECT7263 (BFM), and *Lactobacillus fermentum* CECT5716 (LC40), and butyrate and acetate in genetic hypertension.

5.5 Analyze the role of NO in the antihypertensive effect of *Lactobacillus fermentum* CECT5716 (LC40).

The importance of the endothelium in BP control was describe in the introduction section, and how it produces and releases a great variety of vascular tone modulating factors. Endothelium-derived NO is a key regulator of BP. NO, synthesized by eNOS, is the leading vasodilator factor from the endothelium, having a main role in the control of the HTN and endothelial dysfunction. As was describe previously, probiotics exert antihypertensive effects in human hypertensive patients and also in animals with genetic hypertension. In fact, it has been described how the chronic consumption of *Lactobacillus* strains was able to reduce BP and the damage in the target organs (heart, kidney), and to improve the endothelial dysfunction. The antihypertensive effect seems to be related to the increase in NO bioavailability, as a result of reduced vascular pro-inflammatory and pro-oxidative status. However, whether probiotics are able to induce higher levels of NO is unclear, because there is no information about the antihypertensive effects of probiotic in other non-genetic forms of hypertension. To clarify whether this antihypertensive effect could be related to the capacity of probiotics to increase NO bioavailability, due to an activation of eNOS or maybe only as a result of reduced vascular pro- inflammatory and pro-oxidative status, the last aim of this doctoral thesis will be:

Article 5: Analyze the effects of the probiotic LC40 in hypertension and endothelial dysfunction induced by chronic inhibition of NO synthesis with L-NAME.

ARTICLE 1

Role of the immune system in vascular function and blood pressure control induced by fecal microbiota transplantation in rats

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Short title: **Gut microbiota and blood pressure**

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Abstract

Aim: High blood pressure (BP) is associated with gut microbiota dysbiosis. The aim of this study was to investigate whether changes in gut microbiota induced by exchanging the gut microbiota between spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) alter the gut-immune system interaction inducing changes in vascular function and BP.

Methods: Twenty-week-old recipient WKY and SHR were orally gavaged with donor fecal contents from WKY or SHR. In additional experiments, we used a design to determine whether blockade of B7-dependent costimulation with CTLA4-Ig or blockade of IL-17 with IL-17-neutralizing antibody could prevent hypertension caused by fecal microbiota transplantation (FMT) from SHR to WKY.

Results: Correlation analyses identified the bacterial abundance of *Turicibacter* and *S24-7_g* that, respectively, positively and negatively correlated with systolic BP. FMT from WKY rats to SHR rats reduced basal systolic BP, restored the imbalance between Th17/Treg in mesenteric lymph nodes (MLNs) and aorta, and improved endothelial dysfunction and vascular oxidative status found in SHR transplanted with SHR faeces. FMT from SHR to WKY increased CD80 and CD86 mRNA levels and T cells activation in MLNs, circulating T cells, aortic T cell infiltration, impaired endothelial function and increased basal SBP. These effects were abolished by blockade of B7-dependent costimulation with CTLA4-Ig. IL-17a neutralizing antibody reduced SBP and improved endothelial dysfunction induced by FMT from SHR to WKY.

Conclusion: Gut microbiota is an important factor involved in the control of BP, as a consequence of its effect in T-cell activation in gut immune system and vascular T-cells accumulation.

KEYWORDS

Gut dysbiosis, hypertension, endothelial dysfunction, immune cells.

INTRODUCTION

Numerous studies have speculated or discussed a potential role for gut microbiota in the development of cardiovascular diseases (Ascher et al., 2018). However, there is a limited number of studies pointing to a direct link between hypertension and gut microbiota in both humans and animal models. Karbach et al., 2016 showed that there is no significant difference in blood pressure (BP) between conventionally-raised and germ-free mice. This is in agreement with preciding experiments that describe no effects on BP after a significant decrease in microbial biomass induced by antibiotics (Pluznick et al., 2013). Also, it has been demonstrated that both the abundance levels of selected genera and the *Firmicutes/Bacteroidetes* (F/B) ratio in young spontaneously hypertensive rats (SHR) are not different from their age-matched healthy counterpart, Wistar Kyoto rats (WKY) (Santisteban et al., 2016). Nonetheless, eradicating gut microbiota has a protective effect in mice against angiotensin II-induced vascular dysfunction, hypertension, and hypertension-induced end-organ damage, signaling, for the first time, commensal microbiota as a possible environmental factor promoting angiotensin II-induced high BP (Karbach et al., 2016). Also, in both angiotensin II-induced hypertension and in SHR (Yang et al., 2015), the administration of antibiotics improved BP. Furthermore, a decrease in BP from 160/90 mmHg to 130/60 mm Hg after a treatment consisting of the combination of antibiotics[6] was shown in a case report of resistant hypertensive patients.

A fragile balance in the composition of gut microbiota is basic in maintaining integral homeostasis and intestinal immunity. An imbalance in the composition of microbiota is commonly denominated dysbiosis. Recently, it has been proven that high BP is linked to gut dysbiosis, both in human and animal hypertension (Yang et al., 2015; Durgan et al., 2016; Kim et al., 2018). Gut dysbiosis in hypertension animal models, was characterized by: i) gut microbiota is less rich and less diverse, and shows an increased F/B ratio, due to a contraction of *Bacteroidetes* and an expansion of *Firmicutes*; ii) increased lactate-producing bacteria, and decreased butyrate- and acetate-producing bacteria populations. Still, the link between dysbiosis and hypertension does not necessarily point to a cause-effect relationship. Adnan et al., 2017 using fecal microbiota transplantation (FMT) between WKY and SHR conclude that gut dysbiosis can directly affect systolic BP (SBP). In addition FMT from tacrolimus hypertensive mice increased SBP and induced endothelial dysfunction in control recipient mice (Toral et al., 2018).

Similarly, FMT from hypertensive human donors to germ-free mice elevated BP, showing a direct influence of gut microbiota on the BP of the host (Li et al.,2017). Despite these strong evidences showing that the gut microbiota has a causal role in the development of hypertension, the mechanisms involved in the control of BP are unknown.

Endothelial dysfunction plays a seminal part in the pathogenesis of hypertension. Decreased nitric oxide (NO) bioavailability is the central factor that links oxidative stress to endothelial dysfunction and hypertension (Panza et al., 1993). Both innate and adaptive immune responses participate in the generation of reactive oxygen species (ROS) and inflammatory changes in the kidneys, blood vessels and brain in hypertension (Wenzel et al., 2016). T-cell costimulation via B7 ligands is essential for development of experimental hypertension, and the inhibition of this process could have a therapeutic benefit in the treatment of this disease (Vinh et al., 2010). Increased macrophage infiltration into the peri-adventitial space of vessels and a balance between pro-inflammatory T cells reactivity and inflammatory suppression induced by regulatory T (Tregs) cells determine the development of hypertension (Didion et al., 2009; Kassan et al., 2011; Kasal et al., 2012). Intestinal microbiota has a marked effect on host immune functions (Gensollen et al., 2016), mainly through metabolic products, such as short-chain fatty acids (SCFAs), and structural microbial products, such as lipopolysaccharides (LPS) (Furusawa et al., 2013;Asarat et al., 2016) . In fact, microbiota manipulation with the probiotic *Lactobacillus fermentum* restored the imbalance T helper-17 (Th17)/Treg in mesenteric lymph nodes (MLNs) and inhibited the raise in BP induced by tacrolimus (Toral et al., 2018). In agreement with this, the treatment of mice with *Lactobacillus murinus* prevented high-salt diet-induced generation of Th17 cells and consequently ameliorates salt-sensitive hypertension (Emmensor et al., 1985). However, if changes in T cells activation possibly induced by gut microbiota contribute to control the endothelial function and BP in genetic hypertension, with normal-salt diet, is unknown. The aim of this study was to investigate whether changes in gut microbiota induced by exchanging the gut microbiota between WKY and SHR alter the gut-immune system interaction inducing changes in vascular function and BP.

RESULTS

Blood pressure and target organs morphology are controlled by gut microbiota

FMT from WKY rats to SHR (S-W group) reduced SBP by 38 ± 4 mm Hg basal pressure (199.5 ± 6 mm Hg) (Figure 1A). Similarly, SHR-to-WKY FMT (W-S group) increased basal SBP (149.8 ± 4 mm Hg) by 16 ± 2 mm Hg (Figure 1A). No significant changes in basal SBP were observed in both SHR and WKY after FMT from SHR (S-S group) and WKY (W-W group), respectively. The FMT effects on blood pressure were confirmed by intra-arterial mean arterial BP (MABP) recording (Figure 1B). In addition, renal (Figure 1C) and left ventricular cardiac (Figure 1D) hypertrophy was also reduced in S-W group as compared to S-S group.

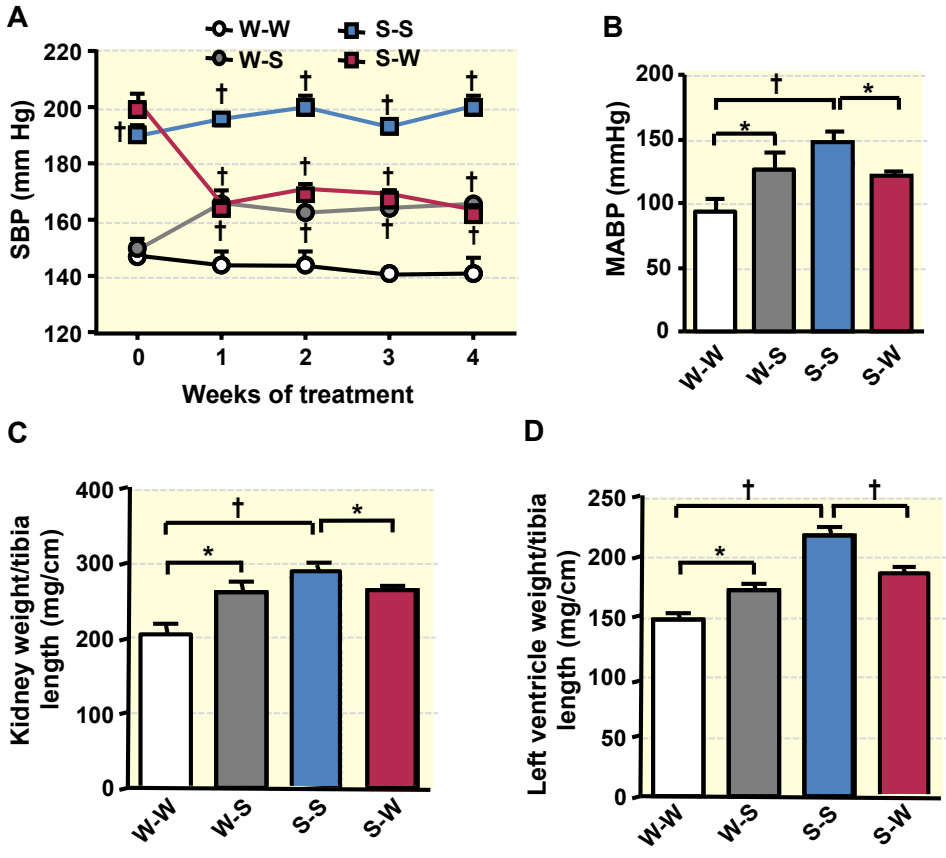


Figure 1. Effects of fecal microbiota transplantation (FMT) on blood pressure and morphological organs change. *Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S) or from Wistar Kyoto rats (WKY) (S-W) and in WKY with stool transplant from WKY (W-W) or from SHR (W-S). The nested design was used to compare the evolution of tail SBP with time, with the rat as random factor and days and treatment as fixed factors.. When the overall difference was significant comparisons were made*

*using Bonferroni´s method with an appropriate error. (**B**) Mean arterial blood pressure (MABP), measured by intra-arterial recording into left carotid artery, at the end of the experimental period One-way ANOVA and Tukey post hoc test. Morphological data in the kidney (**C**) and left ventricle (**D**). One-way ANOVA and Tukey post hoc test. Values are means ± SEM (n = 5-8). *P < 0.05 and †P < 0.01 W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

Changes in gut microbiota after fecal microbiota transplantation

We next examined the microbiota composition of WKY and SHR at time -1, 0, and 4 weeks after FMT (Figure 2). Donors feces (1 week before FMT) from 20 weeks old SHR compared to aged-matched counterpart WKY showed a similar dysbiotic profile than that previously described in 10 weeks old SHR and WKY (Yang et al.,2015), with increased F/B ratio (Figure 2C), and decreased acetate- and increased lactate-producing bacteria (Figure 2D). However, no significant changes in fecal biomass DNA (Figure 2A), microbial richness (Chao 1 index), diversity (Shanon index), and evenness (Pielou index) (Figure S1A) were detected between both strains. When β-diversity between both groups was analyzed through principal coordinate analysis (PCoA) plots, a clear separation was observed between the 2 clusters (Figure 2E). The bacterial load, represented as fecal biomass DNA per milligram of feces (Figure 2A), was reduced by 5 days ceftriaxone treatment (0 week) in ≈ 87% in both SHR and WKY counterparts, with the most suppression of bacteria belonging to the phylum *Bacteroidetes* (Figure 2B, Figure S1B). However, as expected by its antibacterial activity (Emmerson et al.,1985), ceftriaxone enriched the microbiota with resistant bacteria belonging to the *Enterococcus* generus (Figure S1C). The cross FMT during 4 weeks restored the bacterial loads in recipient rats to values similar to that found at -1 weeks, showing the efficiency of the protocol. At this time (4 weeks), the relative abundance of the main phyla in the gut (Figure 2B; Figure S1B) showed that compared with W-W, W-S tended to decreased *Bacteroidetes* (P = 0.054), and increased *Firmicutes* (P = 0.09) abundance, but significantly increased F/B ratio (Figure 2C). Compared to S-S, S-W reduced F/B ratio as a result of significantly reduction of *Firmicutes* abundance. Of note that only a significant reduction of acetate-producing bacteria was found in S-S group as compared to W-W (Figure 2D). PCoA plot illustrates a separation among all experimental groups, showing that W-W microbiota cluster further away from that of group S-S, and both W-S and S-W cluster in the middle of the former groups (Figure 2E).

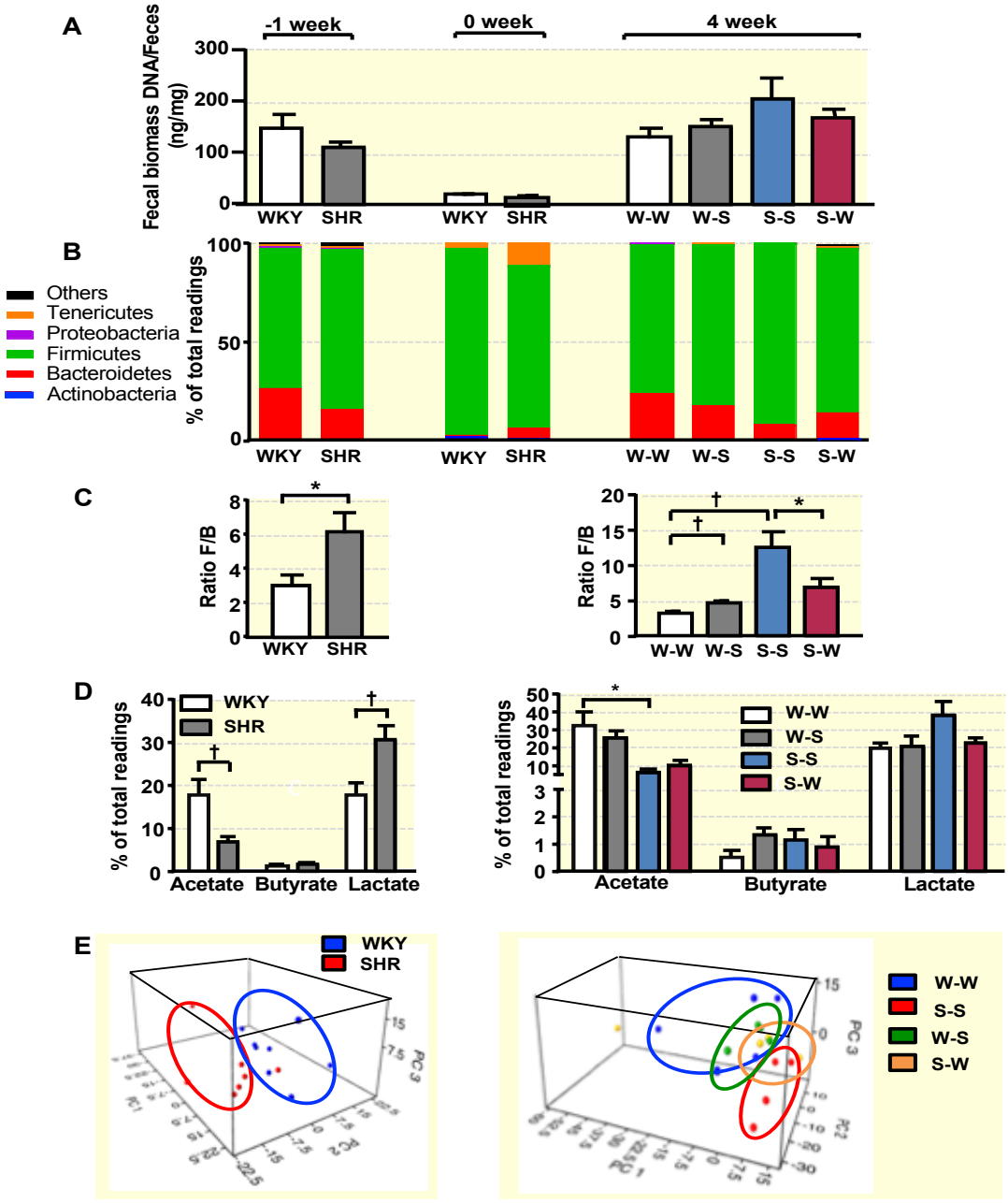
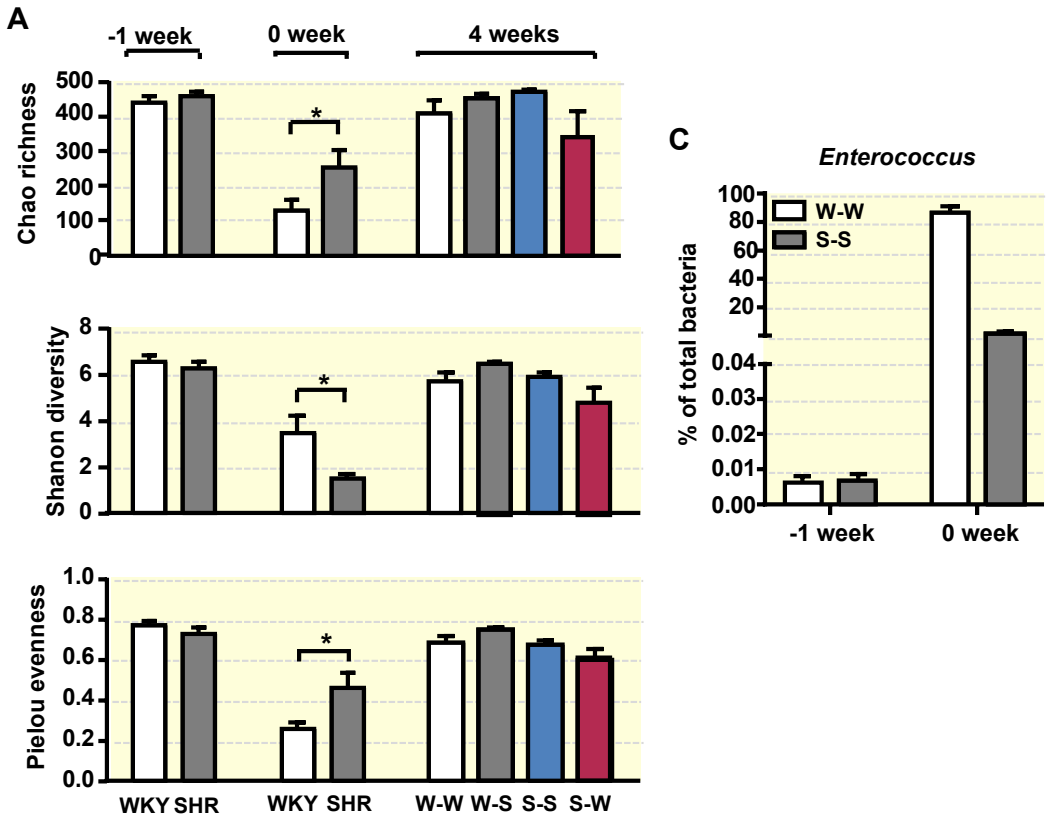


Figure 2. Effects of fecal microbiota transplantation (FMT) on gut microbiota composition. *(**A**) The profile of gut microbiota was measured at -1, 0, and 4 weeks after FMT. At these time fecal biomass, (**B**) percentage of phyla, (**C**) Firmicutes/Bacteroidetes (F/B) ratio, (**D**) the relative proportions of acetate-, butyrate- and lactate-producing bacteria, and (**E**) principal coordinate analysis in the gut microbiota from all experimental groups. Sequence reads were classified according to the primary end product of the assigned bacterial genera. Genera were classified into >1 group correspondingly if they were defined as producers of multiple metabolites. Values are means ± SEM (n = 8, at time -1, and 0; n = 4-5 at 4 weeks). Results at -1 and 0 time point were compared by Student t test and at 4 weeks results were compared by 1-way ANOVA*

*and Tukey post hoc test. Mann-Whitney test was applied in panel C at -1 weeks. *P < 0.05 and †P < 0.01 SRH vs WKY, W-S vs W-W, S-S vs W-W, and S-W vs S-S.*



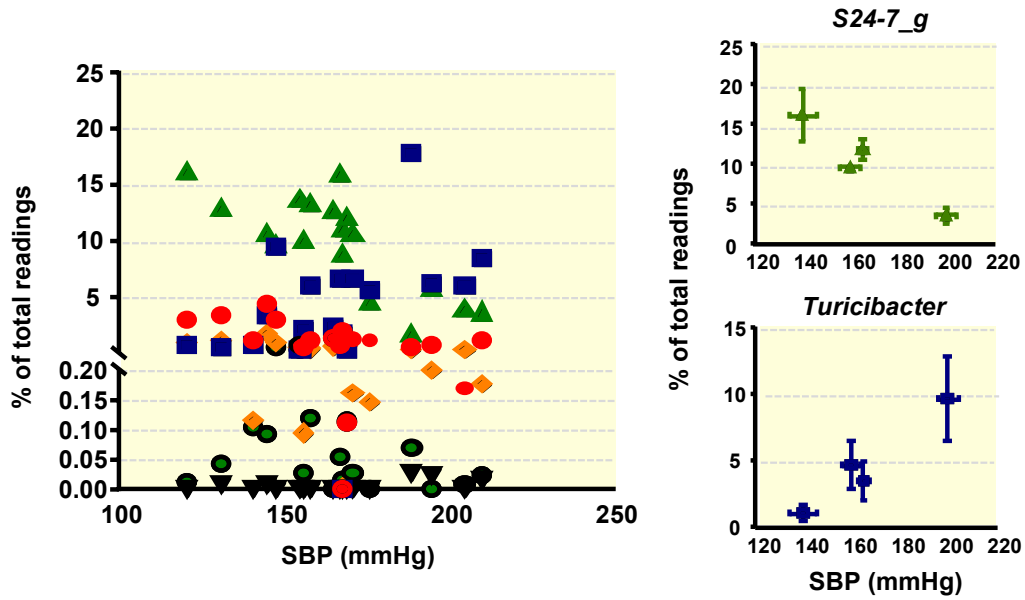
B

	-1 week			0 week		
	WKY (n = 8)	**SHR** (n = 8)	P value (vs WKY)	**WKY** (n = 8)	**SHR** (n = 8)	P value (vs WKY)
Actinobacteria	0.091 ± 0.0	0.26 ± 0.09	0.071	2.03 ± 2.14	1.33 ± 1.02	0.77
Bacteroidetes	26.68 ± 3.4	15.5 ± 1.99	0.009 †	0.4 ± 0.24	4.73 ± 3.03	0.12
Firmicutes	70.94 ± 3.1	81.11 ± 2.01	0.011*	94.5 ± 2.59	82.44 ± 6.59	0.07
Proteobacteria	0.35 ± 0.1	0.3 ± 0.11	0.690	0.003 ± 0.00	0.1 ± 0.069	0.11
Tenericutes	1.15 ± 0.6	0.6 ± 0.11	0.387	2.95 ± 1.68	11.08 ± 7.58	0.25
Others	0.8 ± 0.4	2.23 ± 1.1	0.232	0.081 ± 0.1	0.27 ± 0.2	0.34

	4 weeks						
	W-W (n = 5)	**W-S** (n = 5)	P value (vs W-W)	**S-S** (n = 4)	P value (vs W-W)	**S-W** (n = 5)	P value (vs S-S)
Actinobacteria	0.15 ± 0.03	0.46 ± 0.21	0.690	0.37 ± 0.3	0.884	0.48 ± 0.3	0.983
Bacteroidetes	23.7 ± 2.2	17.23 ± 0.72	0.054	7.74 ± 1.26	0.0001†	15.02 ± 2.43	0.131
Firmicutes	75.45 ± 2.17	81.16 ± 0.56	0.096	89.06 ± 1.2	0.0001 †	81.8 ± 2.48	0.0186*
Proteobacteria	0.33 ± 0.08	0.43 ± 0.17	0.933	0.1 ± 0.03	0.613	0.41 ± 0.18	0.376
Tenericutes	0.15 ± 0.05	0.4 ± 0.14	0.859	0.17 ± 0.05	1.000	0.46 ± 0.46	0.824
Others	0.086 ± 0.04	0.04 ± 0.022	0.999	0.017 ± 0.007	0.999	1.07 ± 1.189	0.589

Figure S1. Effects of fecal microbiota transplantation (FMT) on gut microecological parameters and phyla composition. *(**A**) The profile of gut microbiota was measured at -1, 0, and 4 weeks after FMT. At these points fecal microbial richness, diversity, and evenness were evaluated in the gut microbiota from all experimental groups. (**B**) Phylum breakdown of the most abundant bacterial communities in fecal samples. (**C**) Enterococcus content in fecal samples before and after ceftriaxone treatment. Values are means ± SEM (n = 8, at time -1, and 0; n = 4-5 at 4 weeks). Results at -1 and 0 time point were compared by Student t test; and at 4 weeks results were compared by 1-way ANOVA and Tukey post hoc test. Mann-Whitney test was applied in panel A for Shanon diversity and Pielou evenness at -1 weeks. *P < 0.05 and †P < 0.01 SRH vs WKY, W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

To investigate genera that are more closely related to SBP, correlation analyses were done with SBP and bacterial abundance using Pearson correlation coefficients with $P < 0.05$. Correlation analyses identified the bacterial abundance of *Turicibacter* and *S24-7_g* that, respectively, positively and negatively correlated with SBP (Figure S2).



Gennus increased with SBP	Pearson R	Two-tailed P
Turicibacter	0.9528	0.0239*
YS2	0.8127	0.0985

Gennus decreased with SBP	Pearson R	Two-tailed P
S24-7_g	-0.9259	0.0378*
Bacteroides	-0.7716	0.1216
Odoribacter	-0.7134	0.1554
Parabacteroides	-0.5517	0.2572

Figure S2. Bacterial taxa positively and negatively correlated with systolic blood pressure (SBP). *Bacterial gennus that were significantly positively correlated with SBP were analyzed using the Pearson correlation coefficient. Please note the break in the y-axis. Symbols from panels and correlation coefficients are explained in the tables. *P<0.05.*

Endothelial function, vascular oxidative stress and inflammation are controlled by gut microbiota

Aortic endothelial dysfunction is a key event involved in both vascular tone and BP control (Montezano et al., 2015). Endothelium-dependent relaxant responses to acetylcholine in phenylephrine-contracted aortic segments were impaired in S-S group as compared to W-W group (Emax: 83 ± 5 % vs 54 ± 5%, $P<0.01$). The stool transplant from WKY to SHR improved this relaxant response (Emax: 72 ± 5 %, $p<0.05$ vs S-S group) (Figure 3A), whereas SHR-to-WKY FMT rats reduced the relaxation to acetylcholine (Emax: 64 ± 6 %, $p<0.05$ vs W-W group) (Figure 3A). When incubating with NG-nitro-L-arginine methyl ester (L-NAME) this relaxant response was suppressed in all experimental groups (not shown). In addition, no changes in endothelium-independent relaxation to nitroprusside were observed among all experimental groups (not shown). Within the vascular wall, the levels of ROS, which have been proven to be involved in endothelial dysfunction in SHR (Zarzuelo et al., 2011), were characterized by ethidium red fluorescence in preparations of aorta stained with dihydroethidium (DHE). Nuclear red ethidium fluorescence was quantified and blue fluorescent nuclear counterstains by 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI) were used for normalization, allowing comparisons between different sections. As expected, higher red staining was observed in aorta from S-S group as compared to W-W group (Figure 3B), linked to endothelial dysfunction in S-S group. Rings from W-S group showed a marked staining vascular wall, which was higher than in control W-W rats, whereas FMT from WKY to SHR reduced DHE fluorescence (Figure 3B). Since NADPH oxidase is the principal source of ROS in the vascular wall, we investigated its activity and the mRNA expression of its main subunits in aorta from all groups. As previously described (Duarte et al., 2001; Zarzuelo et al., 2011) both aortic NADPH oxidase activity (Figure 3C) and the levels of mRNA from its catalytic subunits: NOX-1 and NOX-4, and its regulatory subunits: $p22_{phox}$ and $p47_{phox}$ (Figure 3C) were increased in S-S group as compared to W-W group. Once more, SHR-to-WKY FMT increased NADPH oxidase activity and expression of their subunits, whereas WKY-to-SHR FMT reduced the level of these parameters (Figure 3C). As expected, in the presence of the non-selective NADPH oxidase inhibitor apocynin no statistically significant differences were found between groups in the acetylcholine-mediated vasodilation, suggesting the key role of NADPH oxidase in endothelial dysfunction in hypertensive rats (Figure 3C).

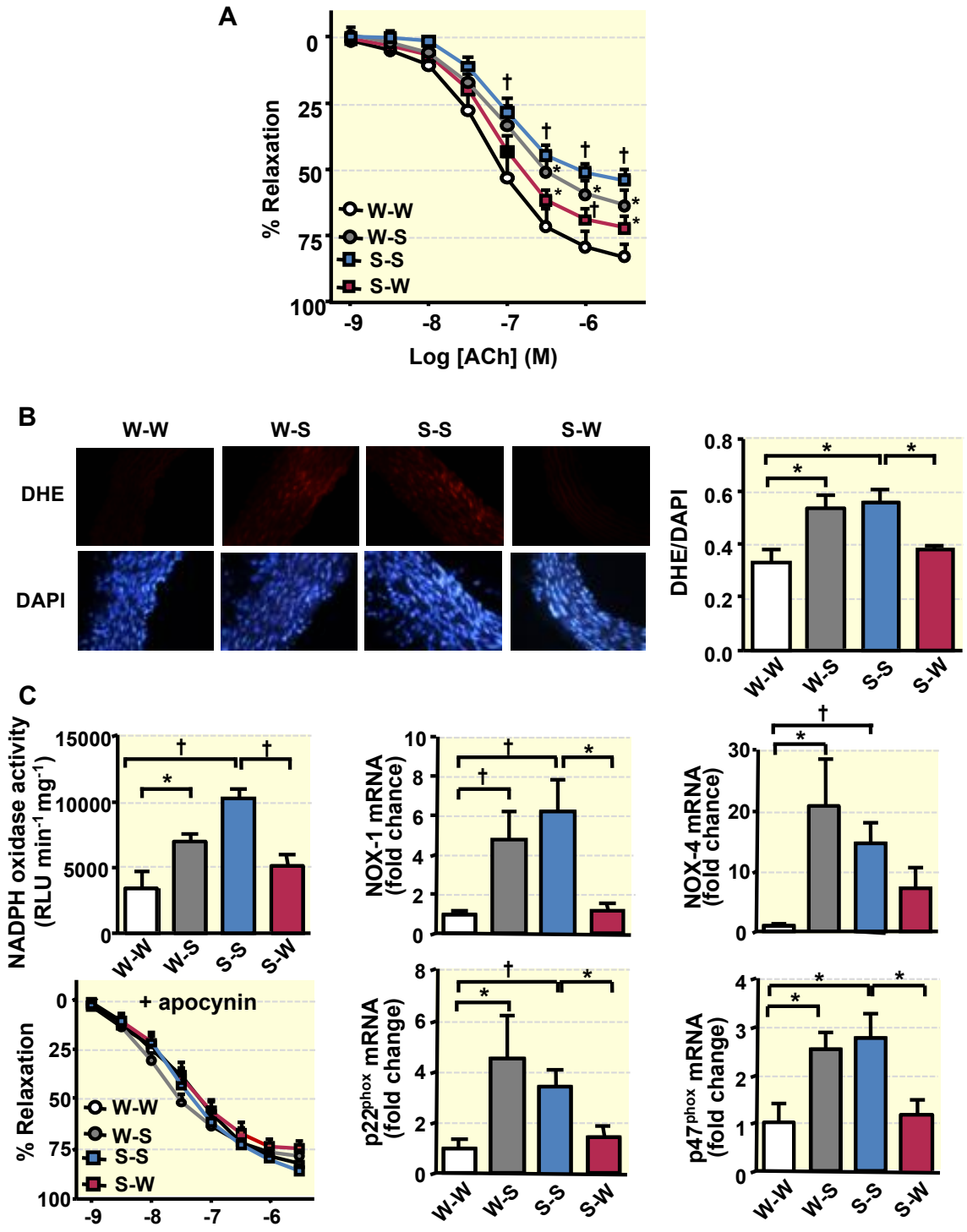


Figure 3. Effects of fecal microbiota transplantation (FMT) on endothelial function and vascular oxidative stress. *(**A**) Vascular relaxant responses induced by acetylcholine (ACh), in endothelium-intact aortae pre-contracted by phenylephrine (Phe, 1 μM) in spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S) or from Wistar Kyoto rats (WKY) (S-W) and in WKY with stool transplant from WKY (W-W) or from SHR (W-S). (**B**) Top pictures show arteries stained with dihydroethidium (DHE), which produces a red fluorescence when oxidized to ethidium by ROS. Bottom pictures show blue fluorescence of the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI) (x 400 magnification). Averaged values, mean ± SEM (n = 8-10*

*rings from different rats) of the red ethidium fluorescence normalized to the blue DAPI fluorescence. One-way ANOVA and Tukey post hoc test. (**C**) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence and expression of NADPH oxidase subunits NOX-1, NOX-4, $p47_{phox}$ and $p22_{phox}$ at the level of mRNA by RT-PCR in all experimental groups. One-way ANOVA and Tukey post hoc test. Endothelium-dependent relaxation induced by ACh in the presence (added 30 min before of Phe) of the NADPH oxidase inhibitor apocynin (10 μM) in aortae pre-contracted by Phe from all experimental groups. Analysis of the nested design was carried out with groups and concentrations to compare the concentration-response curves to acetylcholine (A, C). Values are means ± SEM (n = 5-8). *P < 0.05 and †P < 0.01 W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

Vascular inflammation plays a crucial role in the progression of vascular damage associated with hypertension and has been proposed to be the link between elevated BP and atherosclerosis (Zarzuelo et al., 2011). We found increased aortic mRNA levels of pro-inflammatory cytokines TNF-α and INFγ (Figure 4A) in rats with FMT from SHR as compared to rats transplanted with WKY stool. In addition, aortic infiltration pro-inflammatory cells, such as Th17 cells (Figure 4B), measured by the mRNA expression levels of both IL-17 and RORγ, were also higher in both rats groups (W-S, S-S) transplanted with fecal microbiota from SHR as compared to rats with FMT from WKY (W-W, S-W), whereas, aortic markers of anti-inflammatory Treg cells (FoxP3 and IL-10) were reduced in W-S and S-S groups (Figure 4B). When the ratio between pro-inflammatory/anti-inflammatory cells (Th17/Treg) was analysed, this parameter was higher in W-S and S-S as compared to W-W and S-W (Figure 4C).

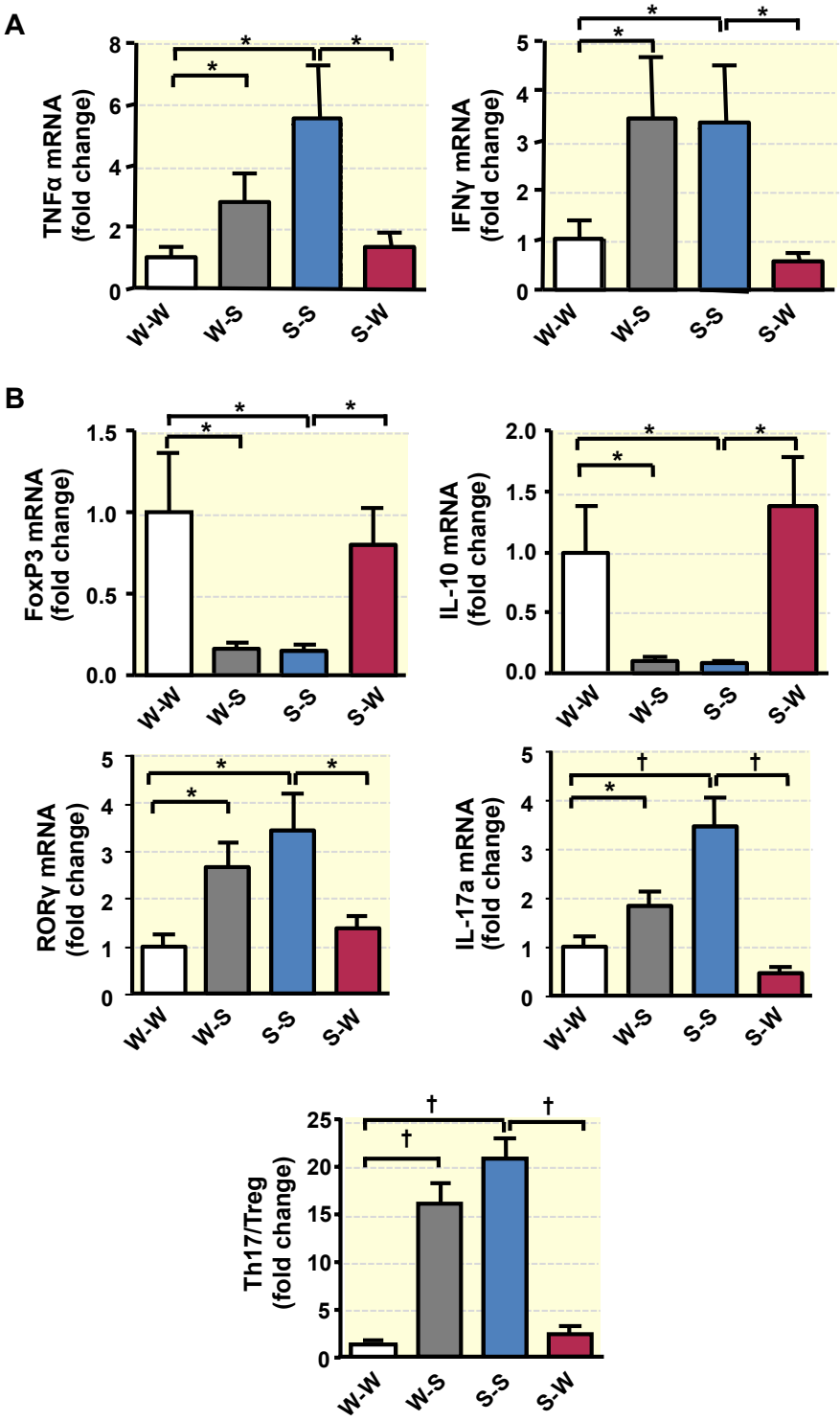


Figure 4. Effects of fecal microbiota transplantation (FMT) on pro-inflammatory markers and T-cells infiltration in aorta. *(A) mRNA levels of pro-inflammatory cytokines TNF-α and INFγ of aorta from all experimental groups. (**B**) T-cells infiltration in aortae from all experimental groups measured by mRNA levels of FoxP3, IL-10, RORγ and IL-17a, and ratio Th17/Treg. Values are means ± SEM (n = 5-8). Results were compared by 1-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

Fecal microbiota transplantation induced changes in mesenteric lymph node cells population

Under impaired gut mucosal integrity states, such as hypertension (Kim et al., 2018), bacteria can translocate through the intestinal barrier causing the activation and migration of CX3CR1+ cells, such as dendritic cells or macrophages, to drain lower intestinal tract lymph nodes (Niess et al., 2005). They also present antigens to naïve CD4+ T lymphocytes, lending to T cell activation. We found increased levels of CX3CR1 in MLNs in rats gavaged with SHR microbiota (W-S, S-S) as compared to rats transplanted with WKY feces (W-W, S-W) (Figure 5A). Consequent to activation, MLNs T lymphocytes up-regulate integrin α4β7 and chemokine receptor CCR9 (Mora et al., 2008). We observed that both Itga4 (Figure 5B), and CCR9 (Figure 5C**)** expression were higher in MLNs from S-S and W-S groups as compared to rats transfected with WKY microbiota (W-W and S-W) suggesting higher activation of T cells in rat with transplanted with SHR feces. No differences in Itgb7 gene expression were observed among groups (Figure 5B). Dendritic cells from hypertensive mice have increased surface expression of the B7 ligands CD80 and CD86, suggestive of dendritic cells maturation and activation (Vinh et al., 2010). In MLNs from rats transplanted with SHR microbiota higher CD80 and CD86 mRNA levels were found than that detected in rats gavaged with WKY feces (Figure 5D). In addition to antigen presentation, and important role of dendritic cells is the release of mediators that promote T cell polarization. It is well known that IL-6 drives the proliferation of Th17 cells and suppression of Treg cells (Kimura et al., 2010). We measured both IL-6 transcript levels in MLNs (Figure 5E) and the IL-6 levels in conditioned media from MLNs (Figure 5F) and observed that both were significantly augmented in MLNs from groups with FMT from SHR donors when compared to those found in groups transfected with WKY microbiota. In addition, no significant changes among groups in total T cells were observed (Figure 6A). However, FMT from SHR to both WKY and SHR reduced the Treg ($CD4^+/FoxP3^+$) cells percentages (Figure 6B) and increased the Th17 ($CD4^+/IL\text{-}17^+$) cells percentage as compared to rats transplanted with WKY microbiota (W-W, S-W groups) (Figure 6C).

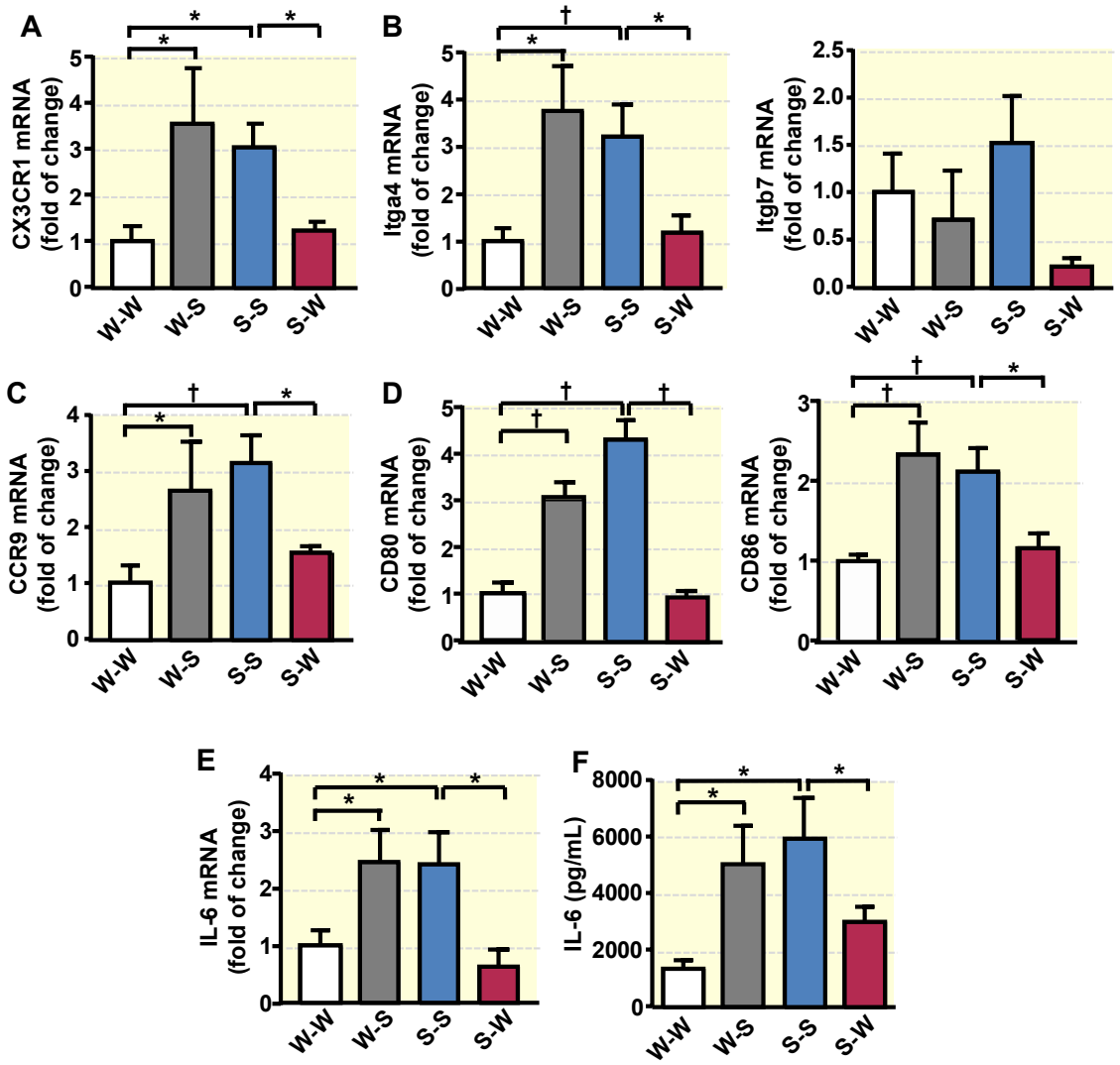


Figure 5. Effects of fecal microbiota transplantation (FMT) on T-cell activation in mesenteric lymph nodes (MLNs). *mRNA levels of CX3CR1 (**A**), α4β7 (Itga4 and Itgb7) (**B**), CCR9 (**C**), B7-lignads (CD80 and CD86) (**D**), and interleukin (IL)-6 (**E**) in MLNs from all experimental groups. IL-6 content of lymphocyte conditioned media from MLNs measured by ELISA (**F**). Values are means ± SEM (n = 5-8). Results were compared by one-way ANOVA and Tukey post hoc test. Kruskal-Wallis with Dunn's multiple comparison test was used in panel B. *P < 0.05 and †P < 0.01 W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

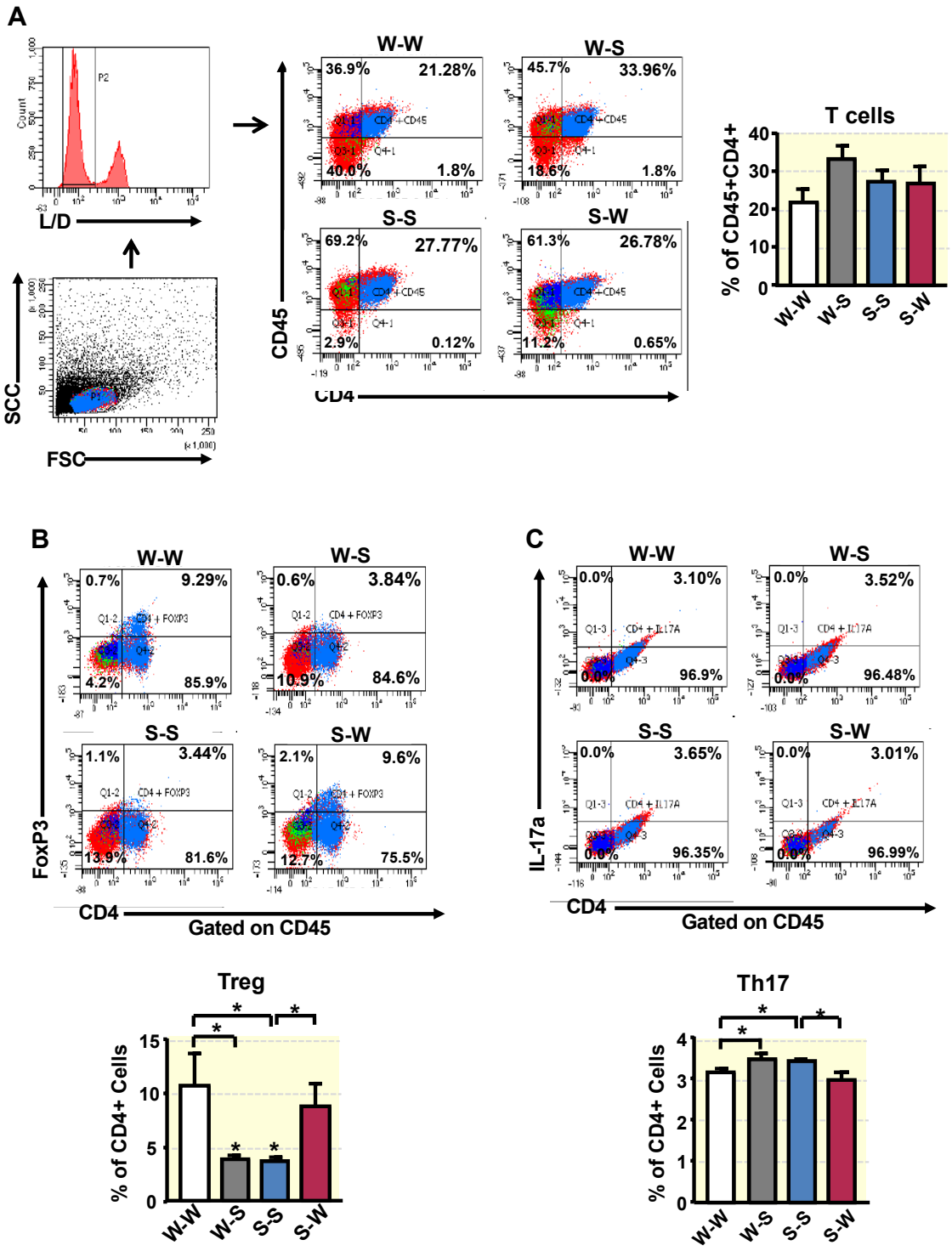


Figure 6. Effects of fecal microbiota transplantation (FMT) on T-cell polarization in mesenteric lymph nodes. *Total T lymphocytes, regulatory T cells (Treg) and Th17 measured in mesenteric lymph nodes from all experimental groups by flow cytometry. Values are means ± SEM (n = 5-8). Results were compared by 1-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, S-S vs W-W, and S-W vs S-S.*

Role of T-cell activation in hypertension- and endothelial dysfunction- induced by fecal microbiota transplantation from SHR to WKY

T-cell costimulation via B7 ligands is essential for the development of experimental hypertension (Vinh et al., 2010). To determine whether T-cell activation in response to stool transplantation from SHR to WKY is involved in hypertension and vascular dysfunction we blockaded B7-dependent costimulation with CTLA4-Ig. This agent abolished both the raise in SBP (Figure 7A), and renal and cardiac hypertrophy (Figure S3) found in W-S group when compared to W-W group. However, CTLA4-Ig did not reduce CX3CR1 mRNA level in MLNs, but suppressed both the increase in T-cell activation markers Itga4 and CCR9 mRNA levels, and T cells polarization, restoring the levels of Treg and Th17 markers (Figure 7B). No significant changes in IL-6 mRNA in MLNs were induced by CTLA4-Ig treatment in W-S group (Figure 7B). The percentages of circulating T cells expressing $CD44_{high}$ (Figure S4), which mimic the phenotype of activated effector T cells, were also increased by stool transplantation from SHR. These responses were eliminated by cotreatment with CTLA4-Ig (Figure S4). In addition, the presence of both total T cells and CD8+ T cells in aortic homogenates were increased in W-S group as compared to W-W group, and normalized after CTLA4-Ig treatment (Figure 8A). No significant changes among groups were observed in aortic CD4+ T cells (not shown). However, CTLA4-Ig reduced the higher aortic mRNA transcript levels of RORγ and IL-17, whereas increased significantly FoxP3 and IL-10 mRNA levels (Figure S5A). The increased ratio Th17/Treg found in W-S group, as compared to W-W, was normalized by CTLA4-Ig treatment (Figure S5A). Moreover, the impaired endothelium-dependent relaxation to acetylcholine found in aorta from W-S group as compared to W-W was also normalized by inhibition of the B7/CD28 T-cell costimulation (Figure 8B), similarly to the *in vitro* incubation with the selective NADPH oxidase inhibitor VAS2870 (Figure 7B). In fact, FMT transplantation from SHR to WKY increased aortic NADPH oxidase activity, which was abolished by CTLA4-Ig (Figure 7C). When aortic rings with perivascular adipose tissue and adventitia were incubated for 6 h in the presence of nIL-17, no significant differences among all experimental groups were found in acetylcholine induced relaxation (Figure S5B) and NADPH oxidase activity (Figure S5C), showing the key role of IL-17 in NADPH oxidase-mediated endothelial dysfunction induced by SHR donors microbiota.

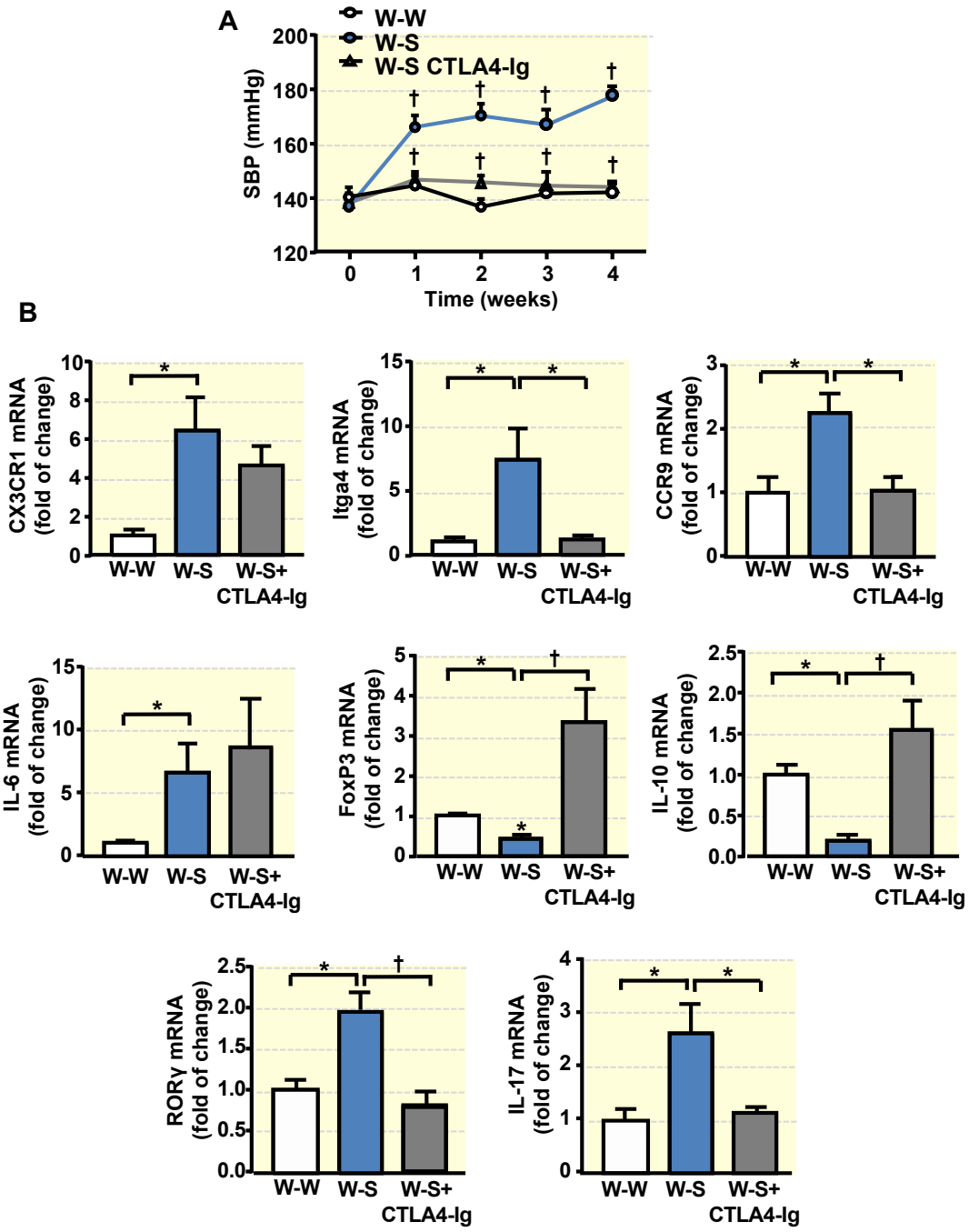


Figure 7. Role of T-cell activation in hypertension induced by fecal microbiota transplantation from SHR to WKY. *Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in WKY with stool transplant from WKY (W-W) or from SHR (W-S) treated with the fusion protein CTLA4-Ig (250 µg/rat) or saline. The nested design was used to compare the evolution of tail SBP with time, with the rat as random factor and days and treatment as fixed factors. When the overall difference was significant comparisons were made using Bonferroni´s method with an appropriate error. (**B**) mRNA levels of CX3CR1, Itga4, CCR9, interleukin (IL)-6, and FoxP3, IL-10, as markers of Treg, and RORγ and IL-17a, as markers of Th17, in MLNs from all experimental groups. Values are means ± SEM (n = 9-10). Results were compared by one-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, and W-S+CTLA4-Ig vs W-S.*

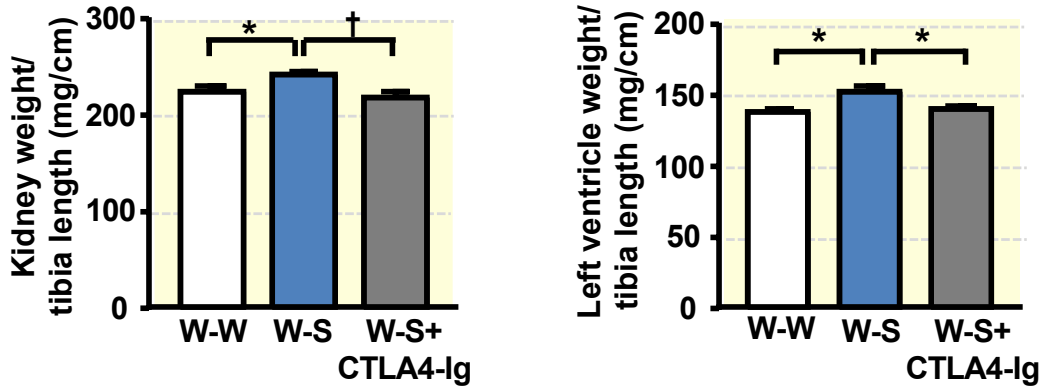


Figure S3. Effect of the blockade of B7-dependent costimulation with CTLA4-Ig in morphological changes induced by fecal microbiota transplantation (FMT) from SHR to WKY. *Kidney and left ventricle hypertrophy in WKY with stool transplant from WKY (W-W) or from SHR (W-S) treated with the fusion protein CTLA4-Ig (250 µg/rat) or saline Values are means ± SEM (n = 9-10). Results were compared by 1-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, and W-S+CTLA4-Ig vs W-S.*

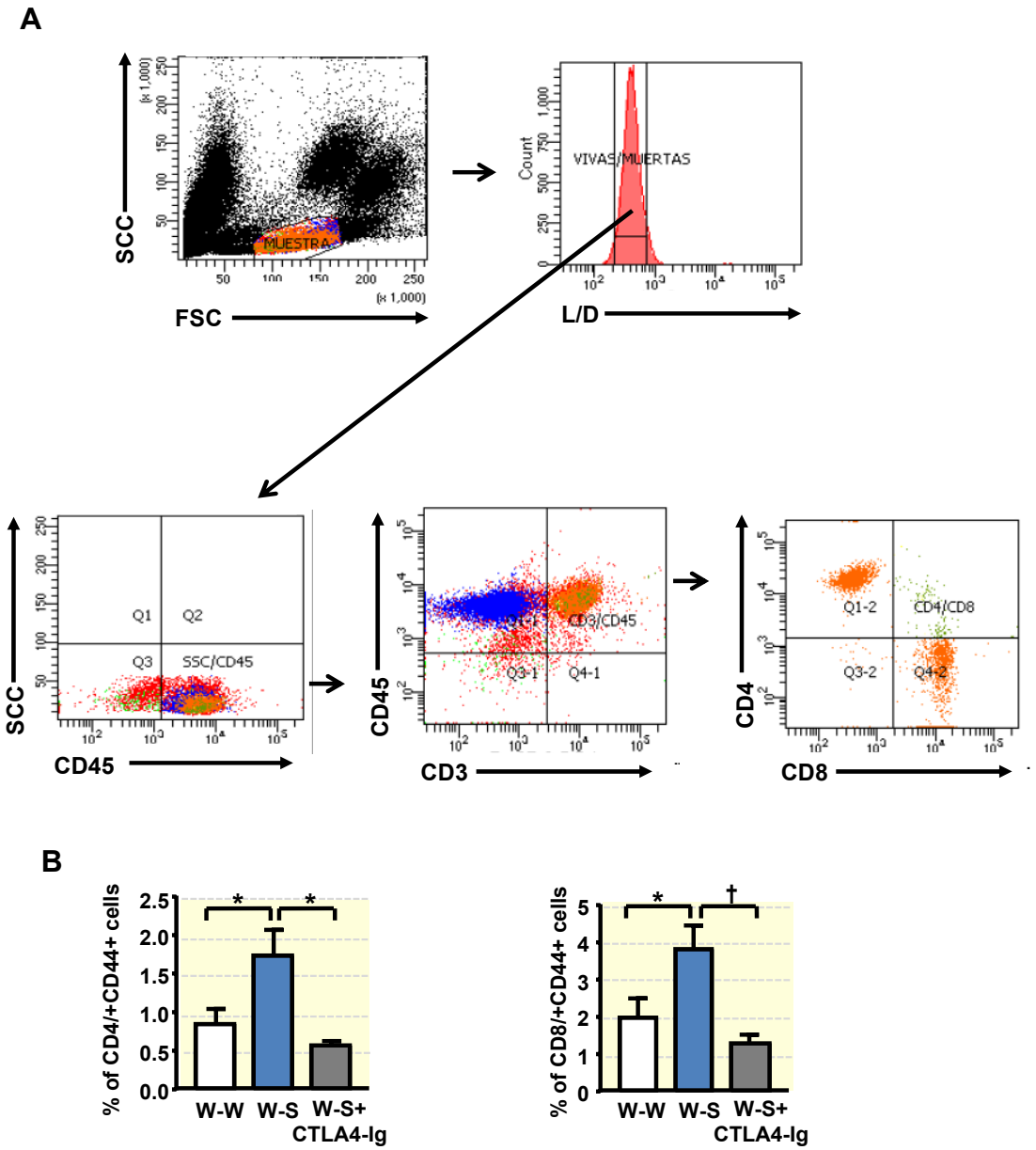


Figure S4. Effect of the blockade of B7-dependent costimulation with CTLA4-Ig in activated circulating T cells induced by fecal microbiota transplantation (FMT) from SHR to WKY. *(**A**) Flow cytometry gating strategy, and (**B**) percentage of circulating CD4+/CD44high and CD8+/CD44high cells in blood from WKY with stool transplant from WKY (W-W) or from SHR (W-S) treated with the fusion protein CTLA4-Ig (250 µg/rat) or saline. Values are means ± SEM (n = 9-10). Results were compared by 1-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, and W-S+CTLA4-Ig vs W-S.*

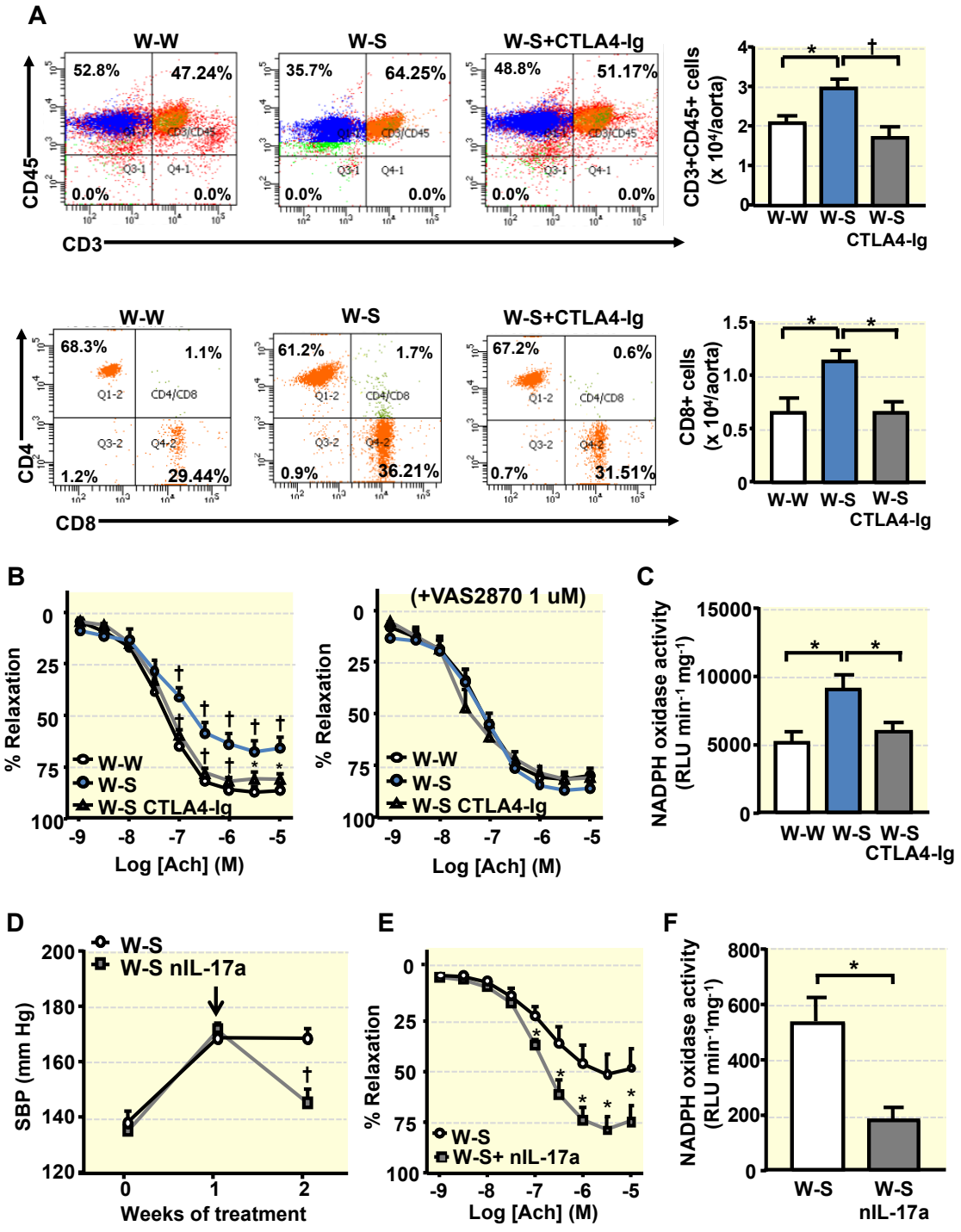


Figure 8. Role of both T-cell activation and IL-17a in endothelial dysfunction induced by fecal microbiota transplantation from SHR to WKY. *(**A**) Representative flow cytometry of aorta from WKY with stool transplantation from WKY (W-W) or SHR (W-S) treated with the fusion protein CTLA4-Ig (250 µg/rat) or saline. Live singlet cells were gated for total leukocytes (CD45+), total T cells (CD3+), CD4+ and CD8+ T cells, and mean data ± SEM are shown. Results were compared by one-way ANOVA and Tukey post hoc test. (**B**) Endothelium-dependent relaxation induced by acetylcholine (ACh) in the absence or in the presence of the selective NADPH oxidase inhibitor VAS2870 (1 µM) in aortae pre-contracted by Phe and (**C**) NADPH oxidase activity measured using a*

*lucigenin-enhanced chemiluminescence protocol in aorta from W-W, W-S and W-S CTLA4Ig groups. Values are means ± SEM (n = 9-10). In order to compare the concentration-response curves to acetylcholine, we carried out an analysis of the nested design and one-way ANOVA and Tukey post hoc test in panel C. (**D**) Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in WKY with stool transplant from SHR (W-S) treated with IL-17–neutralizing antibody (10 µg/rat) or the isotype control (arrow, start of IL-17a administration). (**E**) Endothelium-dependent relaxation induced by acetylcholine (ACh) in aortae pre-contracted by phenylephrine and (**F**) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta from W-S and W-S+IL-17a groups. Values are means ± SEM (n = 5). The nested design was used to compare the evolution of tail SBP with time, with the rat as random factor and days and treatment as fixed factors. When the overall difference was significant comparisons were made using Bonferroni´s method with an appropriate error (panel D). Analysis of the nested design was also carried out with groups and concentrations to compare the concentration-response curves to acetylcholine (panel E). Results were compared by Student t test (panel F). *P < 0.05 and †P < 0.01 W-S vs W-W, W-S+CTLA4-Ig vs W-S, and W-S+nIL17a vs W-S.*

Involvement of IL-17 in hypertension and vascular dysfunction induced by fecal microbiota transplantation from SHR to WKY

To further analyse the participation of IL-17 in the hypertensive effects of SHR microbiota, we administered nIL-17a to hypertensive rats from W-S group. Treatment of these rats with nIL-17 significantly reduced SBP (Figure 8D) as well as improved both aortic endothelium-dependent relaxation to acetylcholine (Figure 8E) and the activity of NADPH oxidase (Figure 8F).

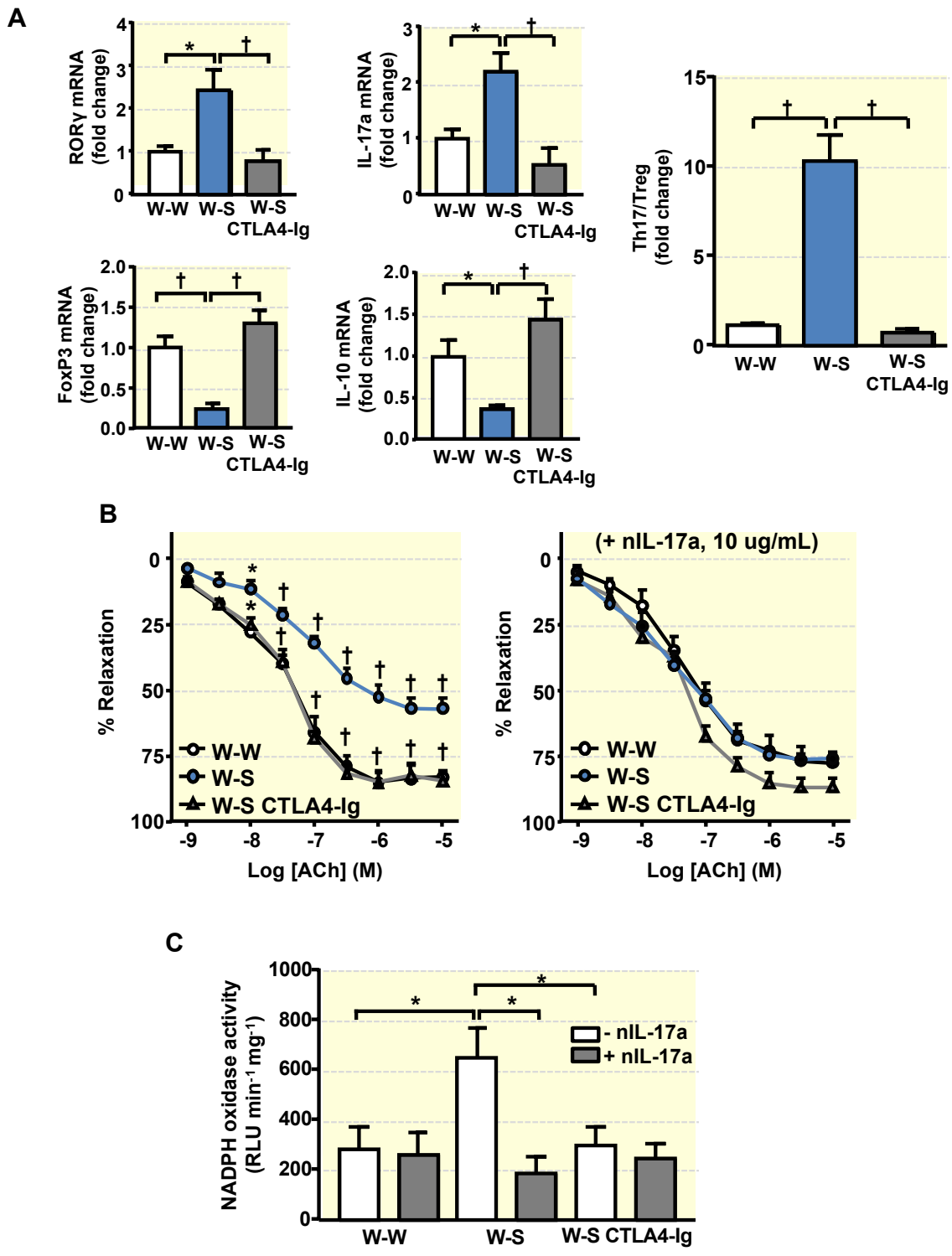


Figure S5. Role of IL-17 in the protective effect of the blockade of B7-dependent costimulation with CTLA4-Ig in endothelial dysfunction induced by fecal microbiota transplantation (FMT) from SHR to WKY. *(A) T-cells infiltration in aortae from all experimental groups measured by mRNA levels of FoxP3, IL-10, RORγ and IL-17a, and ratio Th17/Treg. One-way ANOVA and Tukey post hoc test for RORγ and IL-10, and Kruskal-Wallis with Dunn's multiple comparisons test for IL-17s and FoxP3 (**B**) Endothelium-dependent relaxation induced by acetylcholine (ACh) in aorta with perivascular tissue incubated during 6 h the absence or in the presence of the anti IL-17a*

*(10 μg/mL) in aortae pre-contracted by Phe, and (**C**) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta from WKY with stool transplant from WKY (W-W) or from SHR (W-S) treated with the fusion protein CTLA4-Ig (250 μg/rat) or saline. Values are means ± SEM (n = 9-10). Analysis of the nested design was carried out with groups and concentrations to compare the concentration-response curves to ACh. Results were compared by 1-way ANOVA and Tukey post hoc test. *P < 0.05 and †P < 0.01 W-S vs W-W, and W-S+CTLA4-Ig vs W-S.*

DISCUSSION

The present study demonstrated that alterations in the composition of gut microbiota by FMT from WKY to SHR and viceversa, impacts the gut immune system, thus impacting in vascular tone and BP. The major findings are as follows: (1) Gut dysbiotic microbiome, characterized by increased *Turicibacter* and reduced *S24-7_g*, is an important pathogenic factor for the high BP of the host, independently of genetic charge of the rat strain; (2) changes in gut microbial composition are associated to altered Th17/Treg balance in MLNs, which is likely associated to vascular oxidative stress and endothelial dysfunction. (3) Increased BP induced by dysbiotic microbiota from SHR is a consequence of T cell activation that requires costimulation via B7 ligands (4) The axis Th17/IL17 is essential for the development of endothelial dysfunction and hypertension induced by stool transplantation from SHR to WKY. Our study demonstrated by the first time the essential role of both T cell activation through B7-dependent costimulation and IL-17 in endothelial dysfunction and hypertension induced by dysbiotic microbiota. Our findings confirm previous evidences in genetic hypertensive rats (Toral et al., 2018) and humans (Li et al., 2017), and in hypertension induced by obstructive sleep apnea (Durgan et al., 2016), and in tacrolimus-induced hypertension (Toral et al., 2018), indicating that gut dysbiosis associated to hypertension can increase BP in normotensive animals. Interestingly, chronic microbiota transplant from normotensive to hypertensive rats reduced BP, endothelial dysfunction and target organ hypertrophy. In agreement with this protective effect of normal microbiota some evidence already exists supporting a beneficial role for *Lactobacillus* probiotics in BP regulation in SHR (Gomez-Guzman et al., 2015). An increase in the F/B ratio is considered a hallmark of gut dysbiosis in SHR as compared to WKY (Yang et al., 2015). We observe an increased F/B ratio in W-S as compared to W-W, whereas in S-W was reduced as compared to S-S. However, this F/B ratio was unchanged in DOCA-salt hypertensive mice (Marques et al., 2017) , showing

that the increased F/B is not essential to high BP. However, we found a strong positive correlation between SBP and the lactate-producing genus *Turicibacter*. We did not measure plasma lactate in our experiment, but plasma lactate levels have been shown to be associated with an increase in BP (Juraschek et al., 2015). *Turicibacter* is also linked to the development of colitis in IL-10-deficient mice (Ye et al., 2008) and may have immunomodulatory or host invasion-related traits, such as internalin production (Cuív et al., 2011). However, we did not monocolonize germ-free rats with this bacterium to test if this can evoke a hypertensive phenotype, limiting the pathological value of our finding. By contrast, a negative correlation between SBP and the abundance of genus S24-7_g has been found. The unclassified genus belonging to the S24–7 family, the unidentified taxon from order *Bacteroidales*, was found more abundant in non-diabetic mice also correlated positively with delayed diabetes onset age, and splenic $FoxP3^{+}CD4^{+}$ Treg cells (Krych Ł et al., 2015). Changes in BP are most likely mediated by the metabolome rather than by the bacteria. However, the effects of bacterial-derived products, such as short-chain fatty acids or tryptophan metabolites or LPS, might act in T-cells from MLNs, in the vasculature, heart, kidney, or in central nervous system. Their effects in tissues are also dependent on the level of receptor expression (Gpr109a, Olfr50, Gpr41, Gpr43, TLR4, aryl hydrocarbon receptor). So, it is very difficult to analyze the role of the metabolome in BP, without adding more speculation, and experiments addressed to this specific aim are necessary. Overall, our present study demonstrates that hypertension is, at least partially, the result of pathophysiological variances in gut microbiota, which possibly modulates immune system. However, the mechanisms through which gut microbiota induces pro-hypertensive effects in SHR are still unknown. Endothelial dysfunction is a hallmark underlying vascular disease in hypertension. Conforming to this information, our study shows an impaired endothelium-dependent relaxation to acetylcholine in aorta from hypertensive S-S rats as compared to normotensive W-W rats. FMT from WKY to SHR reduced BP and improved acetylcholine relaxation, whereas FMT from SHR to WKY increased BP and impaired endothelial function. Similarly, in aorta from germ-free mice the relaxant response to acetylcholine was unaltered after angiotensin II-infusion (Karbach et al., 2018), showing the influence of microbiota in the regulation of endothelial function. In addition, *Lactobacillus* strains supplementation reduced BP and improved relaxation to acetylcholine in SHR (Gomez-Guzman et al., 2015), and in tacrolimus-induced hypertension (Toral et al., 2018).NO is the main factor responsible for endothelium-dependent relaxation as denoted by the almost full inhibitory effect of L-

NAME in the aorta. Nevertheless, the relaxant response to nitroprusside, an activator of the soluble guanylyl cyclase, in endothelium-denuded vessels was unchanged in all groups, pointing out that alterations in the cyclic GMP pathway do not contribute to changes in endothelial function after FMT. A fundamental mechanism of endothelial dysfunction includes the vascular synthesis of ROS, and specifically O_2^-, which reacts with NO and inactivates it (Tschudi et al., 1996). In fact, we found increased ROS levels in aortas from W-S and S-S groups linked to impaired relaxation to acetylcholine. The NADPH oxidase is traditionally seen as the main source of O_2^- in the vascular wall (Montezano et al., 2015). We found a marked raise in both the NADPH oxidase activity and the expression of the its subunits NOX-1, NOX-4, $p22_{phox}$ and $p47_{phox}$ in aortas from W-S and S-S groups as compared to W-W, associated to decreased endothelium-dependent relaxation. FMT from WKY to SHR also reduced aortic ROS content, NADPH oxidase activity and NADPH oxidase subunits expression. The non-specific NADPH oxidase inhibitor apocynin abolished the differences among all groups indicating the key role of NADPH oxidase activity in the effects of FMT in endothelial function. The enhancement of the bioavailability of ROS, particularly O_2^-, impacts endothelial function not only by reducing NO bioavailability, but also by promoting inflammation (Crowley et al., 2014). Our data are consistent with this oxidative-pro-inflammatory vascular status, since the gene expression of the pro-inflammatory cytokines TNF-α and IFNγ were higher in aortas from W-S and S-S as compared to W-W and S-W groups. The absence of gut microbiota protects mice from angiotensin II-induced hypertension and vascular dysfunction, at least in part, by inhibition of accumulation of inflammatory cells in the vasculature (Karbach et al., 2016). It is becoming more and more evident that immune cells can affect vascular reactivity and BP regulation (Ryan et al., 2013). We found increased Th17 cells, measured by mRNA levels of RORγ, in aortas with impaired relaxation to acetylcholine from S-S group as compared to W-W group. By contrast, in agreement with Katsuki et al., 2015 Treg accumulation, measured by mRNA levels of FoxP3, was decreased in S-S groups as compared to W-W. Improvement of endothelial function by FMT from WKY to SHR reduced Th17 accumulation and increased Treg in the vasculature, reducing the ratio of pro-inflammatory/anti-inflammatory cells. Our data indicate that increasing vascular inflammatory cells by transplantation with dysbiotic microbiota could contribute to endothelial dysfunction. Various hypertensive stimuli cause T-cell activation and infiltration into target organs such as the vessel and the kidney, which promotes vascular dysfunction and BP elevation. In our study, FMT from

SHR to WKY increased T-cell activation in MLNs, as a result of CX3CR1-expressing cells accumulation, that are mainly antigen presenting cells (APC) (Medina-Contreras et al., 2011;Diehl et al., 2013). These cells can phagocytize bacteria from the gut lumen, translocating them to MLNs, where they present antigens and trigger $CD4^+$ T cell activation, and produce IL-6 that translates into a suppression of Treg, and an increase in Th17 cells. Classically, T-cell activation requires T-cell receptor ligation and costimulation. The latter often involves interaction between B7 ligands (CD80 and CD86) on antigen-presenting cells with the T-cell coreceptor CD28. In the present study, in MLNs FMT from SHR to WKY or SHR increased mRNA levels of costimulatory proteins CD80 and CD86 as compared to rats transplanted with WKY feces. Interestingly, blockade of B7-dependent costimulation with CTLA4-Ig abolished T cells activation and polarization to Th17 in MLNs and the development of hypertension found in W-S rats. Activation of circulating T cells and vascular T-cell accumulation caused by stool transplantation from SHR to WKY were also abrogated by CTLA4-Ig. This protective vascular effect was associated to improvement of endothelium-dependent relaxation to acetylcholine, as a consequence of NADPH oxidase inhibition related to reduced accumulation of IL-17a producing cells in the aorta. Furthermore, administration of IL-17a reduced BP and restored endothelial function in W-S animals, showing the involvement of Th17-IL-17 axis as pro-hypertensive pathway. In overall, the present study therefore provides unequivocal evidence that activation of the adaptive immune response through T-cell costimulation is involved in the pro-hypertensive effects of dysbiotic microbiota. However, other contributing factors (beyond the microbiota) seem to be involved in the development of hypertension in SHR, since FMT transplantation from SHR to WKY increased BP, but not to the level found in SHR, and FMT from WKY to SHR did not normalize BP. Our study demonstrates that microbiota is a crucial factor involved in the control of BP, as consequence of its effect in T-cell activation in gut immune system and vascular T-cells accumulation. Dysbiotic microbiota, characterized by increased *Turicibacter* and reduced *S24-7_g*, strongly correlated to SBP in this genetic model of hypertension. This gut microbiota, generated after establishment of hypertension in SHR, is a pro-hypertensive factor, because induces T-cell activation and polarization in a key immune inductive site, the MLNs, lending to increased Th17 and reduced Treg, increased circulating T cells and vascular T-cell accumulation. In addition, the impaired endothelial function induced by this dysbiotic microbiota was mediated by increased IL-17 production in the vascular tissue. These results confirm

previous studies that suggested a strong link between hypertension and gut microbial dysbiosis, establishing a cause-effect relationship. However, the changes described in our experiment in gut microbiota composition were different to that found in hypertensive patients (Li et al., 2017), which limits their extrapolation to human hypertension. Thus, modifications of gut microbiota may be a novel strategy for hypertension treatment.

MATERIALS AND METHODS

Animals and experimental groups

This research conforms with the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985), with the Good Publishing Practice in Physiology (Persson et al., 2017), and was sanctioned by the Ethics Committee of Laboratory Animals from the University of Granada (Spain) (Ref. 03-CEEA-OH-2013). Male WKY and SHR were purchased from Harlan Laboratories (Barcelona, Spain). Fecal contents were collected fresh from individual rats and pooled from twenty-week-old SHR and WKY rats (n = 8). Twenty-one-week-old recipient WKY and SHR were orally gavaged with donor fecal contents for 3 days, and once every 3 days for a total of 4 weeks. Animals were randomly assigned to four different groups of 5-8 animals each: WKY with WKY microbiota (W-W), SHR with SHR (S-S), WKY with SHR (W-S), and SHR with WKY (S-W). Each rat was housed in a separate cage. The animals were kept in a specific pathogen-free facility and were provided with standard laboratory diet (SAFE A04, Augy, France) and water *ad libitum*. Water was changed daily, and both food and water consumption were documented every day for all groups. We recorded body weight weekly. In additional experiments, we used a design to determine whether blockade of B7-dependent costimulation with CTLA4-Ig could prevent hypertension caused by FMT of SHR to WKY. Recipient WKY rats were assigned to 3 groups: WKY with WKY microbiota (W-W), WKY with SHR microbiota (W-S), and WKY with SHR microbiota plus CTLA4-Ig (W-S CTLA4-Ig). The fusion protein CTLA4-Ig (250 μg/rat), which inhibits T-cell costimulation, was administered intraperitoneally every 3 days for a total of 4 weeks. As a control, saline was administered to W-S rats not receiving CTLA4-Ig. In a separate experiment, we sought to determine whether IL-17 plays a role in hypertension induced by microbiota from SHR. Recipient WKY rats were assigned to 2 groups: WKY

with SHR microbiota (W-S), and WKY with SHR microbiota plus IL-17-neutralizing antibody (W-S nIL-17). IP injections of either IL-17–neutralizing antibody (10 μg/rat; R&D Systems) or the isotype control (10 μg/rat; R&D Systems) were given on days 7, 11, and 14 after FMT.

Fecal microbiota transplantation

We performed the transplant to recipient rats as already described with some modifications (Toral et al., 2018). Briefly, we collected and pooled fecal contents from WKY and SHR rats. Samples were diluted 1:20 in sterile PBS and centrifuged at 800 rpm for 5 minutes. We aliquoted and stored at -80ºC the resulting supernatant. One week previous to the FMT; twenty-week-old recipient rats were orally gavaged with 1 mL sodium ceftriaxone (400 mg/Kg/day) for 5 consecutive days every day, in order to reduce the preexisting microbiota and to facilitate the recovery of gut microbiota populations and diversity from donor rats after FMT (Li et al., 2017). 48 hours after the ceftriaxone treatment, recipient rats were administered with donor fecal preparations (1 mL) as already explained.

Blood pressure measurements

HR and SBP were recorded every week at room temperature in conscious animals by tail-cuff plethysmography as previously described (Zarzuelo et al., 2011). At the end of the experiment, isofluorane was used to anesthesize the animals, in order to monitor intra-arterial BP, a polyethylene catheter with 100U heparin in an isotonic, sterile salt solution was inserted in the left carotid artery in conscious, unrestrained conditions. Direct BP was recorded continuously (MacLab; AD Instruments, Hastings, UK). MABP values obtained during the last 30 min were averaged for intergroup comparisons (Duarte et al., 2011).

Morphological variables

The heart and kidneys were excised, cleaned and weighted. The heart and kidney weight indices were obtained by dividing the heart and kidney weight by the length of the tibia. We froze all tissue samples in liquid nitrogen and then stored them at -80ºC.

Vascular reactivity studies

Thoracic aortic rings were mounted in organ chambers filled with Krebs buffer (composition in mM: KCl 4.75, NaCl 118, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2, and glucose 11) at 37ºC and bubbled with 95% O_2 and 5% CO_2 and adjusted at a basal tension of 2 g. An isometric force-displacement transducer (Letigraph 2000) connected to an acquisition system was used to record isometric tension, as previously described (Gomez-Guzman et al., 2015). The cumulative concentration-relaxation response curves to acetylcholine were performed in rings pre-contracted by 1 μM phenylephrine in the absence or in the presence of eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), or NADPH oxidase inhibitor apocynin (10 μM). The concentration-relaxation response curves to nitroprusside were carried out in the dark in rings without a functional endothelium pre-contracted with 1 μM phenylephrine. In order to study the role of IL-17, we incubated the aortic rings from different experimental groups during 6 hours with anti-IL-17a receptor (10 μg/mL). After incubation, we studied relaxant responses to acetylcholine and NADPH oxidase activity.

NADPH oxidase activity

To determine NADPH oxidase activity in intact aortic rings, we performed a lucigenin-enhanced chemiluminescence assay, as described previously (Gomez-Guzman et al., 2015). Aortic rings from all groups were incubated for 30 minutes at 37 °C in a buffer solution (NaCl 119mM, HEPES 20mM, KCl 4.6mM, $MgSO_4$ 1mM, Na_2HPO_4 0.15mM, KH_2PO_4 0.4mM, $NaHCO_3$ 1mM, $CaCl_2$ 1.2mM and glucose 5.5mM, pH 7.4). The production of O_2^- in aorta was stimulated by addition of NADPH (100 μM). Aortic segments were then put into tubes with the physiological salt solution, in the presence or absence of NADPH, and lucigenin (5 μM) was injected automatically. NADPH oxidase activity was determined by measuring luminescence every 2 s for 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) and was calculated by subtracting the basal

values from those in the presence of NADPH. The aortic rings were then dried for the measurement of dry weight tissue. NADPH oxidase activity is expressed as relative luminescence units (RLU) per min per mg dry aortic tissue.

In situ detection of vascular reactive oxygen species content

Unfixed thoracic aortic rings were cryopreserved (0.1 M PBS plus 30% sucrose for 1-2h), included in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), frozen (-80ºC), and 10 μm cross-sections were obtained in a cryostat (Microm International Model HM500 OM). Aortic sections were incubated for 30 min in HEPES buffer solution, containing DHE (10 μM), counterstained with the nuclear stain 4,6-diamidino-2-phenylindoledichlorohydrate (DAPI, 300 nM) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were visualized and photographed with a fluorescence microscope (Leica DM IRB, Wetzlar, Germany) and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS production was calculated from the ratio of ethidium/DAPI fluorescence (Zarzuelo et al., 2011).

Flow Cytometry

MLN were collected from rats. The tissues were mashed with wet slides to decrease friction and then the solutions were filtered through a 70μM cell strainer. $1\times10_6$ cells were counted and incubated with a protein transport inhibitor (BD GolgiPlug™) for an optimum detection of intracellular cytokines by flow cytometry. For intracellular staining, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate plus 1 μg/mL ionomycin. After 4.5 hours, aliquot cells, of each sample, were blocked with anti-CD32 (clone D34-485) for 30 minutes at 4°C to avoid non-specific binding to mouse Fc-gamma receptors. After that, cells were transferred to polystyrene tubes for the surface staining with mAbs anti-CD4 (PerCP-Vio700, clone REA482**,** Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD45 (APC, clone RA3-6B2 BD Pharmigen™, New Jersey, USA), and viability dye (LIVE/DIED® Fixable Aqua Dead cell Sain Kit, Molecular Probes, Oregon, USA) 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-FoxP3 (PE,clone FJK-16s, eBioscience, San Diego, USA), and anti-IL-17A (PE-Cy7, clone eBio17B7, eBioscience, San Diego, USA) for 30 min at 4°C in the dark. We study T-cell activation phenotypes, for it, total leukocytes were isolated from heparinized blood and red cells were lysed osmotically. The remaining leukocytes were centrifuged (800 x g), washed twice with PBS and containing 1 % BSA. Then, the cells were counted and one million cells re-suspended in 1 % BSA PBS and stored on ice less than 30 minutes. After, aliquot cells, of each sample, were blocked with anti-CD32 (clone D34-485) for 30 minutes at 4°C. After that, cells were transferred to polystyrene tubes for the surface staining with CD45-VioBright FICT (clone REA504, Miltenyi Biotec, Bergisch Gladbach, Germany); CD3-PerCPVio700 (clone REA223, Miltenyi Biotec, Bergisch Gladbach, Germany); CD4-APC (clone REA489, Miltenyi Biotec, Bergisch Gladbach, Germany); CD8-PE (clone REA437, Miltenyi Biotec, Bergisch Gladbach, Germany); CD44-PE-Vio770 (clone REA505, Miltenyi Biotec, Bergisch Gladbach, Germany), and viability dye for 20 min at 4°C in the dark. The lymphocytes were then fixed using 2% PFA. Cells were washed and suspended with PBS. On the other hand, we also analyzed vascular T-cell infiltration in aorta. Following dissection, aortae were digested using collagenase type XI (125 U/mL), collagenase type I-S (450 U/mL), hyaluronidase I-S (60 U/mL) and DNase I (60 U/mL) dissolved in heparinized PBS (20U/mL), for 60 min at 37 °C (modified from Galkina et al., 2006). The tissue was mashed with wet slides to decrease friction and then the solutions were filtered through a 70 μm cell strainer. Cells from aortae were isolated followed by the 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. All samples were analyzed using a flow cytometer CANTO II (BD Biosciences) with BD FACSDIVA™ software[10].

Reverse transcriptase-polymerase chain reaction analysis

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted from the aortae, and mesenteric lymph nodes by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was carried out with a Techne Techgene thermocycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to measure gene expression. The sequences of the sense and antisense primers used for amplification are shown in Table 1. Preliminary experiments

were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study. In order to determine the efficiency of the reaction, we used a standard tissue sample. Quantification was performed by the ΔΔCt method. For normalization, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Zarzuelo et al., 2011) as the housekeeping gene.

16S rDNA V4-V5 region sequencing

We extracted fecal DNA from samples from all experimental groups at -1, 0, and 4 weeks after FMT with the quick-DNA fecal/soil microbe kit (Zymoresearch, Irvine, CA). To amplify bacterial 16S V4-V5 variable regions (Yang et al., 2015), we used primers compatible with illumian Miseq v2 2x250bp kit (Illumina, San Diego, CA). We purified the PCR amplicons by QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and we quantified with Qubit (thermos Fisher Scientific, Waltham, MA). Equal amount of purified PCR product from each sample was pooled together as one library. Real Time PCR was used to quantify the library was quantified (Kapa Biosystems, Wilmington, MA) prior to Miseq sequencing (Illumina, San Diego, CA). The sequencing data obtained a Q30 score $\geq$ 93.5% and 97.17 $\pm$ 0.34% of total cluster passed the filter.

Bioinformatics analysis

We processed the raw paired-reads from Miseq with QIIME 1.9.1. Briefly, in order to remove bases, reads were trimmed using a Phred score lower than 30 and quality-filtered with parameters set as optimized previously (Bokulich et al., 2013). We performed open reference OTU-picking and taxonomical assignment to the generated OTUs with 97% identity versus Greengenes database 13.8. Also, we generated unweighted principal coordinate analyses and alpha diversity plots using the phylogenic tree-based unifrac distance metric with scripts from QIIME package.

Reagents

All reagents were purchased from Sigma-Aldrich (Barcelona, Spain), unless otherwise stated.

Statistical analysis

Results are expressed as means ± SEM of measurements. The nested design was used to compare the evolution of tail SBP in time, with treatment and days as fixed factors and the rats as random factor. Comparisons were made using Bonferroni´s method with an appropriate error when overall differences were significant. Analysis of the nested design was also performed with groups and concentrations to compare the concentration-response curves to acetylcholine. The remaining variables were tested on normal distribution using Shapiro-Wilk normality test and compared using an unpaired t test or 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. $P<0.05$ was considered statistically significant.

We calculated Pielou, Shannon, Chao 1 and observed species with QIIME (PAST 3x). Reads in each operational taxonomic unit (OTU) were normalized to total reads in each sample. Only taxa with reads percentage superior to 0.001% were used for the analysis. Partial Least Square (PLS) analysis was also applied to these data to identify significant differences among all groups.

Table 1. Oligonucleotides for real-time RT-PCR.

mRNA targets	Descriptions	Sense	Antisense
NOX-1	NOX-1 subunit of NADPH oxidase	TCTTGCTGGTTGACACTTGC	TATGGGAGTGGGA
NOX-4	NOX-4 subunit of NADPH oxidase	ACAGTCCTGGCTTACCTTCG	TTCTGGGATCCTC
p22phox	p22phox subunit of NADPH oxidase	GCGGTGTGGACAGAAGTACC	CTTGGGTTTAGGC
p47phox	p47phox subunit of NADPH oxidase	CCCAGCGACAGATTAGAAGC	TGGATTGTCCTTT
IL-6	interleukin-6	GATGGATGCTTCCAAACTGG	AGGAGAGCATTGG
CX3CR1	CX3C chemokine receptor 1	TGAGTGACTGGCACTTCCTG	CGAGGACCACCAA
CCR9	C-C chemokine receptor type 9	CGAGACATGCCCACCATCGCTTCTCCC	ATGCCAGGCCACA
Itgb7	Integrin beta-7	ACCTGAGCTACTCAATGAAGGA	CACCGTTTTGTCC
Itga4	Integrin alpha-4	GGAAGCCCCAGTGGAGAAC	ATTGTCACTCCCA
CD80	CD80	TCGTAGGTGAAACACCTGA	CCGGAAGCAAAG
CD86	CD86	TGGGCGCAGAGAAACTTGAT	AAGCCCGTGTCCT
TNF-α	Tumor necrosis factor-alpha	ACGATGCTCAGAAACACACG	CAGTCTGGGAAGC
IFNϒ	Interferon gamma	GCCCTCTCTGGCTGTTACTG	CCAAGAGGAGGCT
FoxP3	Forkhead box P3	AGGCACTTCTCCAGGACAGA	CTGGACACCCATT
IL-10	Interleukin-10	GAATTCCCTGGGAGAGAAGC	GCTCCACTGCCTT
RORϒ	ROR-gamma	GCCTACAATGCCAACAACCACACA	TGATGAGAACCAA
IL-17a	Interleukin-17a	CTTCACCTTGGACTCTGAGC	TGGCGGACAATAC
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	ACCACAGTCCATGCCATCAC	TCCACCACCCTGT

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ARTICLE 2

Critical role of the interaction gut microbiota - sympathetic nervous system in the regulation of blood pressure

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ABSTRACT

Association between gut dysbiosis and neurogenic diseases, such as hypertension, has been described. The aim of this study was to investigate whether changes in the gut microbiota alter gut-brain interactions inducing changes in blood pressure (BP). Recipient normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) were orally gavaged with donor fecal contents from SHR and WKY**.** We divided the animals into four groups: WKY transplanted with WKY microbiota (W-W), SHR with SHR (S-S), WKY with SHR (W-S) and SHR with WKY (S-W). Basal systolic BP (SBP) and diastolic BP (DBP) were reduced with no change in heart rate as a result of fecal microbiota transplantation (FMT) from WKY rats to SHR. Similarly, FMT from SHR to WKY increased basal SBP and DBP. Increases in both NADPH oxidase-driven reactive oxygen species production and proinflammatory cytokines in brain paraventricular nucleus linked to higher BP drop with pentolinium and plasmatic noradrenaline (NA) levels, were found in the S-S group as compared to the W-W group. These parameters were reduced by FMT from WKY to SHR. Increased levels of pro-inflammatory cytokines, tyrosine hydroxylase mRNA levels and NA content in the proximal colon, whereas reduced mRNA levels of gap junction proteins, were found in the S-S group as compared to the W-W group. These changes were inhibited by FMT from WKY to SHR. According to our correlation analyses, the abundance of *Blautia* and *Odoribacter* showed a negative correlation with high SBP. In conclusion, in SHR gut microbiota is an important factor involved in BP control, at least in part, as consequence of its effect on neuroinflammation and the sympathetic nervous system activity.

KEY WORDS

Gut dysbiosis, hypertension, oxidative stress, neuroinflammation, sympathetic nervous system.

INTRODUCTION

Abundant evidence has demonstrated the association between gut dysbiosis and neurogenic diseases, such as hypertension (Mell *et al.,* 2015; Yang *et al.,* 2015). A common characteristic of resistant hypertension is chronically elevated sympathetic nervous system (SNS) activity accompanied by a high release of noradrenaline (NA) (Tsioufis *et al.,* 2011), which indicates a neurogenic component that contributes to the initiation, maintenance and progression of hypertension (Yang and Zubcevic, 2017).

The factors that stimulate sympathetic tone in human essential hypertension are poorly understood. A newly identified interaction between the brain, gut and bone has been identified as a possible mechanism in the pathogenesis of hypertension (Santisteban *et al.,* 2016). For instance, an increase in sympathetic drive to bone marrow (BM) and the gut may also trigger a sequence of signalling events that can, ultimately, contribute to an overall increase in blood pressure (BP) and the establishment of hypertension, by affecting the structure and function of BP target organs, such as vasculature, kidney, heart, and brain. Increased sympathetic activity to the gut could result in dysbiosis, increased gut permeability and inflammatory status, leading to an imbalance in the gut content of short-chain fatty acids (SCFAs)-producing bacteria and in the plasma levels of lipopolysaccharide (LPS). These metabolic and structural microbial products, working together, elevate sympathetic drive to the BM and other lymphoid organs, and may act as modulators for BM cell activity by increasing the proliferation and release of myeloid progenitors and other pro-inflammatory cells. This increase in myeloid progenitor cells contributes to an increase in peripheral and central inflammation that could be a critical event for the establishment of hypertension.

The immune and sympathetic systems are recognized to contribute to the development of hypertension (Yang *et al*, 2017), while there exists a bidirectional signalling between the brain and gut microbiota which can regulate the BP through the modulation of the interaction between SNS and immune system (Yang and Zubcevic, 2017). Therefore, since a link between hypertension and gut dysbiosis has recently been suspected (Mell *et al.,* 2015; Yang *et al.,* 2015), several groups are investigating this interaction. In this way, different studies have shown that fecal microbiota transplantation (FMT) from hypertensive human and rat donors elevate the BP of the host, normotensive mice and rats, respectively (Adnan *et al.,* 2017; Li *et al.,* 2017; Toral *et al.,* 2018),

pointing out that gut dysbiosis plays a possible contributing or even causal role in hypertension. However, the communication between gut microbiota and the SNS in hypertension is not completely understood. We hypothesize that an increased sympathetic activity contributes to gut dysbiosis, which then increases inflammation and sympathetic activity. Because of this, in the present study, we tested whether changes in gut microbiota composition induced by reciprocal fecal microbiota transplantation from Wistar-Kyoto (WKY) to spontaneously hypertensive rats (SHR), could decrease neuroinflammation and sympathetic activity and thereby lower BP. We used SHR as a model of neurogenic hypertension characterized by sustained age-dependent elevation in sympathetic activity and dysbiosis (Judy *et al.,* 1976; Yang and Zubcevic, 2017).

RESULTS

BP is controlled by gut microbiota

Fecal exchange from SHR to WKY enhanced basal SBP (149.8 ± 4 mm Hg) by 16 ± 2 mm Hg ($p_{FMT} < 0.01$), measured by tail-cuff plethysmography. Similarly, FMT from WKY rats to SHR lowered SBP by 38 ± 4 mm Hg ($p_{FMT} < 0.01$) the basal (199.5 ± 6 mm Hg, $p_{strain} < 0.01$) (**Figure 1A**) resulting in a significant strain versus FMT interaction (pi < 0.05). In conscious rats, direct SBP and DBP values were also increased by FMT from SHR to WKY as compared to the W-W group ($p_{FMT} < 0.01$), and reduced in SHR after FMT from WKY as compared to FMT from SHR to SHR ($p_{FMT} < 0.01$), leading to a significant strain versus FMT interaction (pi < 0.05). No significant changes ($p_{FMT} = 0.62$, $p_{strain} = 0.44$) were observed among all experimental groups in HR obtained by direct register (**Figure 1B**). No interaction was observed between strain and FMT (pi = 0.39).

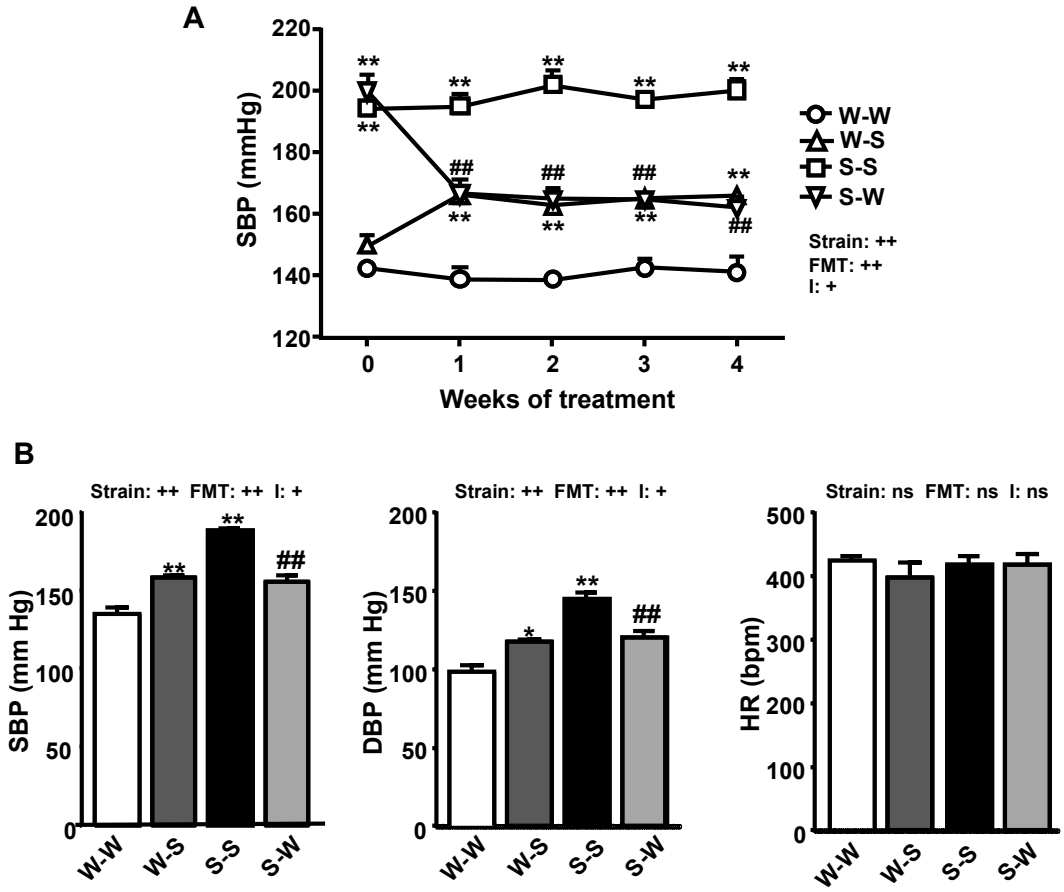


Figure 1. Effects of fecal microbiota transplantation (FMT) on blood pressure. *Systolic blood pressure (SBP), measured by tail-cuff plethysmography during 4 weeks of FMT* ***(A),*** *and SBP, diastolic blood pressure (DBP), and heart rate (HR), measured by direct register (**B**), in spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S) or from Wistar Kyoto rats (WKY) (S-W) and in WKY with stool transplant from WKY (W-W) or from SHR (W-S) at the end of the experimental period. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01, +p < 0.05 and ns (not significant) for the probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs W-W; ##P<0.01 vs S-S statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

Sympathetic activity, brain PVN NADPH oxidase, inflammation, and macrophages and T cells infiltration are regulated by gut microbiota

We used a ganglionic blocker, pentolinium, in conscious rats to determine the effect of FMT on sympathetic outflow. The reduction on SBP after ganglionic blockade was higher in the S-S group as compared to the W-W group (-113.3 ± 5.3 mm Hg vs. -59.8 ± 2.5 mm Hg, $p_{strain} < 0.01$). FMT from WKY to SHR inhibited of the decay in SBP after pentolinium (-83.6 ± 4.1 mm Hg, $p_{FMT} < 0.01$) as compared to the S-S group, whereas in W-S rats this reduction was also higher (-79.4 ± 3.6 mm Hg, $p_{FMT} < 0.05$) than that found in the W-W group (**Figure 2A**), which led to a significant strain versus FMT interaction ($pi < 0.05$). Similar qualitative changes among groups in DBP reductions after pentolinium injection were observed ($p_{strain} < 0.01$, $p_{FMT} < 0.01$), without interaction between strain and FMT ($pi = 0.83$). No changes in HR reductions ($p_{FMT} = 0.24$, $p_{strain} = 0.63$) and no interaction were observed between strain and FMT ($pi = 0.42$) (**Figure 2A**). Plasma NA concentration ($p_{strain} < 0.01$), another marker of sympathetic nerve activity, was reduced ≈ 65 % by FMT from WKY to SHR ($p_{FMT} < 0.05$) and increased ≈ 2.5 times by FMT from SHR to WKY rats ($p_{FMT} < 0.05$) (**Figure 2B**). PRA was found ≈ 2 times higher in the S-S groups as compared to the W-W group ($p_{strain} < 0.05$). However, neither FMT from SHR to WKY increased PRA, as compared with W-W, nor FMT from WKY significantly reduced PRA as compared with S-S group ($p_{FMT} = 0.52$) (**Figure 2C**), which not led to a significant strain versus FMT interaction (NA concentration: $pi = 0.46$; PRA: $pi = 0.55$). We found that ROS production ($p_{strain} < 0.01$) (**Figure 3A**), NADPH oxidase activity ($p_{strain} < 0.01$) (**Figure 3B**) and the mRNA levels of NADPH oxidase subunits, NOX-1, NOX-4, $p47_{phox}$, and $p22_{phox}$ ($p_{strain} < 0.01$) (**Figure 3C**) and pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-17a, and interferon (IFN)-γ ($p_{strain} < 0.01$) (**Figure 4A-E**) in brain PVN were higher in the S-S group than those found in the W-W group, and were reduced by FMT from WKY rats to SHR ($p_{FMT} < 0.05$). However, these patterns did not led to a significant strain versus FMT interaction. By contrast, the anti-inflammatory cytokine IL-10 was reduced in the S-S group as compared to the W-W group ($p_{strain} < 0.05$) and increased after FMT from WKY to SHR ($p_{FMT} < 0.05$) (**Figure 4F**) leading to a significant strain versus FMT interaction ($pi < 0.05$). In addition, the mRNA levels of CCL2 ($p_{strain} < 0.05$) (**Figure 4G**) and CD11b ($p_{strain} < 0.01$) (**Figure 4H**) were also increased after FMT from SHR to WKY and decreased after FMT from WKY to SHR ($p_{FMT} < 0.05$), without significant

interaction (pi = 0.10). We sought to determine whether there were alterations in the genetic expression of olfactory receptors at the PVN. Interestingly, we found an increase of Olfr59 ($p_{strain} < 0.01$) in PVN accompanied by a downregulation of GPR-41 and GPR-43 ($p_{strain} < 0.05$) (**Figure 5A-B**) in SHR as compared to the W-W group. FMT from WKY to SHR restored the mRNA levels of these receptors ($p_{FMT} < 0.01$, pi = 0.37; $p_{FMT} < 0.05$, pi = 0.08; $p_{FMT} < 0.05$, pi < 0.03; respectively) (**Figure 5A-B**).

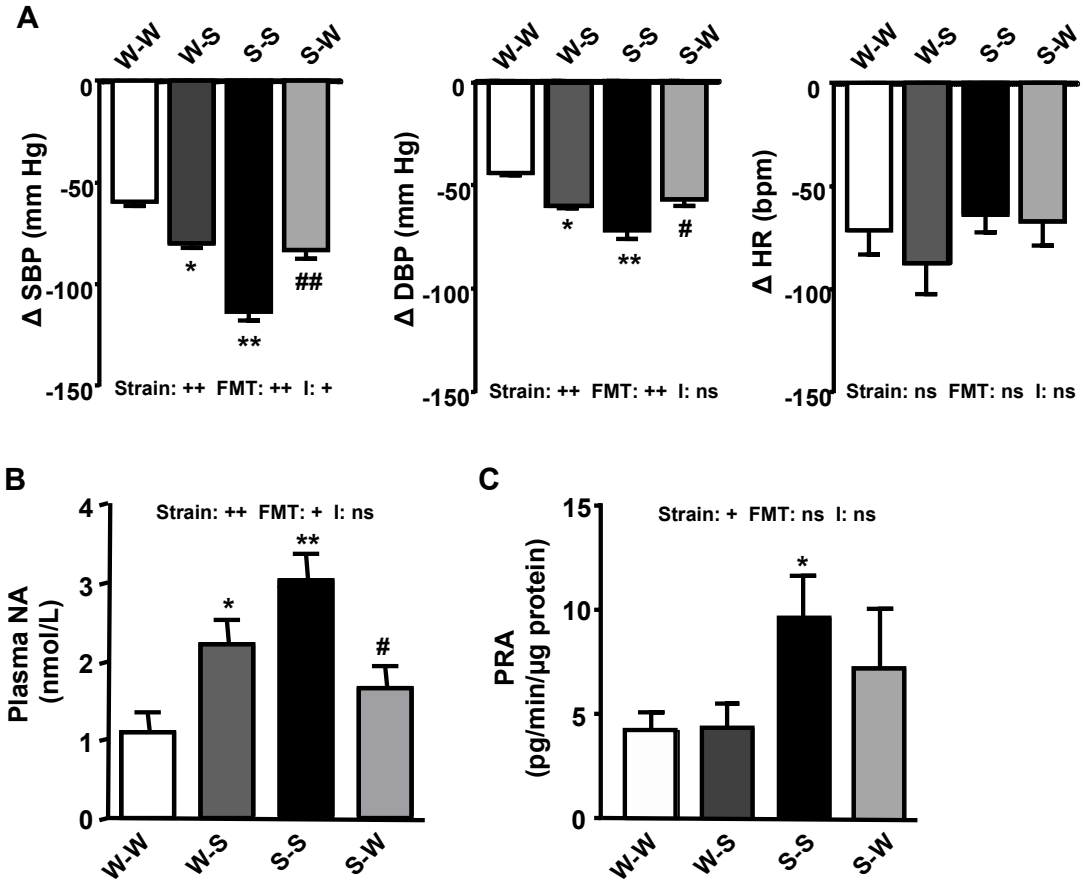


Figure 2. Effects of fecal microbiota transplantation (FMT) on sympathetic tone. *Decrease induced by acute intravenous administration of pentolinium (10 mg/kg) on systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR), in conscious rats (**A**). Plasma noradrenaline (NA) levels (**B**), and plasma renin activity (PRA) (**C**) found in all experimental groups. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01, +p < 0.05 and ns (not significant) for the probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs Wistar-Kyoto (WKY) with stool transplant from WKY (W-W); #P<0.05 and ##P<0.01 vs spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S), statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

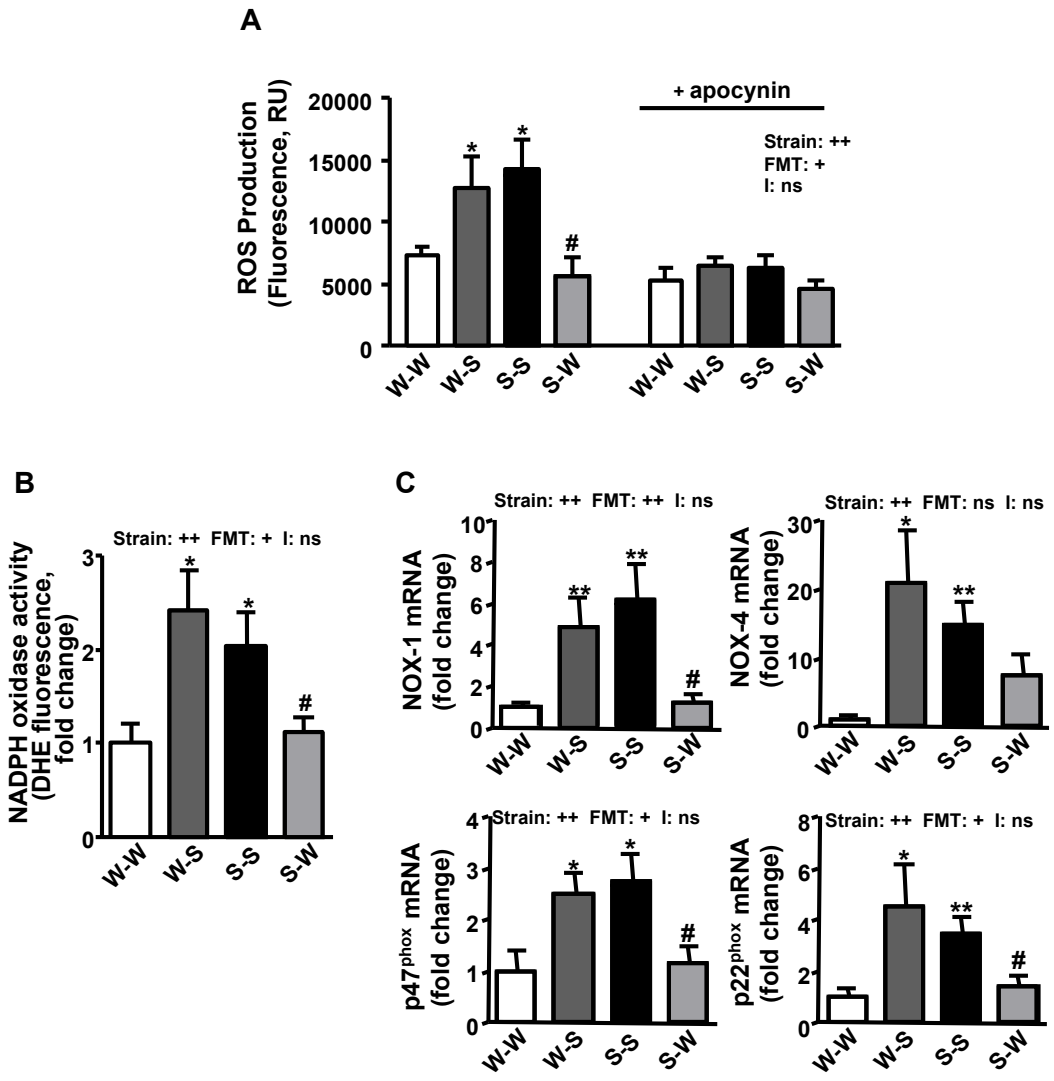


Figure 3. Effects of fecal microbiota transplantation (FMT) on ROS production and NADPH oxidase pathway in the brain PVN. *CM-H2DCFDA-detected intracellular ROS in absence and presence of NADPH oxidase inhibitor apocynin (50 µM) (**A**) and NADPH oxidase activity measured by DHE fluorescence measured in the microplate reader (**B**) in homogenates from brain PVN. mRNA levels of NADPH oxidase subunits NOX-1, NOX-4, $p47_{phox}$ and $p22_{phox}$ (**C**) in the brain PVN from all experimental groups. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01 and ns (not significant) for the probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs Wistar-Kyoto (WKY) with stool transplant from WKY (W-W); #P<0.05 vs spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S), statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

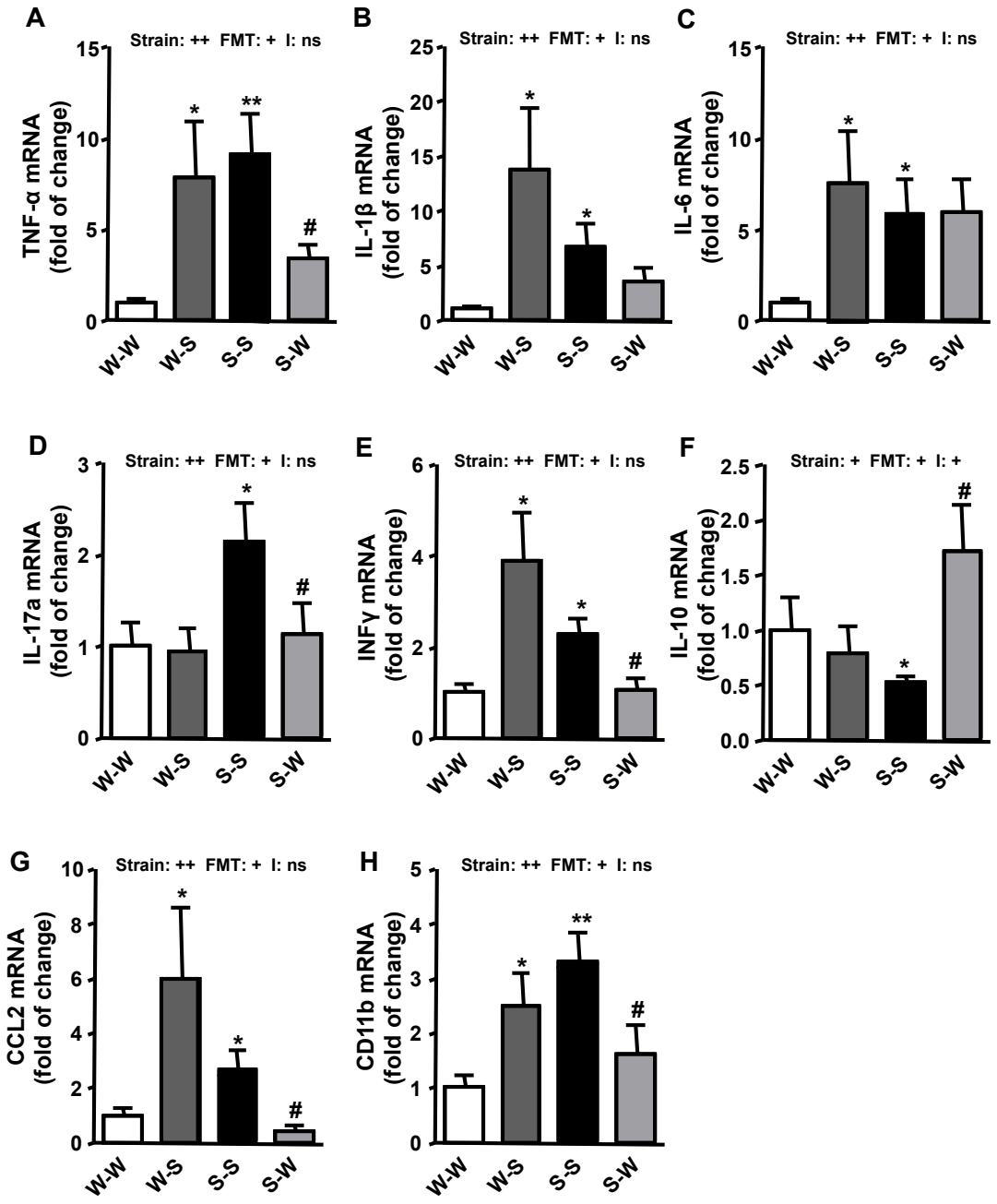


Figure 4. Effects of fecal microbiota transplantation (FMT) on brain PVN pro-inflammatory markers expression. *mRNA levels of TNF-α (**A**), IL-β (**B**), IL-6 (**C**), IL-17a (**D**), interferon-γ (IFNγ) (**E**), IL-10 (**F**), C-C chemokine ligand 2 (CCL2) (**G**) and macrophage marker CD11b (H) measured by RT-PCR in brain PVN from all experimental groups. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01, +p < 0.05 and ns (not significant) for the probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs Wistar-Kyoto (WKY) with stool transplant from WKY (W-W); #P<0.05 vs spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S), statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

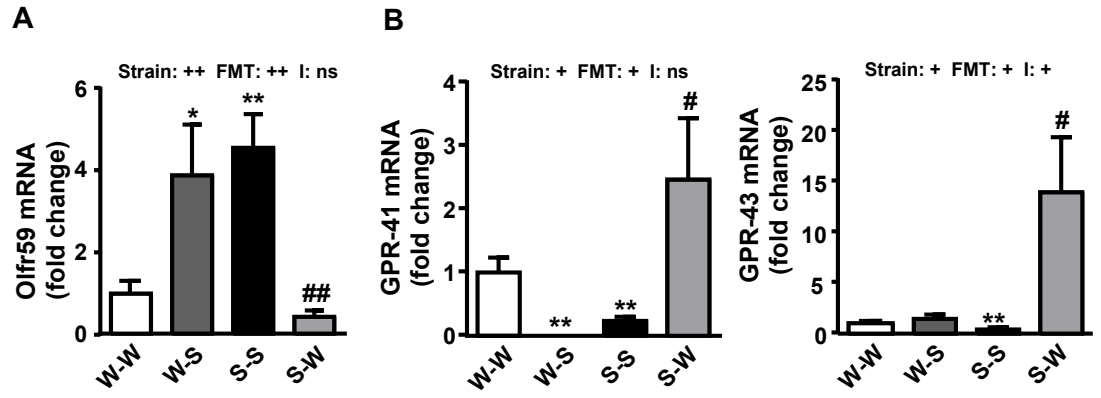
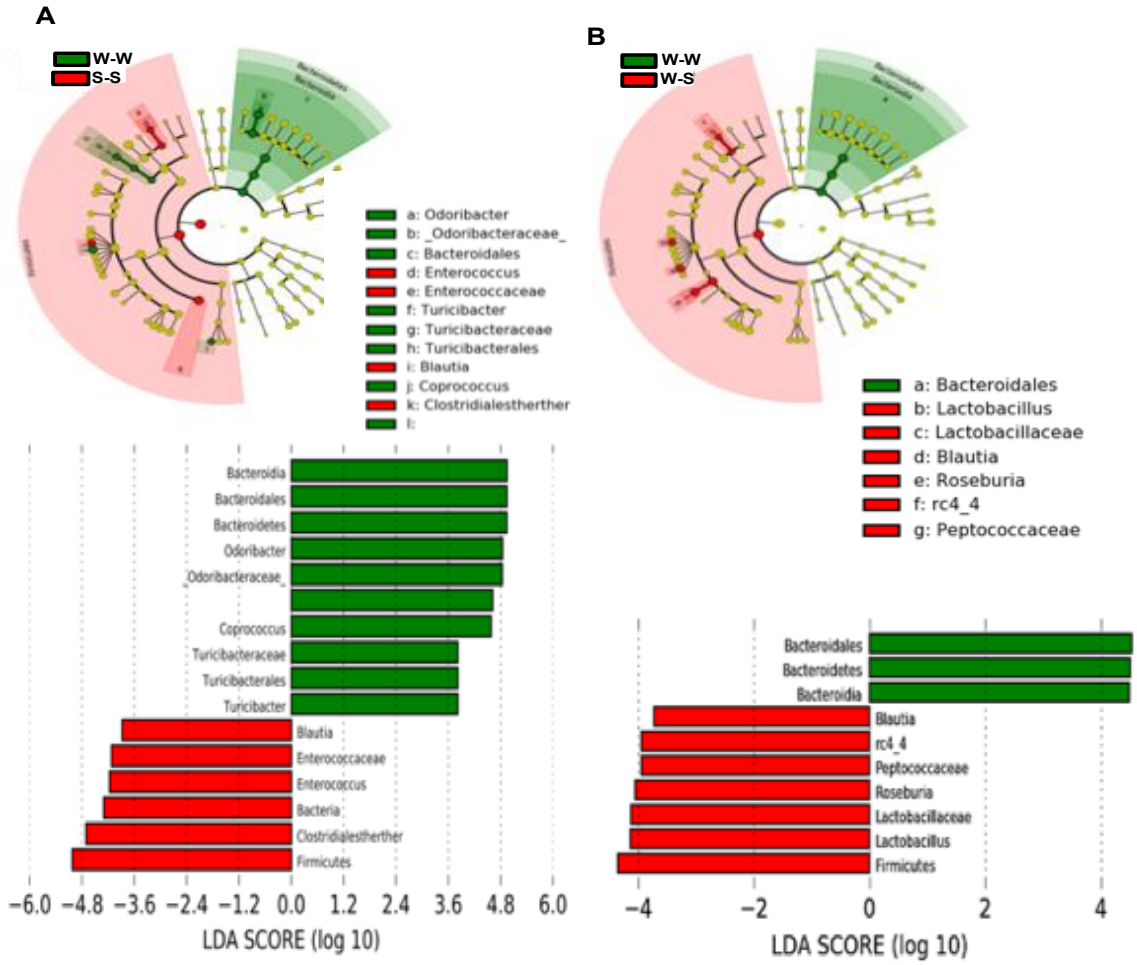


Figure 5. Effects of fecal microbiota transplantation (FMT) on brain PVN SCFA-sensing receptors expression. *mRNA levels of Olfr 59 (A), GPR-41 and GPR-43 (**B**) measured by RT-PCR in brain PVN from all experimental groups. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01, +p < 0.05 and ns (not significant) for the probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs Wistar-Kyoto (WKY) with stool transplant from WKY (W-W); #P<0.05 and ##P<0.01 vs spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S), statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

FMT induced changes in the gut microbiota composition

To determine the dynamics of gut microbiota during the exchange of gut microbiota between SHR and WKY, we analyzed fecal DNA isolated from all experimental groups. **Figure 6A** shows the bacterial taxa (class, order, family, and genus) that were altered by fecal exchange from SHR to WKY, according to LEfSe analysis. Prominent shifts in bacterial community were observed after four weeks of treatment between the W-W and S-S groups, with an increase in the relative abundance of 10 bacterial taxa (green) and a decreasing of 6 taxa (red) comparing with the W-W group. Several changes in microbial taxa were also driven by fecal exchange from SHR to WKY with an increase in the relative abundance of 3 bacterial taxa (green) and a reduction of 7 taxa (red) (**Figure 6B**). While S-W compared to the S-S group, only relative abundance of 2 taxa were increased (green) and 22 were decreased (red) (**Figure 6C**). Interestingly, we found an association between a major abundance of butyrate-producing family *Odoribactereae* and the genus of *Odoribacter* with low BP levels present in the W-W and S-W groups. In contrast, we observed a correlation between *Blautia* and *Peptococcaceae* abundance and elevated BP in the S-S and W-S groups (**Figure 6**). When comparing the

bacterial composition evolution, at the family level, in the gut microbiota between all experimental groups, we found that W-S had a significantly lower abundance of *Bacteroidaceae*, and greater abundance of *Clostridiacea* in the gut microbiota than the W-W group at 4 weeks of treatment. While S-W showed a depletion of abundance of *Lactobacillales* and an increase of *Eryspelotrichaceae* compared to the S-S group at the end of the experiment (**Figure 7A**).



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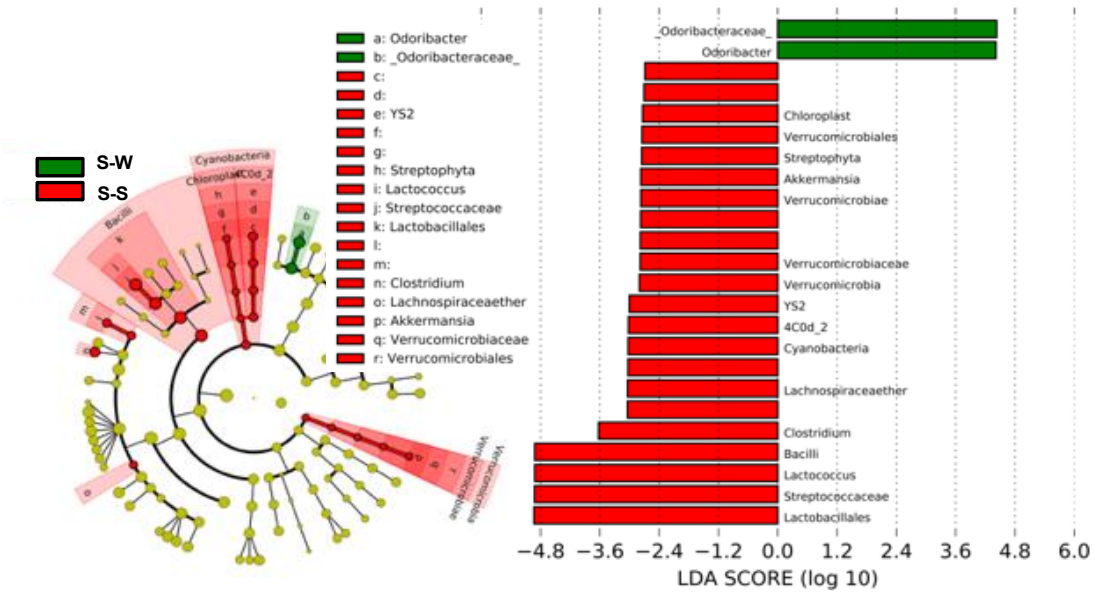

Figure 6. Effects of fecal microbiota transplantation (FMT) on changes of the gut microbiome. *Comparisons of microbiome changes in Wistar-Kyoto (WKY) with stool transplant from WKY versus spontaneously hypertensive rats (SHR) with stool transplant from SHR (**A**) WKY-WKY versus WKY-SHR (**B**) and SHR-SHR versus SHR- WKY (**C**). Cladograms (top panes) show the significantly enriched taxa, the taxa are identified in the key to the right of each pane. The larger the circles the greater the difference in abundance between the groups. The lower panels show the results of linear discriminant analysis effect size at p <0.05 and LDA score of >2.5 and detail the taxa most enriched by WKY, SHR or SHR-WKY. n = 6 animals per treatment group in each comparison.*

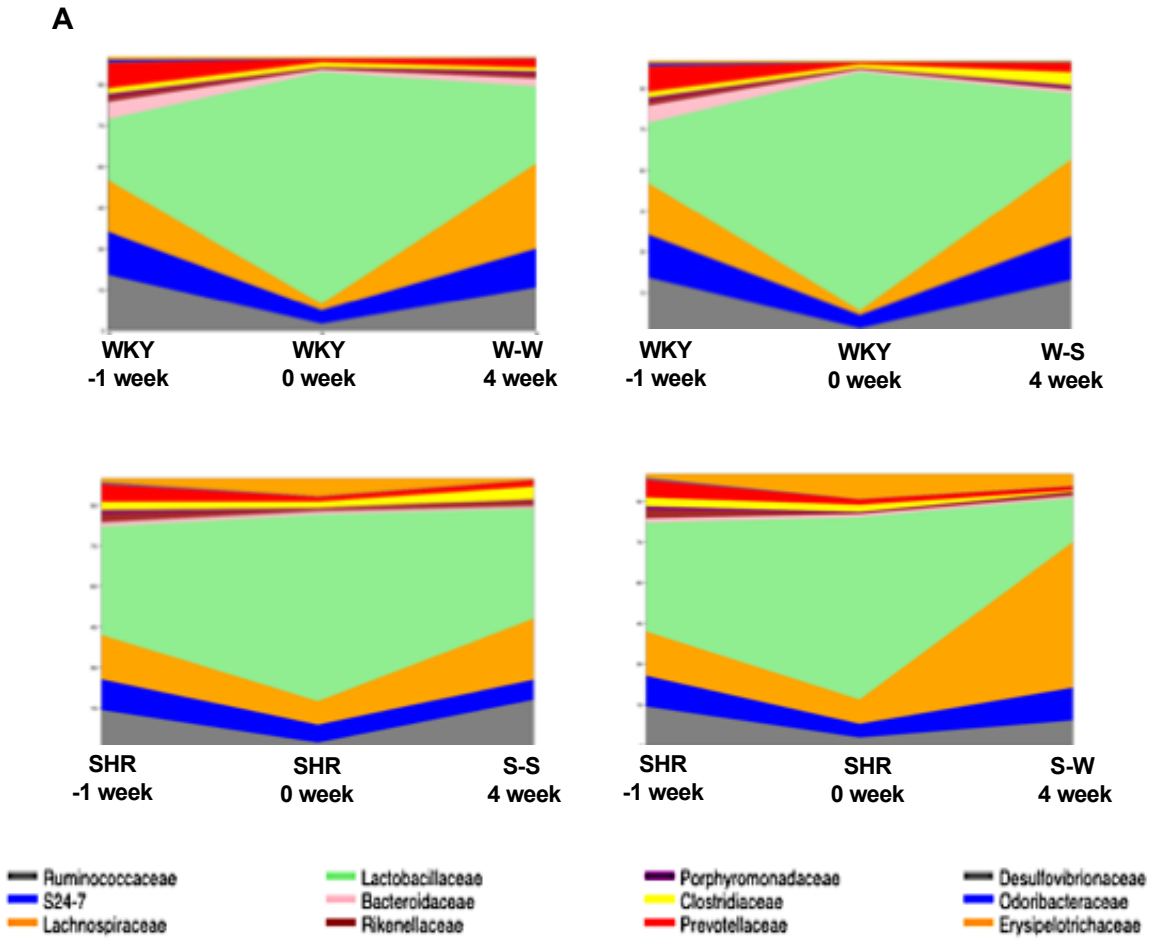

Figure 7. Effects of fecal microbiota transplantation (FMT) on changes of composition of gut microbiota. *Time-dependent changes of fecal microbiota upon in all experimental groups (A) (n = 6 rat per group).*

Gut integrity and inflammation are regulated by gut microbiota

In concurrence with previous data (Santisteban *et al.,* 2017) the levels of occludin ($p_{strain} < 0.05$) (**Figure 8A**), and zonula occludens (ZO)-1 ($p_{strain} < 0.01$) (**Figure 8B**), and mucin (MUC)-2 ($p_{strain} < 0.05$) (**Figure 8C**) were significantly reduced in the S-S group as compared to the W-W group. The expression of colonic ZO-1 and MUC-2 ($p_{FMT} < 0.05$) were also reduced when FMT from SHR to WKY was performed, which were accompanied of a significant strain versus FMT interaction ($pi < 0.05$), whereas FMT from WKY to SHR tended to increase these parameters but without statistical significance, as compared to the S-S group. Similarly, increased mRNA expression of pro-inflammatory TNF-α ($p_{strain} < 0.05$) (**Figure 8D**) and IL-6 ($p_{strain} < 0.05$) (**Figure 8E**), without significant change in IL-1β (**Figure 8F**) was also observed in the S-S group as compared to the W-W group. FMT from WKY to SHR reduced the levels of these pro-inflammatory cytokines ($p_{FMT} < 0.05$, $pi < 0.05$; $p_{FMT} < 0.01$, $pi < 0.01$; respectively). We found reduced GPR-43 mRNA levels in the gut from both SHR groups as compared to the W-W group ($p_{strain} < 0.05$) (**Figure 8 G**). GPR-43 transcript level tended to be reduced in WKY rats after FMT from SHR ($p_{FMT} = 0.057$) without interaction between strain and FMT ($pi = 0.11$). In addition, an increase in intestinal permeability has been proposed as a crucial mechanism for the development of endotoxemia (Cani *et al.,* 2008). In fact, we found increased plasma LPS levels **Figure 8H**) in rats with high BP (W-S and S-S groups) ($p_{strain} < 0.05$; $p_{FMT} < 0.05$; $pi = 0.19$), qualitatively associated with impaired colonic integrity. Interestingly, FMT transplantation from WKY to SHR reduced plasma LPS levels despite no significant increase in gut integrity. Furthermore, both the colonic expression of tyrosine hydroxylase (TH) ($p_{strain} < 0.05$) (**Figure 8I**) and the colonic NA concentration ($p_{strain} < 0.01$) (**Figure 8J**) were significantly up-regulated in both groups that received fecal contents from SHR. Interestingly, the long-term treatment with stool from WKY significantly decreased both TH levels ($p_{FMT} < 0.01$; $pi = 0.62$) and NA content ($p_{FMT} < 0.01$; $pi < 0.05$) in the S-W group.

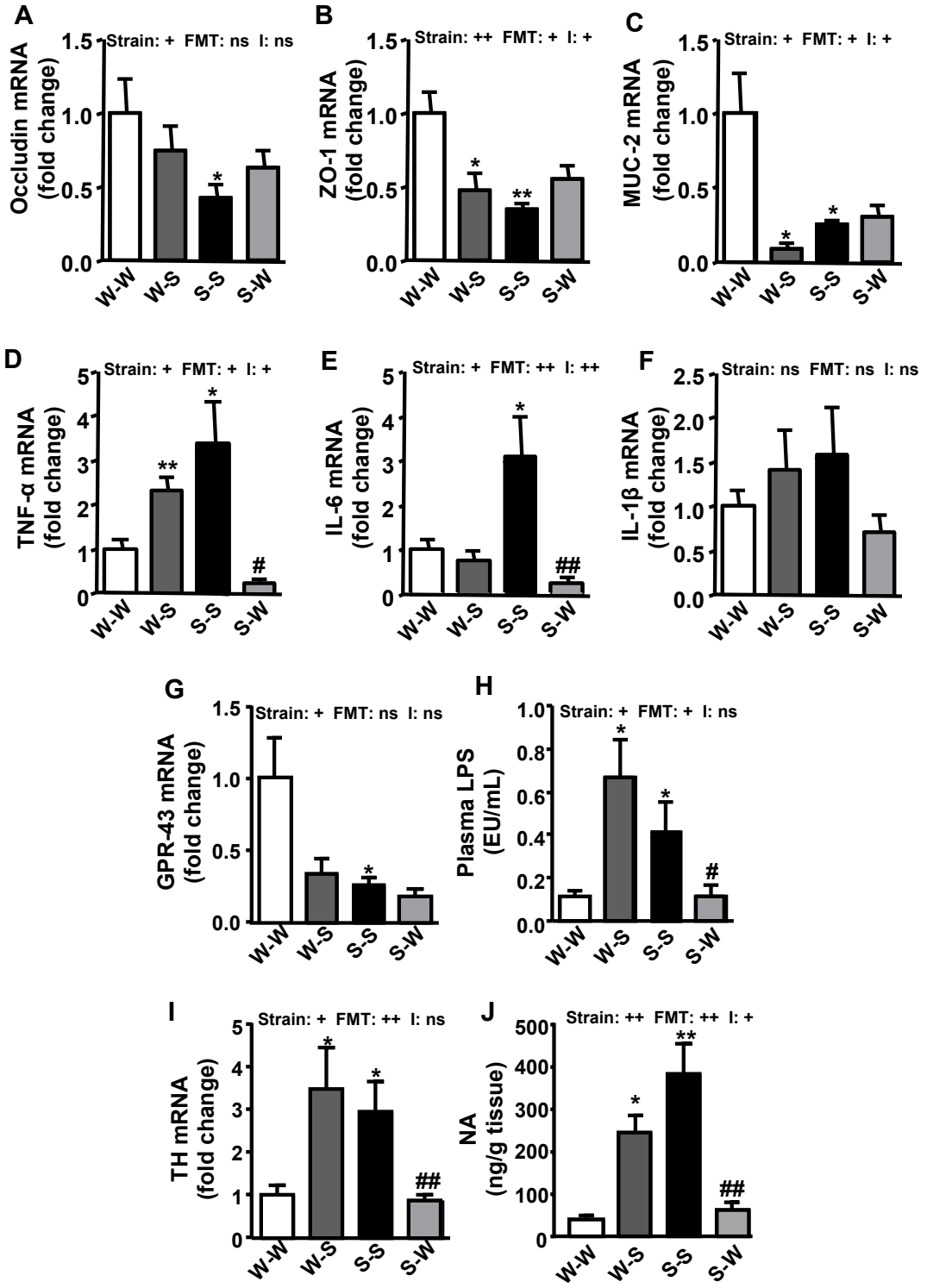

Figure 8. Effects of fecal microbiota transplantation (FMT) on colonic pro-inflammatory, epithelial integrity, and sympathetic activity markers. *Colonic occludin (**A**), zonula occludens-1 (ZO-1) (**B**), mucin (MUC)-2 (**C**), TNF-α (**D**), IL-6 (**E**), IL-β (**F**) and GPR-43 (**G**) mRNA levels, plasma endotoxin concentrations (EU/mL, endotoxin units/mL) (**H**), mRNA levels of tyrosine hydroxylase (TH) (**I**), and noradrenaline (NA) content (**J**), in all experimental groups. Strain factor, FMT factor and I interaction between strain and FMT factors. ++p < 0.01, +p < 0.05 and ns (not significant) for the*

*probability based on a two-way analysis of variance. Values are means ± SEM (n= 5-8). *P<0.05 and **P<0.01 vs Wistar-Kyoto (WKY) with stool transplant from WKY (W-W); #P<0.05 and ##P<0.01 vs spontaneously hypertensive rats (SHR) with stool transplant from SHR (S-S), statistical significance for the probability based on a Sidak's correction multiple comparisons test.*

DISCUSSION

Fecal transplantation from animals (Adnan *et al.,* 2017; Toral *et al.,* 2018) and subjects (Li *et al.,* 2017) with hypertension to normotensive animals can elevate BP. However, the mechanisms by which bacteria control BP have not been elucidated. Our results demonstrate, for the first time, that alteration of gut microbiota composition induced by reciprocal FMT between WKY and SHR influences the brain, and SNS impacting BP. The most significant findings of this study are; 1) Microbiota affects brain PVN NADPH oxidase activity, neuroinflammation and sympathetic activity in both strain of rats, 2) Lower *Blautia* and *Odoribacter* content in feces is inversely correlated with high SBP, and 3) Loss of gut integrity in SHR seems to be independent of sympathetic tone, BP, and microbiota composition. It is pertinent to note that elevated SNS activity is a hallmark of both animal and human hypertension (Grassi *et al.,* 2015; DiBona, 2013). Santisteban *et al*., (Santisteban *et al.,* 2017) observed enhanced gut-neuronal communication in hypertension originating from the PVN of the hypothalamus and presenting as increased sympathetic drive to the gut. Previous studies have also characterized hypertension with alterations in the gut microbiota and sympathetic dysregulation (Zubcevic *et al.,* 2014; Yang *et al.,* 2015; Santisteban *et al.,* 2017). Our current study, demonstrating both higher BP reductions after pentolinium administration and plasma NA levels, both markers of increased sympathetic drive, associated with microbial dysbiosis, provides new evidence in support of that proposal. This higher SNS activity correlates with higher SBP and DBP in the W-S group compared to W-W. Similarly, FMT from SHR to SHR showed higher levels of these sympathetic drive parameters than that found in the W-W group. The changes induced by FMT from WKY to SHR and vice versa in BP seem to be independent of systemic renin-angiotensin system, since PRA was not significantly affected. Moreover, increased catecholaminergic neurotransmission has been reported in SHR, characterized by increased TH activity as well as gene and protein expression (Yu *et al.,* 1996; Reja *et al.,* 2002; Lopez Verrilli *et al.,* 2009), suggesting that TH plays a key role in the genesis,

development and/or maintenance of hypertension. In agreement with this information, our data showed increased colonic expression of TH and NA content from the S-S and W-S groups compared to W-W, showing increased sympathetic activity in this tissue. The gut microbiota influences the hosts inflammatory response (Cani *et al.,* 2007) and inflammation induces oxidative stress and vice versa. In brain, angiotensin II via an AT1 receptor mechanism activates the sympathetic outflow by stimulation of the NADPH oxidase-dependent ROS production (Gao *et al.,* 2005). In brain from the S-S group we found increased NADPH oxidase activity driven-ROS production, expression of NADPH oxidase subunits, pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and sympathetic activity, as compared to W-W rats. These data could be related to higher PRA found in the S-S group as compared to the W-W group. Conversely, FMT from SHR to WKY also resulted in higher PVN inflammation, NADPH-oxidase activity and sympathetic outflow than induced by FMT from WKY to WKY. Taken together, our data suggest that gut microbiota is involved in the regulation of brain inflammatory and oxidative status, and the subsequent sympathetic activity. The increased sympathetic activity also affects the BM resulting in an increase in inflammatory cells, which migrate to the PVN and enhance neuroinflammation (Santisteban *et al.,* 2016). Accordingly, we found increased inflammation in brain PVN from the S-S and W-S groups, associated to an increased expression of CCL2, which facilitates BM cells entering the brain's parenchymal space; CD11b, a macrophage marker; and IL-17a, mainly produced by Th17 cells, that might contribute to neuroinflammation. Overall, all this data showed a gut-brain communication characterized by increased pro-oxidant, pro-inflammatory and immune cell infiltration profile in brain PVN after FMT from SHR to WKY. Interestingly, chronic normal microbiota transplantation to hypertensive rats induced a stable BP reduction linked to reduced brain PVN inflammation, NADPH oxidase activity and sympathetic excitation. Taken together, our present results demonstrate that hypertension is, at least in part, a result of pathophysiological changes in the gut microbiota, affecting brain areas of cardiovascular control such as PVN. The mechanisms involved in the pro-hypertensive effects of gut microbiota from SHR are unknown. There is growing evidence that gut microbiota has emerged as an important factor that can influence the host's physiology through bacterial metabolic products such as SCFAs (Pluznick *et al.,* 2013). Gut dysbiosis in SHR is characterized by reduced acetate- and butyrate-producing bacteria than their WKY normotensive counterparts (Yang *et al.,* 2015). These SCFAs induced anti-inflammatory effects in the gut, mediated by GPR-43 activation (Yang *et al.,* 2018).

SCFAs, in addition to providing energy to gut epithelium and peripheral tissues, also promote intestinal epithelial integrity and aid in the repair of wounded epithelium, mainly throught GPR-43 activation (D'Souza *et al.,* 2017). We can hypothesize that insufficient signalling through SCFAs-activated GPR-43 pathway in the gut, mediated by both lower SCFAs-producing bacteria and lower colonic GPR-43 expression in SHR, can lead to compromised gut integrity, dysregulated inflammation, and passage of substances such as LPS into the blood. The increase in BP in SHR was associated with gut pathology that included increased intestinal permeability and decreased tight junction proteins (Santisteban *et al.,* 2017). We analysed the integrity of the gut epithelial barrier measuring the mRNA levels for tight junction proteins and mucins, which are involved in mucus production, in the proximal colon. We found that FMT from SHR to WKY significantly reduced colonic ZO-1 and MUC-2, increased colonic TNF-α and plasma levels of LPS. LPS might be involved in the pathogenesis of hypertension, through toll-like receptor (TLR)-4 stimulation in the vasculature (Liang *et al.,* 2013), and by inducing systemic inflammation, accompanied by microglia activation, oxidative stress in cardiovascular regions of the brain, such as rostral ventrolateral medulla (Wu *et al.,* 2012) and PVN (Zhang *et al.,* 2010). However, high levels of plasmatic LPS, as a consequence of an infection, can lead to septic shock and hypotension (Bermejo *et al.,* 2003). On the other hand, butyrate, acetate and propionate are metabolic products of the gut bacteria that, in addition to their gut anti-inflammatory effects, have anti-hypertensive properties (Natarajan and Pluznick, 2014; Natarajan *et al*., 2016; Pluznick, 2014). SCFAs modulate BP through the renal and vascular Olfr and GPRs. SCFAs modulate BP through the renal and vascular olfactory receptor (Olfr) 59 (its stimulation elevates BP), G-protein-coupled receptor (GPR)-41, and GPR-43 (their stimulation both lower BP) (Pluznick *et al.,* 2013). In addition to the vasculature (Pluznick *et al.,* 2013; Pluznick, 2014), the SCFA-sensing receptors Olfr78 and GPR-43, as well as GPR-41, are present on the sympathetic ganglia (Kimura *et al.,* 2011; Nøhr *et al.,* 2015). We also found expressional changes in PVN of the hypothalamus (a major brain region involved in regulation of the sympathetic output) of receptors for SCFAs, suggesting a probable connection with BP control. Prior works have demonstrated that a high abundance of butyrate-producing genus *Odoribacter,* in the gut microbiota in pregnant women at 16 weeks gestation is associated with decreased BP (Gomez-Arango *et al.,* 2016). As butyrate is able to cross the blood brain barrier (Bergersen *et al.,* 2002; Vijay and Morris, 2014; Sun *et al.,* 2016) circulating butyrate may thus have direct effects on regions of the brain that regulate BP. Our data showed

that a significant depletion of *Odoribacter* in the W-S and S-S groups were noted, consequently, this depletion might contribute to the dysregulation of the expression Olfr59, GPR-41, and GPR-43 found in PVN from hypertensive groups. Because Olfr59 elevates BP and GPR-41 opposes this action to lower BP, these data support the suggestion that the altered expression of SCFA receptors in the PVN may play a role in elevated BP of the SHR. Several groups have reported the presence of SCFA-sensing receptors (i.e. Olfr59, GPR-41) in multiple organs and neural tissues (Kimura *et al.,* 2011; Nøhr *et al.,* 2015). These studies also show that expression levels of SCFA-sensing receptors in the whole brain are relatively low (Kimura *et al.,* 2011), but no studies to date have specifically examined the expression of these receptors in cardioregulatory brain regions such as the PVN of the hypothalamus. Moreover, the effects of SCFAs on modulation of sympathetic activity and BP have also previously been suggested. Kimura *et al*. (Kimura *et al.,* 2011) observed that intraperitoneal administration of propionate caused a significant increase in heart rate in the wild type, but not the Gpr41-/- mice. It is tempting to hypothesize that the possible low plasma butyrate levels and lower GPR-41, and GPR-43 expression found in PVN from rats transplanted with SHR microbiota as compared to that transplanted with WKY feces, could also contribute to increased neuroinflammation and sympathetic outflow in these animals. However, neither plasma nor brain levels of SCFAs have been measured in the present study, which limit the real role of SCFAs in the control of sympathetic output. Clearly, additional studies are necessary to confirm this hypothesis and provide a mechanism for the differential regulation of these SCFA receptors throughout the SNS. Furthermore, we found in the W-S group a depletion of *Peptococcaceae,* a sulfate-reducing bacteria considered to be beneficial in the regulation of BP (Ahmad *et al.,* 2012; Yu *et al.,* 2018). Sulfate-reducing bacteria, and hydrogen sulfide released in the colon may also contribute to the control of arterial BP, being antihypertensive, at least in part, through suppression of sympathetic outflow (Duan *et al.,* 2015). The genus *Blautia*, which is the main bacterial group in *Clostridium* coccoides-group, was also found to decrease in Japanese patients with type 2 diabetes, as compared with control subjects (Sato *et al.,* 2014). Interestingly, transplantation of gut microbiota from WKY to SHR reduced gut inflammation, plasma LPS levels, brain PVN inflammation, NADPH oxidase activity and sympathetic excitation, leading to a stable drop of BP. However, no significant improvement of colonic integrity was found in this group as compared to the S-S group. Sympathetic activity influenced these gut parameters (ZO-1, occludin, MUC-2), via top-down

signaling (Santisteban *et al.,* 2017). However, normalized sympathetic activity to the gut (lower TH transcript level and NA content) without correction of gut parameters, in the SHR with a WKY microbiota, is in conflict with this hypothesis. The lack of protective effects in the gut integrity could be related to the lower expression of GRP-43 found in colonic tissue from this group, and subsequent lower GRP-43 pathway activation despite the possible increase in gut SCFAs induced by FMT from WKY. A similar loss of gut integrity has been found in young prehypertensive SHR despite no gut dysbiosis being detected in these animals (Santisteban *et al.,* 2017). Overall, gut integrity in SHR seems to be independent of sympathetic tone, BP, and microbiota composition. In conclusion, in this genetic model of hypertension (SHR) gut dysbiosis correlates to sympathetic outflow via stimulation of NADPH-oxidase-derived ROS in the brain. These central effects seem to be associated with reduced expression of butyrate-sensing receptors in the hypothalamus, Th17 and macrophages infiltration in PVN and higher plasma levels of LPS. These results are congruent with evidence from previous studies suggesting a strong correlation between hypertension and gut microbiota dysbiosis, establishing a cause-effect relationship between elevated BP and altered gut microbiota. We can speculate that changes in gut microbiota through the use of probiotics in treating gut dysbiosis could have positive effects on neurogenic hypertension, through modulation of central sympathetic activity.

MATERIALS AND METHODS

Animals and experimental groups

This research was performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and approved by the Ethic Committee of Laboratory Animals of the University of Granada, Spain (Ref. 03-CEEA-OH-2013). Male SHR and WKY were obtained from Harlan Laboratories (Barcelona, Spain). All rats were fed standard rat chow (Harlan global diet 2014, Harlan Laboratories, Inc., Milan, Italy) ad libitum for the duration of the experiment. Stool samples were collected and pooled from twenty-week-old WKY and SHR rats. Donor fecal contents were administered through oral gavage to twenty-five-weeks-old WKY and SHR rats for 3 consecutive days, and once every 3 days for a total extension of 4 weeks. Animals were randomly assigned to four different groups of 5-8 animals each: WKY with WKY

microbiota (W-W), WKY with SHR (W-S), SHR with SHR (S-S) and SHR with WKY (S-W). Rats were kept in individually ventilated cages in a pathogen-free animal facility. Body weight, food and water intake were recorded weekly for all groups. During the experimental periods, rats had free access to tap water and chow. FMT to recipient rats were carried out as previously reported with several modifications (Bruce-Keller *et al.,* 2015).

Fecal microbiota transplantation (FMT)

FMT to recipient rats were carried out as previously reported with several modifications (Toral *et al.,* 2018). Briefly, fecal contents were isolated and pooled from WKY rats and SHR (n = 5). Fecal contents were diluted 1:20 in sterile PBS and centrifuged at 800 rpm for 5 minutes. The supernatant was aliquoted and stored at -80ºC. Starting 1 week before the administration, recipient rats were administered with 1 mL ceftriaxone sodium (400 mg/Kg/day) daily for 5 consecutive days by oral gavage. The purpose of the antibiotic treatment was to reduce the preexisting microbiota and to facilitate the recovery of the population and diversity of intestinal microbiota from donor rats after FMT (Li *et al.,* 2017). Forty-eight hours after the last antibiotic treatment, recipient rats were orally gavaged with donor fecal contents (1 mL) as explained above.

Blood pressure measurements

Systolic blood pressure (SBP) and heart rate (HR) was measured weekly at room temperature using tail-cuff plethysmography as described previously (Zarzuelo *et al.,* 2011). At the end of the experimental period, animals were subjected to isofluorane anesthesia, a polyethylene catheter containing 100U heparin in isotonic, sterile NaCl solution was inserted in the left carotid artery to monitor intra-arterial BP. Twenty-four hours after the implantation of the catheter, we recorded intra-arterial BP uninterruptedly for 60 min with a sampling frequency of 400/s (McLab; AD Instruments, Hastings, UK). For intergroup comparisons, BP values recorded during the last 30 min were averaged.

Evaluation of the contribution of sympathetic activity

Acute BP responses to intravenous injection of pentolinium (10 mg/Kg) were analyzed in conscious rats. Before pentolinium administration, arterial blood samples (0.2 mL) were drawn via the catheter to measure NA levels and plasma renin activity (PRA). The pentolinium dose was selected because it produces maximal sympathetic inhibition (Pechánová *et al.,* 2004). Finally, the rats were subjected to isofluorane anesthesia and were killed by complete exsanguination, then the brain was removed, snap-frozen in liquid nitrogen, and stored at -80°C until processed for the reverse transcriptase-polymerase chain reaction (RT-PCR) measurement (Romero *et al.,* 2016).

Plasma and colonic determinations

Blood samples were cooled in ice and centrifuged for 10 min at 3,500 rpm at 4 ºC, and the plasma was frozen at -80 °C. Plasma LPS concentration was measured using the Limulus Amebocyte Lyste (LAL) chromogenic endotoxin quantitation Kit (Lonza, Valais, Switzerland), according to the instructions of the manufacturer. We used enzyme-linked immunosorbent assay kits (IBL International, Hamburg, Germany) to measure both plasma and colonic NA concentrations following the manufacturer's protocol. Colon samples were collected and immersed in the appropriate conservation solution. EDTA 1 mM and sodium metabisulfite 4 mM were added to prevent the catecholamine degradation, then plasma and tissue samples were stored at -80ºC for later use. The Rat Renin Activity Fluorometric Assay Kit (BioVision, Milpitas, CA, USA; K806-100) was used to measure the renin activity following the manufacturer's protocol.

Measurement of intracellular reactive oxygen species (ROS) concentrations

ROS production was measured in homogenates from brain paraventricular nucleus (PVN) using the fluorescent probe 5-(and-6-)chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Brain PVN was homogenized in lysis buffer composed of 50 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM PMSF. Fresh homogenates (10 μg of protein) in 96-well plates were incubated with 5 μmol/L CM-H2DCFDA for 30

min at 37 ºC, in the absence or in the presence of the NADPH oxidase inhibitor apocynin (50 μM). The fluorescent intensity was measured using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany) (Toral *et al.*, 2015).

NADPH oxidase activity

The NADPH oxidase activity in homogenates from brain PVN was measured by dihydroethidium (DHE) fluorescence assay in the microplate reader, as described previously (Fernandes *et al*., 2007; Toral *et al.*, 2015). Fresh homogenates (10 μg of protein) were incubated with DHE (10 μM) and deoxyribonucleic acid (DNA, 1.25 μg/mL) in PBS (100 mM), pH 7.4, containing 100 μM DTPA with the addition of NADPH (50 μM), at a final volume of 120 μL. Incubations were performed for 30 min at 37ºC in the dark. Total fluorescence was followed in a microplate reader using a rhodamine filter (excitation 490 nm and emission 590 nm) in a spectrofluorometer (Fluorostart, BMG Labtechnologies, Offenburg, Germany).

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from the colon, and brain PVN by homogenization and converted to cDNA by standard methods. PVN and colon tissue was homogenized in 1 ml of TRI Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA isolation was performed with traditional methods using sequential washes with bromochloroporopane, isopropanol and ethanol 75%. RNA concentrations were measured with a NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A Techne Techgene thermocycler (Techne, Cambridge, UK) was used to perform the polymerase chain reaction. mRNA expression was analysed through quantitative real-time RT-PCR. RNA was reverse transcribed using oligo (dT) primers, Recombinant RNasin® Ribonuclease, dNTP (10mM) and M-MLV reverse Transcriptase (Promega, Southampton, UK). Reverse resulting cDNA (2 ng) was amplified on optical grade 48-well plates in an EcoTM Real-Time PCR System (Illumina, CA, USA), using GoTaq® qPCR Master Mix, 2x (Promega, Southampton, UK). In Table 1 are listed the sequences of both sense and antisense primers used for amplification. In order to determine nonsaturating conditions of PCR amplification for all genes studied,

preliminary experiments with various amounts of cDNA were performed. Under these conditions, RT-PCR method was used to assess the relative quantification of mRNA. A standard tissue sample was used to determine the efficiency of the PCR reaction. The ΔΔCt method was performed for quantification. The housekeeping gen glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for internal normalization (Romero *et al.,* 2016).

16S rDNA V4-V5 region sequencing

Fecal DNA was extracted from the samples collected from 5-6 animals per group by using a quick-DNA fecal/soil microbe kit (Zymoresearch, Irvine, CA). Primers compatible with illumian Miseq v2 2x250bp kit (Illumina, San Diego, CA) were used to amplify bacterial 16S V4-V5 variable regions (Robles-Vera *et al.,* 2018). The PCR amplicons were purified using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified by Qubit (thermos 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 ≥93.5% and 97.17±0.34% of total cluster passes the filter.

Bioinformatics analysis

The raw paired-reads from Miseq were processed using QIIME 1.9.1. Briefly, reads were trimmed to remove bases with Phred scores lower than 30 and quality-filtered with parameters set as previously optimized [ref: Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing]. Open reference OTU-picking was performed and taxonomical assignment to the generated OTUs were performed with 97% identity against Greengenes database 13.8. Alpha diversity and unweighted principal coordinate analyses plots using the phylogenic tree-based unifrac distance metric were generated using scripts from QIIME package.

Reagents

All reagents were purchased from Sigma-Aldrich (Barcelona, Spain) unless otherwise specified.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 software. Results are expressed as means ± SEM. To test if the values come from a gaussian distribution a Shapiro-Wilk normality test was used. For comparisons of the four groups with two variables (strain and treatment) we used a two-way ANOVA (with Sidak's correction for comparison of multiple means). Factors were partitioned into strain (WKY-SHR) and FMT (from WKY/from SHR). A p value of less than 0.05 was considered significant considering the main effects of the strain (WKY-SHR), FMT (from WKY/from SHR) and their interaction (I; strain vs. FMT).

ABBREVIATIONS

BP, blood pressure; BM, bone marrow; DBP, diastolic blood pressure; DHE, dihydroethidium; DNA, deoxyribonucleic acid; FMT, fecal microbiota transplantation; GPR, G-protein-coupled receptor; HR, heart rate; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; MUC, mucin; NA, noradrenaline; O_2^-, superoxide anion; Olfr, olfactory receptor; PRA, plasma renin activity; PVN, paraventricular nucleus; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SBP, systolic blood pressure; SCFAs, short-chain fatty acids; SHR, spontaneously hypertensive rats; SNS, sympathetic nervous system; of TH, tyrosine hydroxylase; TNF-α; tumor necrosis factor-α, TLR, toll-like receptor; WKY, Wistar-Kyoto; ZO, zonula occludens.

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Table 1. Oligonucleotides for real-time RT-PCR.

mRNA targets	Descriptions	Sense	Antisense
NOX-1	NOX-1 subunit of NADPH oxidase	TCTTGCTGGTTGACACTTGC	TATGGGAGTGGGAATCTTGG
NOX-4	NOX-4 subunit of NADPH oxidase	ACAGTCCTGGCTTACCTTCG	TTCTGGGATCCTCATTCTGG
p22phox	p22phox subunit of NADPH oxidase	GCGGTGTGGACAGAAGTACC	CTTGGGTTTAGGCTCAATGG
p47phox	p47phox subunit of NADPH oxidase	CCCAGCGACAGATTAGAAGC	TGGATTGTCCTTTGAGTCAGG
TNF-α	Tumor necrosis factor-alpha	ACGATGCTCAGAAACACACG	CAGTCTGGGAAGCTCTGAGG
IL-6	Interleukin-6	GATGGATGCTTCCAAACTGG	AGGAGAGCATTGGAAGTTGG
IL-10	Interleukin-10	GAATTCCCTGGGAGAGAAGC	GCTCCACTGCCTTGCTTTTA
IL-17a	Interleukin-17a	CTTCACCTTGGACTCTGAGC	TGGCGGACAATAGAGGAAAC
IFNϒ	Interferon gamma	GCCCTCTCTGGCTGTTACTG	CCAAGAGGAGGCTCTTTCCT
cd11b	Cd11b	GAGAACTGGTTCTGGCTTGC	TCAGTTCGAGCCTTCTT
CCL2	C-C chemokine ligand 2	CCTCCACCACTATGCAGGTC	CAGCCGACTCATTGGGATCA
Olfr59	Olfactory receptor 59	CTGCTAGTCATGGGTGTAGATG	CAAGGGTGATAGAACGGTAAGG
GPR-41	G-protein-coupled receptor-41	TGACGGTGAGCATAGAACGTTT	GCCGGGTTTTGTACCACAGT
GPR-43	G-protein-coupled receptor-43	TCGTGGAAGCTGCATCCA	GCGCGCACACGATCTTT
Occludin	Occludin	AGCCTGGGCAGTCGGGTTGA	ACACAGACCCCAGAGCGGCA
Muc2	Mucin-2	CGATCACCACCATTGCCACTG	ACCACCATTACCACCACCTCAG
ZO-1	Zonula occludens-1	GCCAGCCAGTTCCGCCTCTG	AGGGTCCCGGGTTGGTG
IL-1β	Interleukin-1 beta	GTCACTCATTGTGGCTGTGG	GCAGTGCAGCTGTCTAATGG
TH	Tyrosine hydroxylase	GATTGCTACCTGGAAGGAGGT	AGTCCAATGTCCTGGGAGAAC
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

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ARTICLE 3

Title: "Losartan-induced gut microbial changes has an antihypertensive effect"

Running title: Losartan restored gut microbiota in SHR

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Abstract

Background and Purpose: Hypertension is associated with gut dysbiosis. We aimed to evaluate the effects of the angiotensin receptor blocker losartan on gut microbiota in spontaneously hypertensive rats (SHR), and to test if this modification contributes to its blood pressure (BP) reducing properties.

Experimental Approach: Twenty-weeks old Wistar Kyoto rats (WKY) and SHR were assigned to three groups: untreated WKY (WKY), untreated SHR (SHR), and SHR treated with losartan for 5 weeks (SHR-losartan). A faecal microbiota transplantation (FMT) experiment was also performed by gavage of faecal content from donor SHR-losartan group to SHR recipient.

Key results: Faeces from SHR showed gut dysbiosis, characterised by higher *Firmicutes/Bacteroidetes* ratio, lower acetate- and higher lactate-producing bacteria, and lower strict anaerobic bacteria, which was restored by losartan. The improvement of gut dysbiosis was linked to higher colonic integrity and lower sympathetic drive in the gut. In contrast, hydralazine reduced BP but it did neither restore gut dysbiosis nor colonic integrity. Interestingly, FMT from SHR-losartan to SHR reduced BP, improved the aortic endothelium-dependent relaxation to acetylcholine and reduced NADPH oxidase activity. These vascular changes were linked to both increased Treg and decreased Th17 cells population in the vascular wall.

Conclusions and Implications: Losartan treatment reduced gut dysbiosis in SHR. This effect seems to be related to its capacity to reduce sympathetic drive in the gut, improving gut integrity. The changes induced by losartan in gut microbiota contributed, at least in part, to protect the vasculature and reduce BP, possibly by modulating gut immune system.

Introduction

The gut harbours trillions of bacteria that modulate the host homeostasis within and outside the intestinal tract. Gut microbiota is commonly referred to as an essential acquired organ because its composition and richness are constantly adapting to the challenges occurring in the environment or in the host, such as age, diet, and lifestyle modifications. In addition, it has been commonly observed that a change in the host health status has been accompanied by a shift in the gut microbiota (Marques et al., 2017).

In the periphery, gut microbiota plays an important role in shaping a robust systemic and intestinal immune system (Chow et al., 2010; McDermott and Huffnagle, 2014). Recent data suggest that gut microbiota may also play a role in the development and maintenance of cardiovascular disease and metabolic disorders, such as obesity, diabetes mellitus, and metabolic syndrome (Tang et al., 2013; Tilg and Kaser A, 2011; Everard and Cani, 2013; Howitt and Garrett, 2012). Recently, it has been demonstrated that the normal gut microbiota may influence blood pressure (BP). A direct association between gut microbiota and hypertension in both animal models and humans has been described (Yang et al., 2015; Kim et al., 2018, Toral et al., 2019, Sun et al., 2019). In contrast, Karbach et al. (2016) showed that BP was not different between germ-free and conventionally raised mice, which is consistent with previous observations describing no effect on BP after dramatic reduction in faecal microbial biomass induced by antibiotic treatment (Pluznick et al., 2013). Similarly, it has been described that both the *Firmicutes*/*Bacteroidetes* (F/B) ratio and the abundance levels of selected genera in pre-hypertensive spontaneously hypertensive rats (SHR) were not significantly different from the age matched Wistar Kyoto rats (WKY) (Santisteban et al., 2016). Interestingly, transplantation of microbiota from SHR to WKY (Adnan et al., 2016, Toral et al, 2019a), or from hypertensive human to germ-free mice (Li et al., 2017) increased BP, showing the role of dysbiotic microbiota in the development of hypertension. In human, overgrowth of bacteria such as *Prevotella* and *Klebsiella,* have been described in both pre-hypertensive and hypertensive populations as compared to healthy (Li et al., 2017). Moreover, seven bacterial species (*Parabacteroides johnsonii, Klebsiella unclassified, Anaerotruncus unclassified, Eubacterium siraeum, Prevotella bivia,* and *Rumminococcus torques*) positively, and three (*Bacteroides thetaiotaomicron, Paraprevotella clara,* and *Paraprevotella unclassified*) negatively correlated with systolic BP (SBP) (Kim et al., 2018). In SHR, a strong positive correlation between SBP and the lactate-producing

genus *Lactobacillus* (Adnan et al., 2017), or *Streptococcus* and *Turicibacter* (Yang et al., 2015, Toral et al., 2019b), and a negative correlation between butyrate-producing bacteria genus *Odoribacter* or acetate-producing bacteria genus *Bautia*, with SBP (Toral et al., 2019a) has been described. However, the absence of gut microbiota protects mice from angiotensin II-induced hypertension, vascular dysfunction, and hypertension-induced end-organ damage, showing for the first time that commensal microbiota, an ecosystem acquired after birth, could represent an environmental factor promoting angiotensin II-induced high BP (Karbach et al. 2016). Similarly, oral administration of antibiotics improved BP in angiotensin II-induced hypertension and in SHR (Yang et al., 2015). The BP reduction by minocycline was able to rebalance the hypertension-related dysbiotic gut microbiota by reducing the F/B ratio in angiotensin II-induced hypertension (Yang et al., 2015). Similarly, the consumption of probiotic *Lactobacillus* strains reduced BP in SHR (Gómez-Guzmán et al., 2015), in tacrolimus-induced hypertension in mice (Toral et al., 2018) and in high salt diet-induced hypertension in mice (Wilck et al., 2017), showing that restoring the microbiota composition to a similar composition as that found in normotensive animals led to the improvement of BP. In addition, increased permeability of gut epithelial barrier, intestinal inflammatory status and dysbiosis are associated with BP elevation in angiotensin II-infused mice (Kim et al., 2018). In fact, the inhibition of the renin-angiotensin system (RAS) by the angiotensin-converting enzyme (ACE) inhibitor captopril reduces BP, reverses gut pathology (Santisteban et al., 2017), and shifts the gut microbiota composition in SHR (Yang et al., 2019). However, whether BP reduction by other RAS inhibitors, such as the angiotensin receptor blocker losartan, shifts the gut microbiota composition is unclear. Whether changes in microbiota induced by these drugs are involved in their antihypertensive effects is also unknown.

Normal microbiota regulates immune homeostasis, such as the balance between T helper 17 (Th17) and regulatory T cells (Treg) in gut lymph organs and in the vascular wall, which is involved in BP regulation (Karbach et al., 2016; Toral et al., 2018). Furthermore, a critical role of the interaction gut microbiota-sympathetic nervous system in the regulation of BP has been recently described (Toral et al., 2019). We hypothesised that BP reduction in established hypertension might contribute to correct gut dysbiosis in SHR, which also contribute to maintain low BP through change T cell populations in the vascular wall. Therefore, the aim of this study was to evaluate the effects of losartan on

gut microbioma in SHR, and the role of gut microbiota in the antihypertensive effect of losartan.

Results

Losartan treatment reduces gut dysbiosis in SHR

The compositions of bacterial communities were evaluated by calculating three major ecological parameters, including Chao richness, Pielou evenness, and Shannon diversity and the number of observed species. Reduced richness and diversity without changes in evenness and observed species were found in gut microbiota from SHR compared to those found in WKY. Losartan did not restore these changes despite its antihypertensive effect (Figure S1A). We performed a three-dimensional PCA of the bacterial community, which measures microorganism diversity between samples, i.e. β-diversity, at the level of the different taxa (phylum, class, order, family, genus and species), in an unsupervised manner. This analysis at the phylum level showed a perfect clustering of the animals into the SHR and WKY groups, with no perfect clustering of the animals into SHR-losartan group (Figure S1B). The composition of the faecal microbial communities of the WKY and SHRs was found to be distinct from the previously reported composition (Yang et al., 2015). A clear separation was observed in the PCA between the 2 clusters representing the microbial compositions of WKY and SHR, indicating 2 extremely different gut environments. However, the cluster SHR-losartan is closer to WKY than to SHR, being the key bacterial populations that are responsible for discriminating among groups the phylum *FIrmicutes* (loading 0.84). The Kaiser-Meyer-Olkin (KMO) test, that measure sampling adequacy, was 0,777, indicating a middling sampling. The Barlett's test of sphericity was < 0.05. The analysis of the phyla composition showed that *Firmicutes* was the most abundant phylum in the rat faeces, followed by *Proteobacteria*, *Actinobacteria, Bacteroidetes,* and *Verrucomicrobia* (Figure 1A). The proportion of bacteria from the *Fimicutes* phylum was significantly higher in SHR than in WKY and SHR-losartan, whereas the proportions of *Actinobacteria* and *Bacteroidetes* were lower in SHR than in the other groups. Losartan treatment restored bacteria from the *Firmicutes* and *Bacteroidetes* phyla to levels similar to those found in WKY. The F/B ratio, a signature of gut dysbiosis in hypertension (Yang et al., 2015), was

≈ 3-fold higher in SHR than in WKY, and this ratio returned to normal values by the action of losartan (Figure 1B). In addition, significant lower percentages of acetate- and propionate-producing bacteria, and a higher percentage of lactate-producing bacteria, without significant differences in the percentage of butyrate-producing bacteria, were found in SHR compared to WKY; these effects were abolished by losartan treatment (Figure 1C). Moreover, the percentage of strict anaerobic bacteria was significantly lower in SHR compared to WKY, no significant differences in strict aerobic bacteria were found between SHR and WKY. Losartan restored this change in the percentage of anaerobic bacteria (Figure 1D). Figure S2 shows the bacterial taxa (class, order, family, and genus) that were altered in SHR according to LEfSe. Prominent changes in bacterial taxa occurred, where the relative abundance of 51 taxa was increased (green) and 19 taxa were decreased (red) in WKY compared to SHR. Losartan treatment of SHR also induced several changes in the microbiota taxa compared to SHR, where the relative abundance of 41 taxa was increased (green) and 16 taxa was decreased (red) (Figure S3). We also identified what bacterial families (Figure S4) and genera (Figure S5) were associated with changes in the composition of microbiota. The key bacterial populations that are responsible for discriminating among groups was the familia *Clostridiaceae* (loading 0.38), and the genus *Oscillospira* (loading 0.78). The KMO test was 0,84, and 0,82, indicating in both cases a meritorious sampling for PCA at the family and genus levels, respectively. The Barlett's test of sphericity was < 0.05 in both cases. A significant depletion of *Verrucomicrobiacea, Pedobacter,* and *Akkermansia*, with a higher abundance of *Lactobacillaceae*, and *Lactobacillus* was found in the microbiota of SHR group compared to WKY. Losartan treatment tended to restore changes in *Verrucomicrobiacea, Pedobacter,* and *Akkermansia*, and significantly restored changes in *Lactobacillaceae and Lactobacillus*.

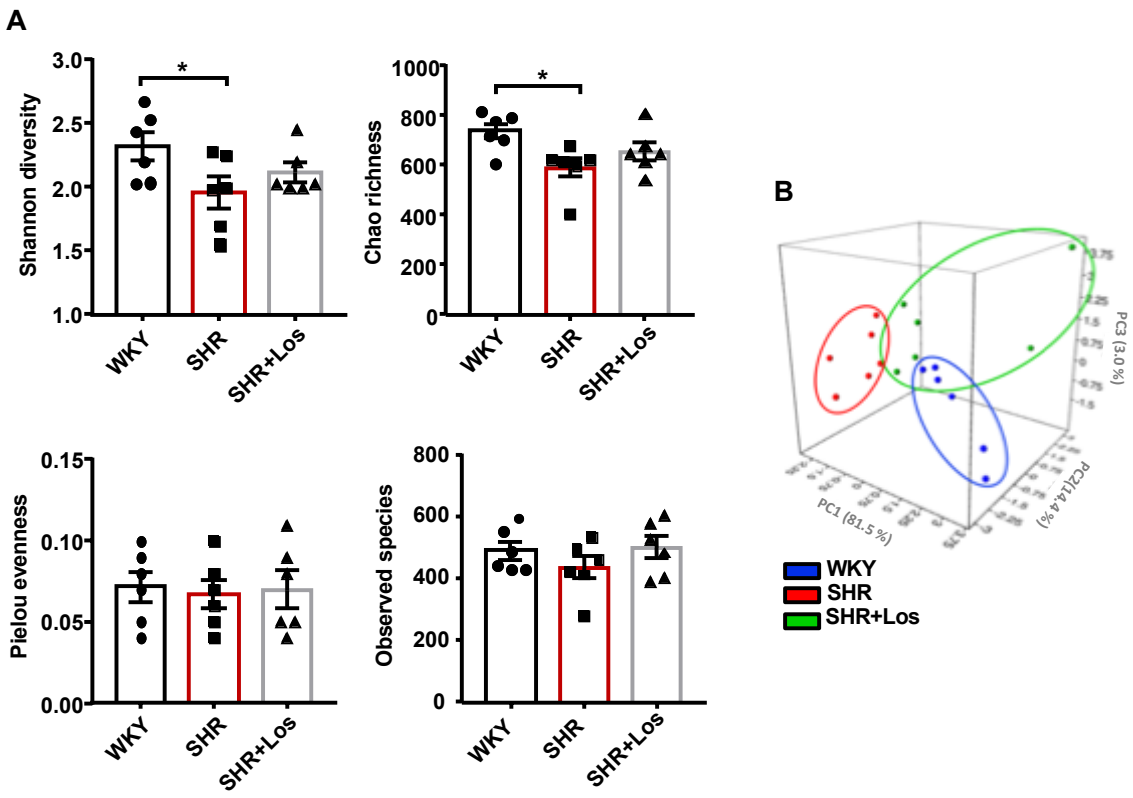


Figure S1. Losartan (Los) does not induce changes in the microecological parameters of gut microbiota of spontaneously hypertensive rats (SHR). *Changes in alpha-diversity parameters (i.e., Shannon diversity, Chao richness, Pielou evenness, and the observed species) (**A**), and the principal coordinate analysis of the Phyla (**B**) were examined in the gut microbiota of Wistar Kyoto rats (WKY), SHR and SHR treated with Los (SHR-Los). Data are expressed as mean ± SEM. *P< 0.05 significant differences compared with WKY.*

A

Phylum	WKY (n=6)	SHR (n=6)	SHR+Los (n=6)
Firmicutes	72.6 ± 5.0	86.4 ± 1.4*	74.8 ± 4.3#
Proteobacteria	11.0 ± 4.8	5.8 ± 1.4	7.5 ± 2.3
Actinobacteria	6.1 ± 1.0	3.5 ± 0.6*	4.4 ± 1.2
Bacteroidetes	5.1 ± 1.4	1.7 ± 0.3*	7.7 ± 1.5#
Verrucomicrobia	1.9 ± 0.6	0.8 ± 0.3	2.5 ± 1.2

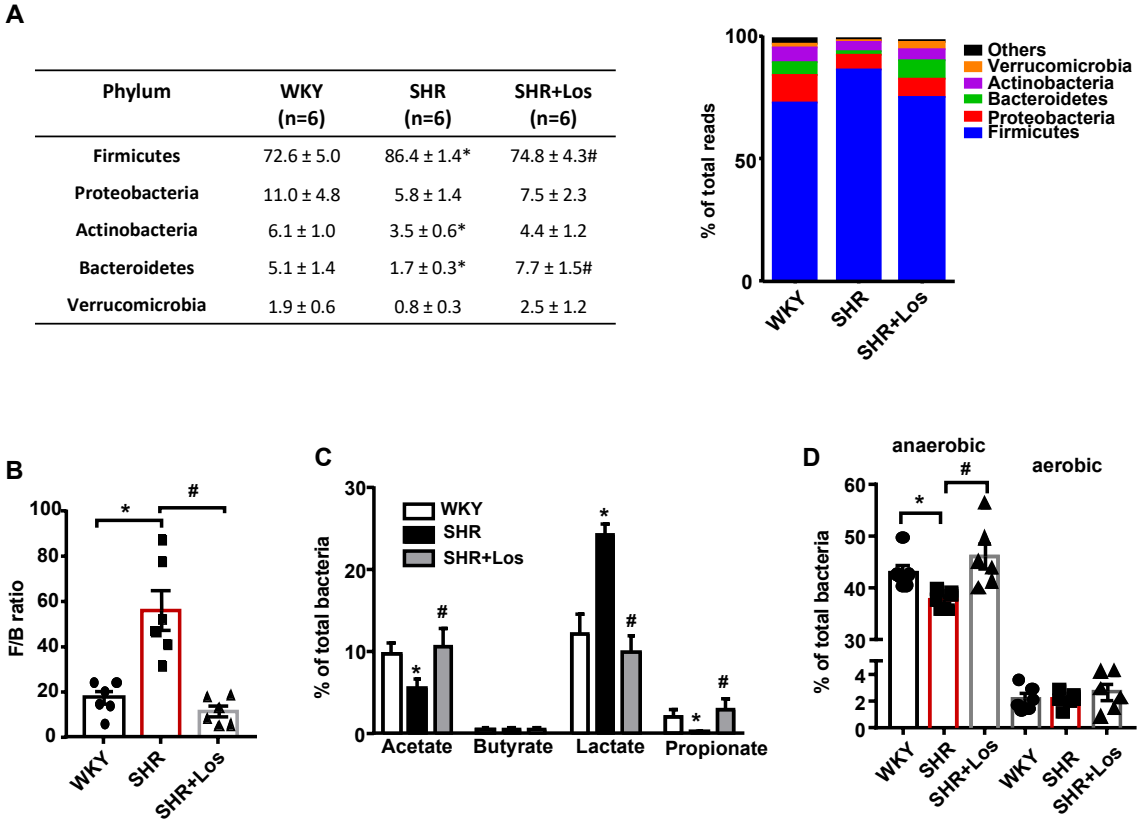
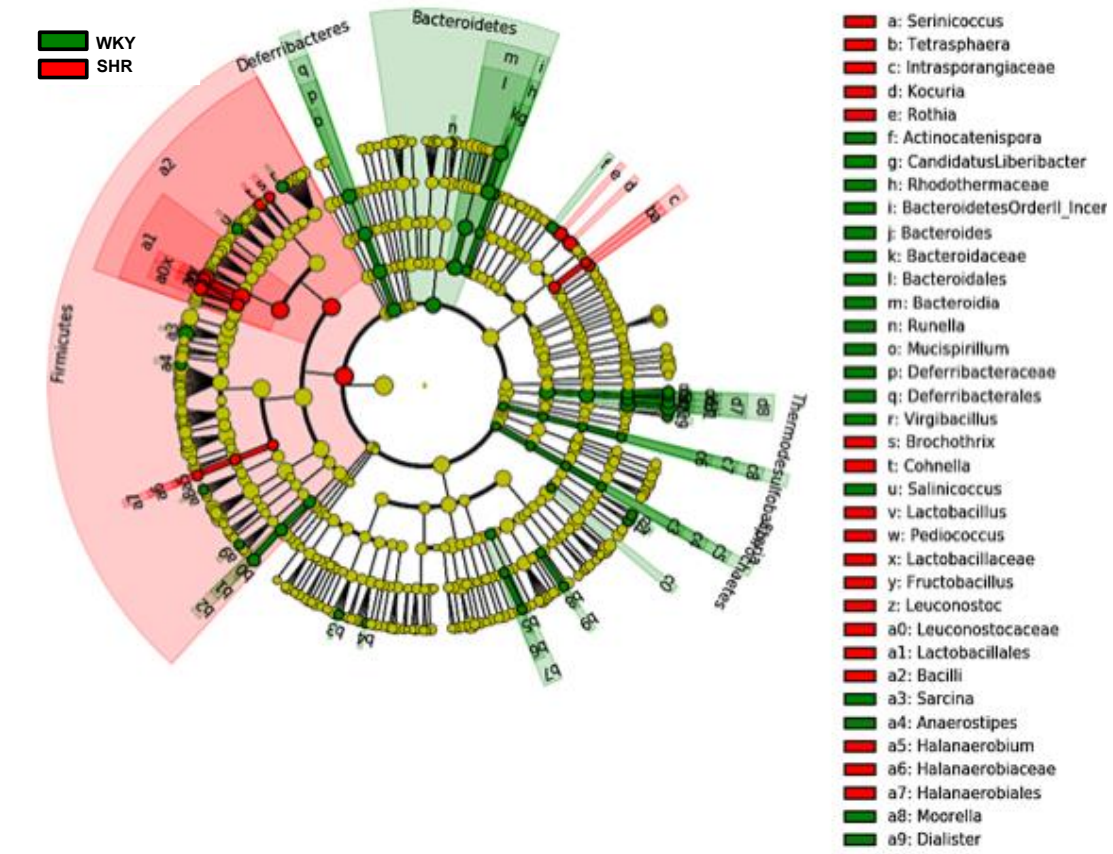


Figure 1. Losartan (Los) induces different behaviours of the gut microbiota of spontaneously hypertensive rats (SHR). *Faecal samples were collected and bacterial 16S ribosomal DNA was amplified and sequenced to analyse the composition of microbial communities. Phylum breakdown of the 5 most abundant bacterial communities in the faecal samples obtained from all experimental groups (**A**). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis (**B**). Relative proportions of acetate-, butyrate-, lactate-, and propionate-producing bacteria (**C**). Anaerobic and aerobic bacteria expressed as relative proportions (**D**) in the gut microbiota in Wistar Kyoto rats (WKY), untreated SHR (SHR) and SHR treated with losartan (SHR-Los). Values are expressed as mean ± SEM (n = 6). *P< 0.05 significant differences compared with WKY rats. #P< 0.05 significant differences compared with the untreated SHR.*

A


WKY
SHR
Deferribacteres
Bacteroidetes
Firmicutes
Thermodesulfobacteria
a: Serinicoccus
b: Tetrasphaera
c: Intrasporangiaceae
d: Kocuria
e: Rothia
f: Actinocatenispora
g: CandidatusLiberibacter
h: Rhodothermaceae
i: BacteroidetesOrderII_Incer
j: Bacteroides
k: Bacteroidaceae
l: Bacteroidales
m: Bacteroidia
n: Runella
o: Mucispirillum
p: Deferribacteraceae
q: Deferribacterales
r: Virgibacillus
s: Brochothrix
t: Cohnella
u: Salinicoccus
v: Lactobacillus
w: Pediococcus
x: Lactobacillaceae
y: Fructobacillus
z: Leuconostoc
a0: Leuconostocaceae
a1: Lactobacillales
a2: Bacilli
a3: Sarcina
a4: Anaerostipes
a5: Halanaerobium
a6: Halanaerobiaceae
a7: Halanaerobiales
a8: Moorella
a9: Dialister

B

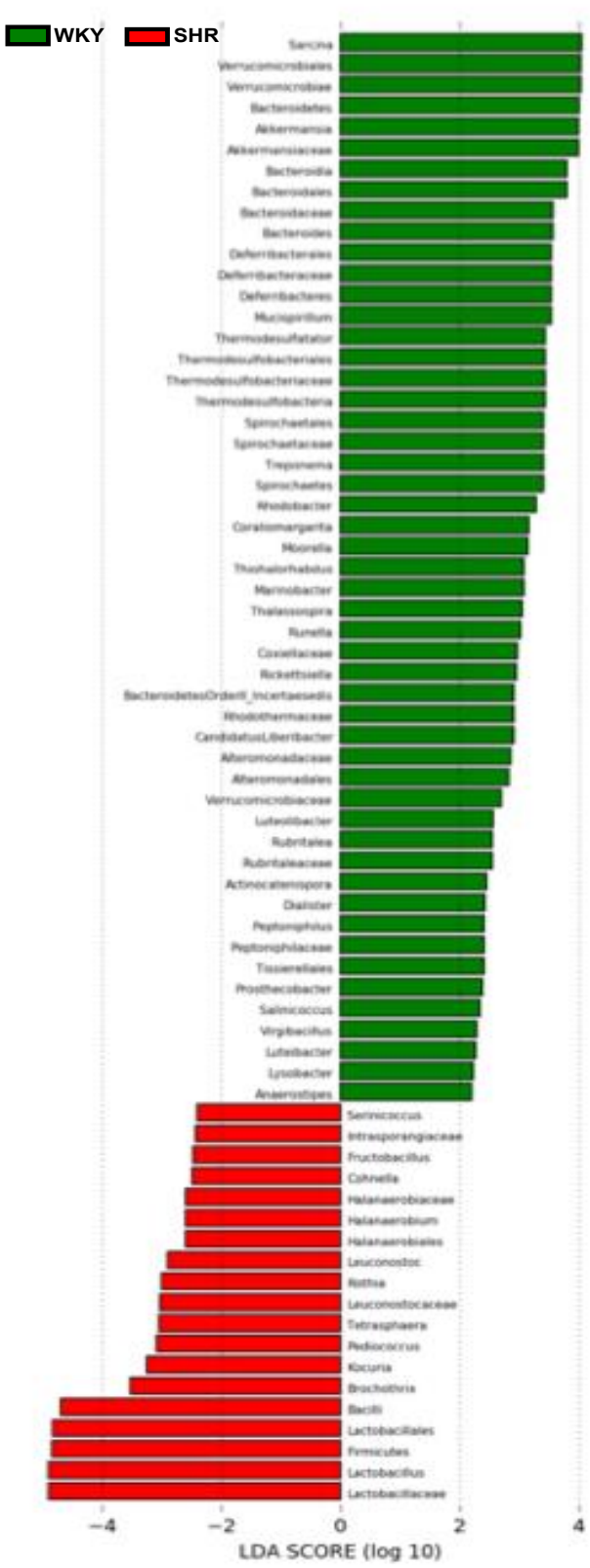
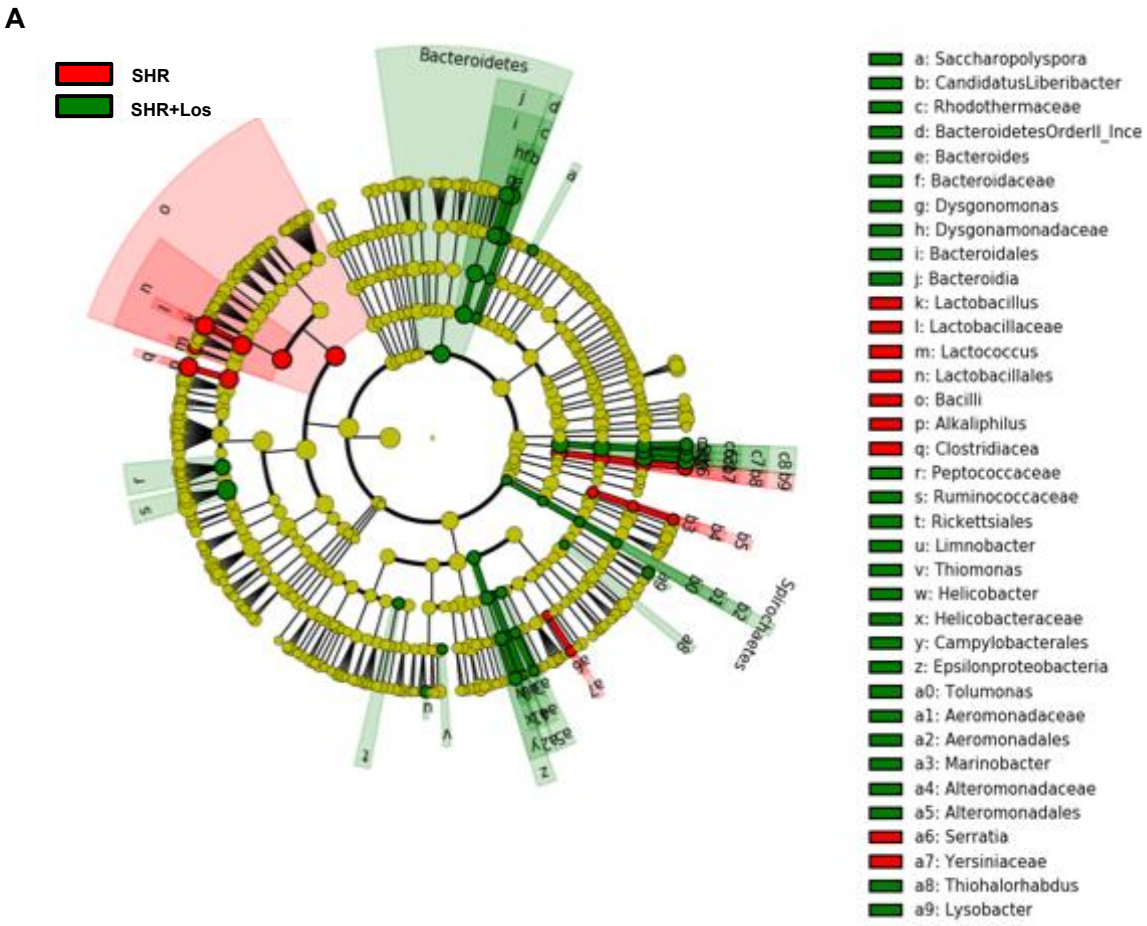

WKY
SHR
Sarcina
Verrucomicrobiales
Verrucomicrobiae
Bacteroidetes
Akkermansia
Akkermansiaceae
Bacteroidia
Bacteroidales
Bacteroidaceae
Bacteroides
Deferribacterales
Deferribacteraceae
Deferribacteres
Mucispirillum
Thermodesulfatator
Thermodesulfobacteriales
Thermodesulfobacteriaceae
Thermodesulfobacteria
Spirochaetales
Spirochaetaceae
Treponema
Spirochaetes
Rhodobacter
Coraliomargarita
Moorella
Thiohalorhabdus
Marinobacter
Thalassospira
Runella
Coxiellaceae
Rickettsiella
BacteroidetesOrder8_Incertaesedis
Rhodothermaceae
CandidatusLiberibacter
Alteromonadaceae
Alteromonadales
Verrucomicrobiaceae
Lutaribacter
Rubritalea
Rubritaleaceae
Actinocatenospora
Okalister
Peptoniphilus
Peptoniphilaceae
Tissierellales
Prosthecobacter
Salmicoccus
Virgibacillus
Lutaibacter
Lysobacter
Anaerostipes
Sennicoccus
Intrasporangiaceae
Fructobacillus
Colwellia
Halanaerobiaceae
Halanaerobium
Halanaerobiales
Leuconostoc
Rothia
Leuconostocaceae
Tetrasphaera
Pediococcus
Kocuria
Brochothrix
Bacilli
Lactobacillales
Firmicutes
Lactobacillus
Lactobacillaceae
−4
−2
0
2
4
LDA SCORE (log 10)

Figure S2. Distinct changes in the gut microbiota between Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). *Cladograms show the significantly enriched taxa, the taxa are identified on the key to the right of each panel. Larger circles represent greater differences in abundance between groups (**A**). Comparisons of microbiome changes in WKY versus SHR. Linear discriminant analysis effect size (LEfSe) identified significantly different bacterial taxa enriched in each cohort at LDA Score > 2, p<0.05 (green bars represent WKY-enriched taxa, red bars represent SHR-enriched taxa) (**B**). n = 6 rats per experimental group in each comparison.*



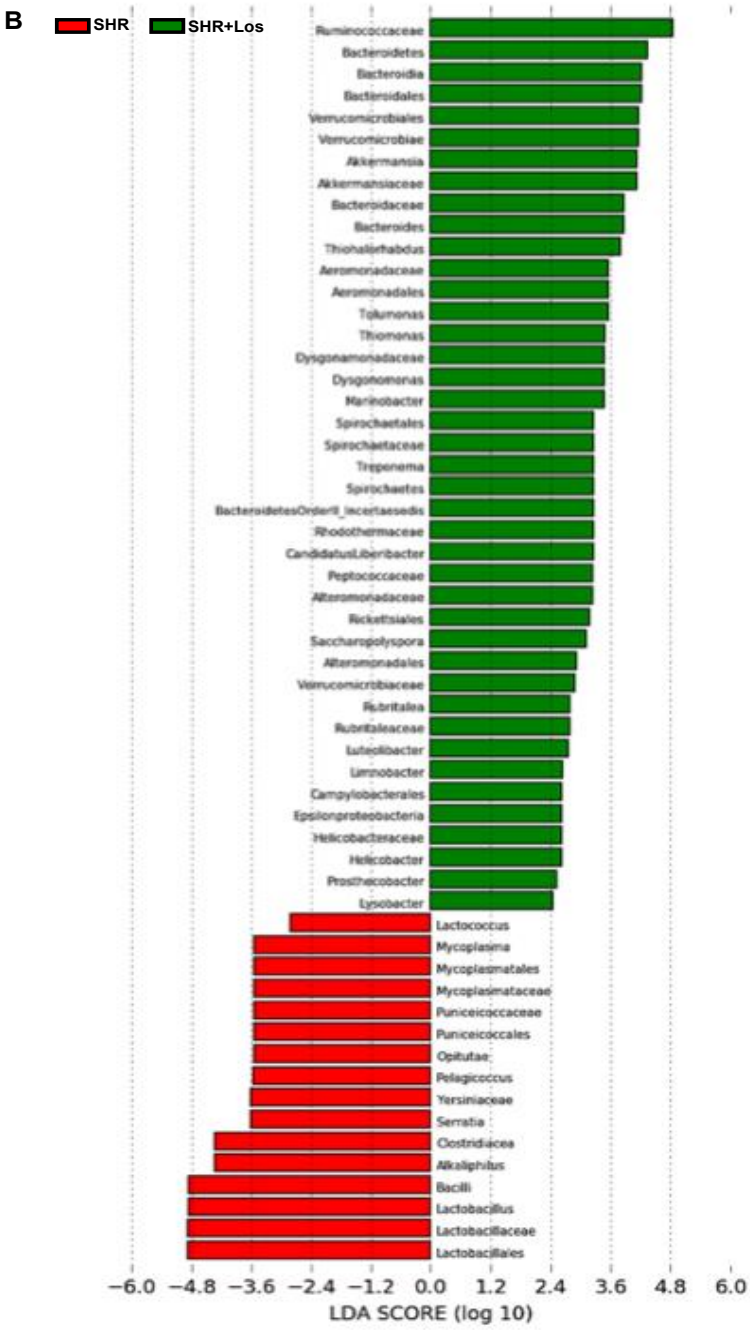


Figure S3. Losartan (Los) induced different behaviors of gut microbiota in spontaneously hypertensive rats (SHR). *Comparisons of microbiome changes in SHR versus SHR-Los. Cladograms show the significantly enriched taxa, the taxa are identified on the key to the right of each panel. Larger circles represent greater differences in abundance between groups (**A**). Linear discriminant analysis effect size (LEfSe) identified significantly different bacterial taxa enriched in each cohort at LDA Score > 2, $p<0.05$ (red bars SHR enriched, green bars SHR-Los enriched) (**B**). n = 6 rats per experimental group in each comparison.*

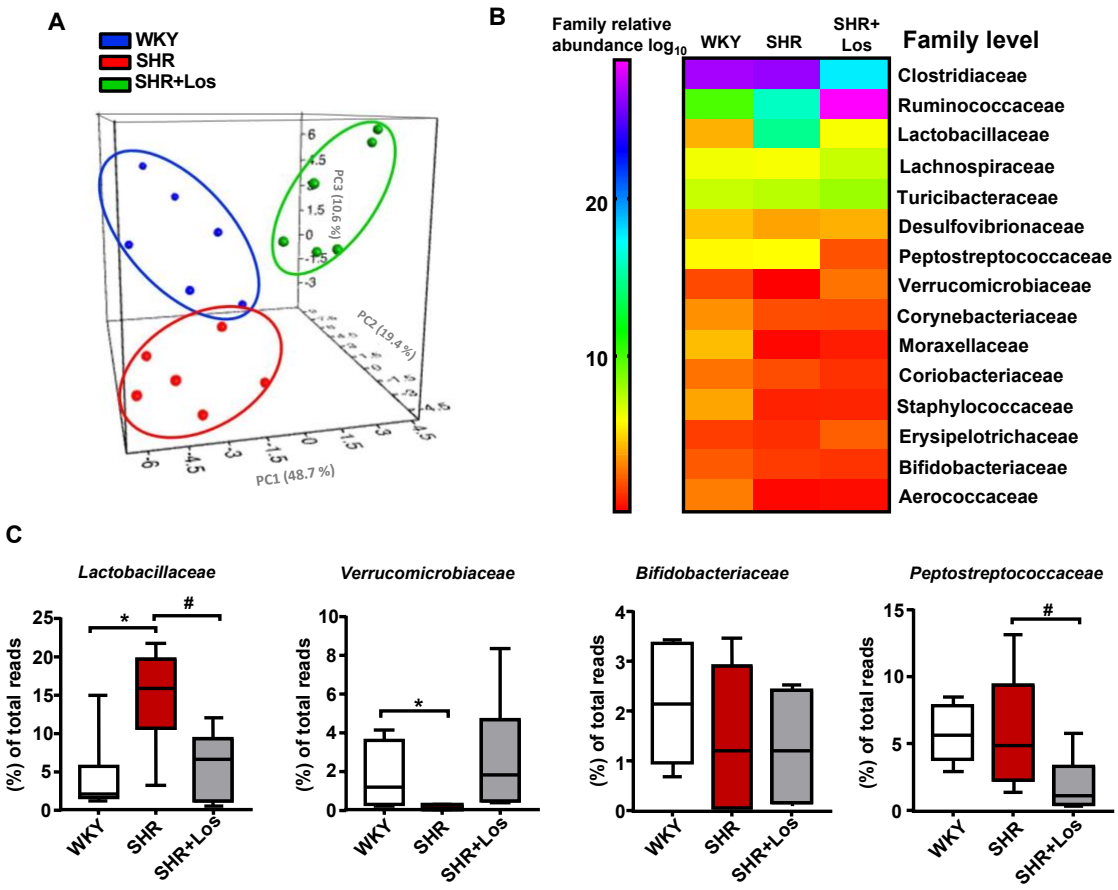


Figure S4. Losartan (Los) induced changes at the family level in the gut microbiota of spontaneously hypertensive rats (SHR). *Principal coordinate analysis of families (**A**) in the gut microbiota from all experimental groups. Samples clustered by experimental group demonstrated that the experimental conditions resulted in distinct populations of bacteria at the family level. Heat map showing differences in the relative abundance of the bacterial families between the three groups studied (**B**). The colours of the heat map represent the relative percentage of microbial family assigned within each sample. The most significantly modified bacterial families (**C**) in the gut microbiota in Wistar Kyoto rats (WKY), SHR and SHR treated with Los (SHR-Los). Values are expressed as mean ± SEM (n = 6). *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with untreated SHR.*

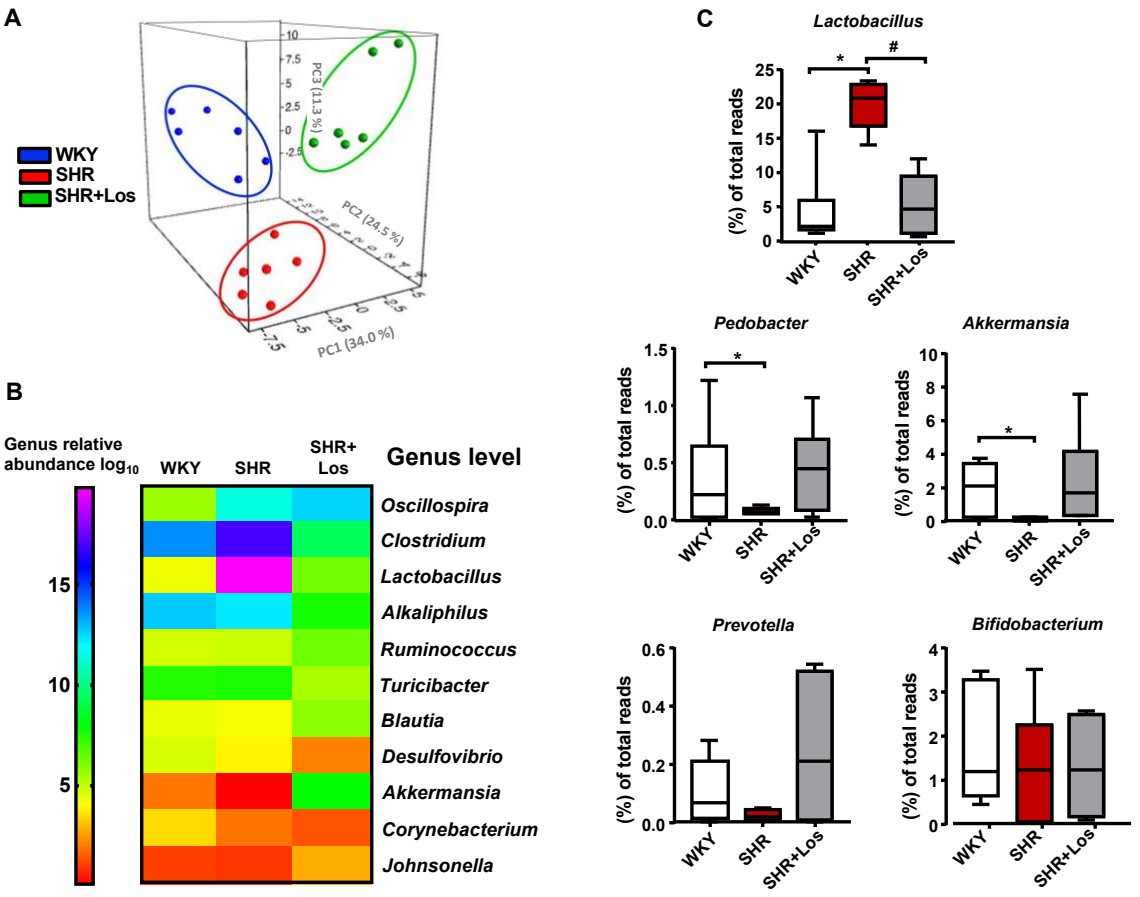


Figure S5. Losartan (Los) induced changes at the genus level in the gut microbiota of spontaneously hypertensive rats (SHR). *Principal coordinate analysis of genera (**A**) in the gut microbiota from all experimental groups. Samples clustered by experimental group demonstrated that the experimental conditions resulted in distinct populations of bacteria at the genus level. Heat map showing differences in the relative abundance of the bacterial genera between the three groups studied (**B**). The colours of the heat map represent the relative percentage of microbial genus assigned within each sample. The most significantly modified bacterial genera (**C**) in the gut microbiota in Wistar rats (WKY), SHR and SHR treated with Los (SHR-Los). Values are expressed as mean ± SEM (n = 6). *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with untreated SHR.*

Losartan improved intestinal integrity, α-defensins production, gut sympathetic tone, and changed MLNs T cell population in SHR

Hypertension is associated with the altered expression of gut tight junction proteins, increased permeability and gut pathology. The ACE inhibitor captopril, which lowers BP in SHR, reverses gut pathology (Santisteban et al., 2017). We also found reduced mRNA levels of barrier-forming junction proteins (occludin and zonula

occludens-1 (ZO-1)) in the colon of SHR compared to WKY (Figure 2A). Losartan treatment restored occludin and ZO-1 mRNA levels in the colon, suggesting an enhanced barrier function. In addition, increased gut permeability in adult hypertensive SHR correlates with reduced goblet cells (Santisteban et al., 2017). Goblet cells produce mucins, which protect the gut from pathogen invasion, thereby regulating gut immune response (Mowat AM, Agace WW, 2014). We have also found downregulation of mucin (MUC)-2 transcripts, without change in MUC-3 in SHR than in WKY, which was restored by losartan (Figure 2A), suggesting reduced gut permeability. We measured endotoxin levels in plasma, and found them to be significantly higher in SHR compared with the WKY group (Figure 2B). Interestingly, the long-term treatment with losartan significantly decreased 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. Furthermore, the increased mRNA levels of the colonic pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-6 (Figure 2C) in SHR were significantly reduced by losartan administration. RAS participates in the regulation of gastrointestinal inflammation (Garg et al., 2012; Brzozowski, 2014) and has been linked to human inflammatory bowel diseases (IBD) (Hume et al., 2016). In fact, pharmacological inhibition of classical RAS with losartan is capable to alleviate murine models of IBD, independently of their antihypertensive effects (Wengrower et al., 2012; Liu et al, 2016). However, we did not found any changes in the mRNA levels of several RAS components, such as angiotensinogen (AGT), ACE and ACE2 (Figure 3A), in colonic samples from all experimental groups, suggesting that the protective effects of losartan in both colonic integrity and inflammation seems to be independent of local RAS inhibition. Santisteban et al., (2017) showed that the increase in the sympathetic nerve activity to the gut lead to alteration of gut junction proteins in SHR. We found an increase in the expression of tyrosine hydroxylase (TH), a key enzyme involved in NA generation and NA content in the gut from SHR compared to WKY, which was abolished by chronic losartan treatment (Figure 3B). These results suggest that reduced sympathetic tone in the gut could be involved in the protective effects of losartan in the gut. Epithelial intestinal cells are able to secrete antimicrobial peptides, α-defensins, which are cysteine-rich cationic peptides with antibiotic activity against a wide range of bacteria and other microbes (Bevins, 2005), to maintain the intestinal microbiota composition (Hashimoto et al., 2012). We found reduced mRNA levels of the α-defensin RNP1-2, and increased mRNA levels of RNP3 and RNP4, without significant change of RNP5 in colon from

SHR compared to the WKY group. Again, losartan treatment restored the mRNA levels of defensins to levels similar to those found in WKY (Figure 3C). Under altered gut mucosal integrity, bacteria are able to translocate across the intestinal epithelium leading to activation and migration of CX3CR1+ cells, such as macrophages and dendritic cells, to draining lymph nodes of the lower intestinal tract (Niess et al., 2005). They also present soluble antigen to naïve CD4+ T cells, leading to T cell activation. We found that the number of total lymphocytes in MLNs was higher in SHR compared to WKY (Figure 4). Losartan treatment did not change the content in lymphocytes in MLNs. The percentage of Treg (CD4+/FoxP3+) was reduced, whereas the percentage of Th17 (CD4+/IL-17+) lymphocytes was significantly increased in MLNs in SHR compared to WKY (Figure 4). Losartan increased only Treg population in MLNs without affecting Th17 population.

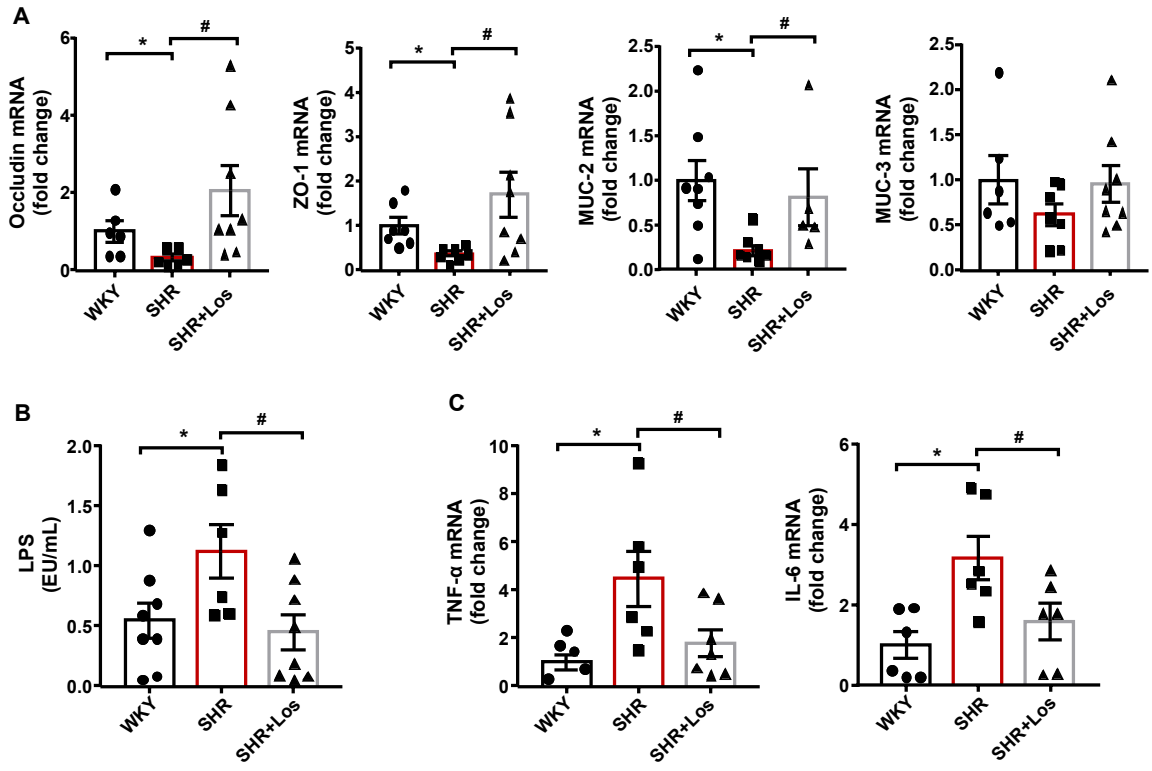


Figure 2. Losartan (Los) induces improvement of gut inflammation and permeability in spontaneously hypertensive rats (SHR). *Colonic mRNA levels of occludin, zonula occludens-1 (ZO-1), mucin (MUC)-2, and MUC-3 (**A**). Plasma endotoxin concentrations (endotoxin units/mL (EU/mL)) (**B**). Pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 (**C**) in Wistar Kyoto rats (WKY), untreated SHR (SHR) and SHR treated with losartan (SHR-Los). Values are expressed as mean ± SEM (n = 8). *P< 0.05 significant differences compared with WKY rats. #P< 0.05 significant differences compared with the untreated SHR rats.*

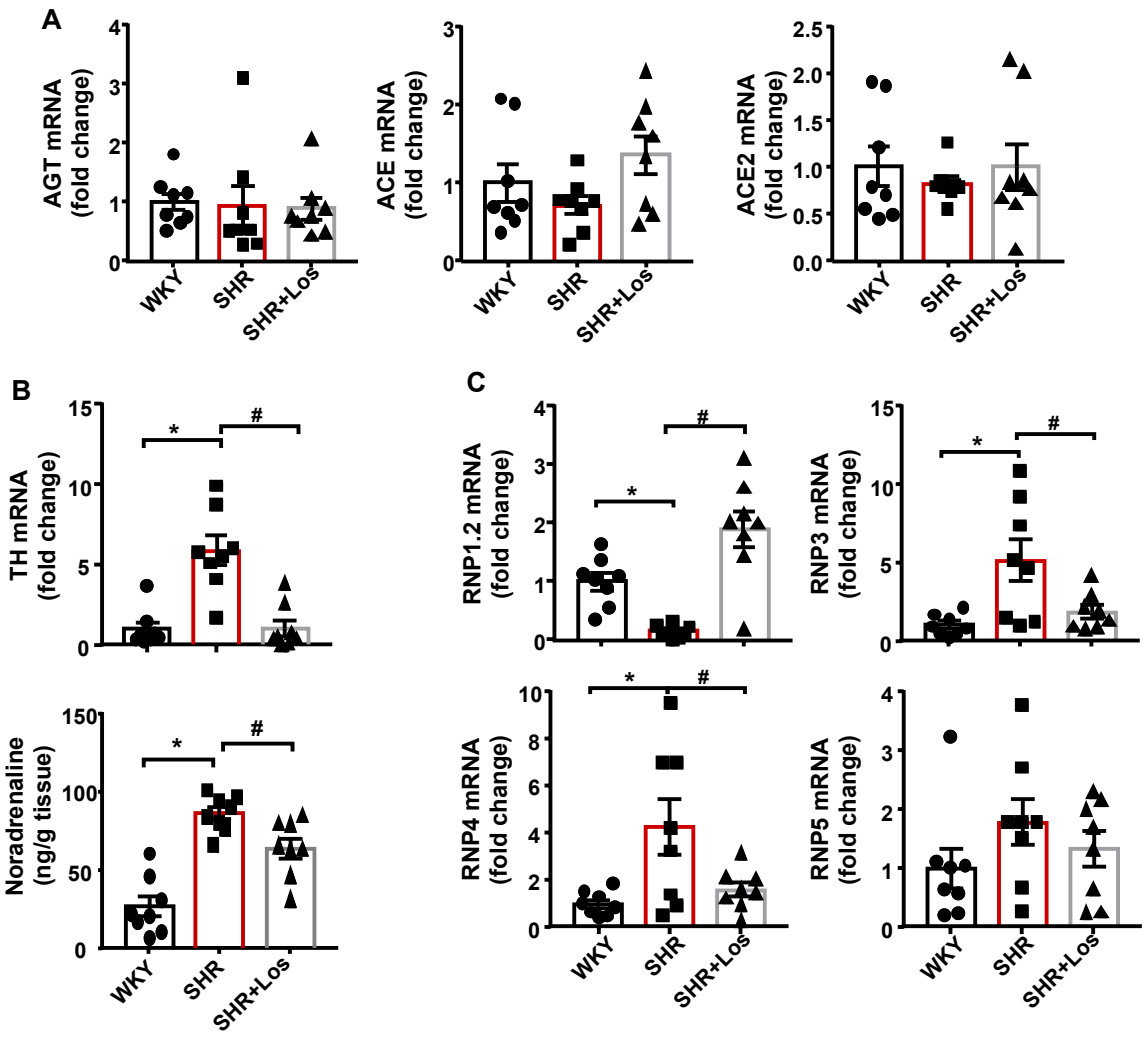


Figure 3. Losartan (Los) induces improvement of α-defensins production and gut sympathetic tone in spontaneously hypertensive rat (SHR). mRNA levels of several renin angiotensin system such as angiotensinogen (AGT), angiotensin-converting enzyme (ACE) and ACE2 (**A**). mRNA levels of tyrosine hydroxylase (TH) expression and noradrenaline content (**B**). mRNA levels of α-defensins (RNP1.2, RNP3, RNP4, and RNP5) in colon from Wistar Kyoto rats (WKY), untreated SHR (SHR) and SHR treated with losartan (SHR-Los). Values are expressed as mean ± SEM. *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with the untreated SHR.

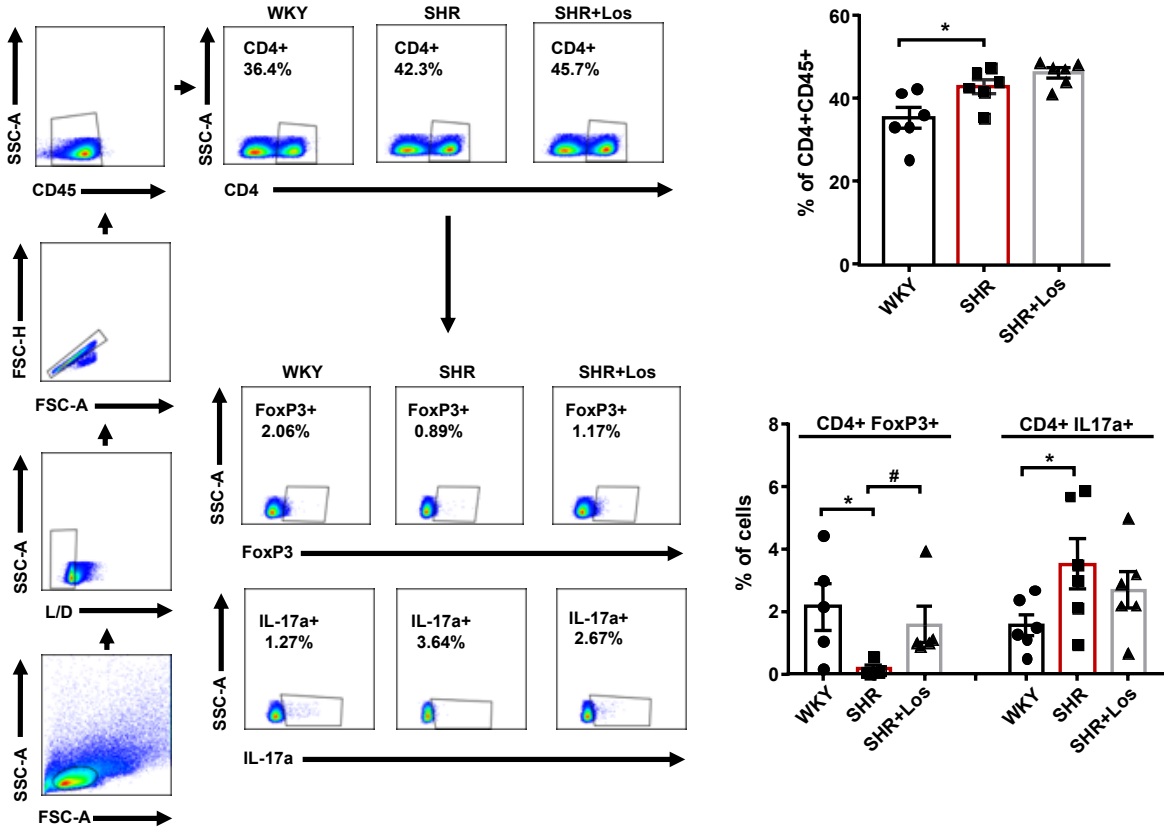


Figure 4. Losartan (Los) induces changes in T-cell polarization in mesenteric lymph nodes in spontaneously hypertensive rats (SHR). *Total T cells (CD4+CD45+), regulatory T cells (Treg; CD4+ FoxP3+), and T helper (Th)-17 (CD4+ IL17a+) cells measured in mesenteric lymph nodes in Wistar Kyoto rats (WKY), SHR and SHR treated with losartan (SHR-Los). Values are expressed as mean ± SEM. *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with untreated SHR.*

Losartan reduces blood pressure and organ target damage and improves vascular nitric oxide (NO) pathway, oxidative stress and inflammation

As expected, SHR receiving chronic losartan treatment showed a progressive decrease in SBP (Figure S6A), which was already significant after the first week, and was almost normalised after 5 weeks (a decrease of 54.7 ± 9.4 mmHg). No significant differences were found among groups in heart rate (not shown). At the end of the study period, losartan prevented the increase in the left ventricular weight index (≈ 12 %) and in urine protein excretion (≈ 51 %) found in SHR compared with the WKY counterparts (data not shown). Aortas from the SHR control group showed strongly reduced endothelium-dependent vasodilator responses to acetylcholine compared to aortas from

the WKY group, which was restored after losartan treatment (Figure S6B). The incubation with L-NAME in the organ bath abolished the relaxation response to acetylcholine in all experimental groups, involving NO in this relaxation (data not shown). The presence of the non-selective NADPH oxidase inhibitor apocynin in the organ bath increased the relaxation response to acetylcholine in untreated SHR until reaching similar relaxation percentages to those found in WKY (Figure S6B), suggesting that an increased NADPH oxidase activity is involved, at least in part, in the impaired relaxation to acetylcholine in aorta from SHR. In addition, no differences were observed among groups in the endothelium-independent vasodilator responses to the NO donor sodium nitroprusside in aortic rings, excluding changes in NO pathway in smooth muscle (data not shown). Rings from SHR showed both marked increased ROS content (Figure S6C), measured by red staining to ethidium in vascular wall (≈ 27 %), and NADPH oxidase activity (Figure S6D) compared to WKY, which were suppressed by losartan. The infiltration of T cells and macrophages measured by immunofluorescence was higher in aorta from SHR than from WKY counterparts, and was inhibited by losartan (Figure 5A). In addition, we also analysed the transcript level of transcription factor FoxP3, retinoid-related orphan receptor-γ (RORγ), T-bet and GATA3, as markers of accumulation of Treg, Th17, Th1 and Th2, respectively, in aorta from all experimental groups (Figure 5B). Aortic Th17, Th1, and Th2 contents were increased in SHR group, whereas Treg content was reduced. Aorta from the SHR-losartan group showed levels of T cells similar to those found in WKY (Figure 5B). The mRNA expression of pro-inflammatory cytokines IL-17a, IFN-γ and TNF-α was higher, whereas the mRNA levels of anti-inflammatory IL-10 were lesser in aortic homogenates from SHR than in WKY. Losartan treatment significantly suppressed these changes (Figure 5C).

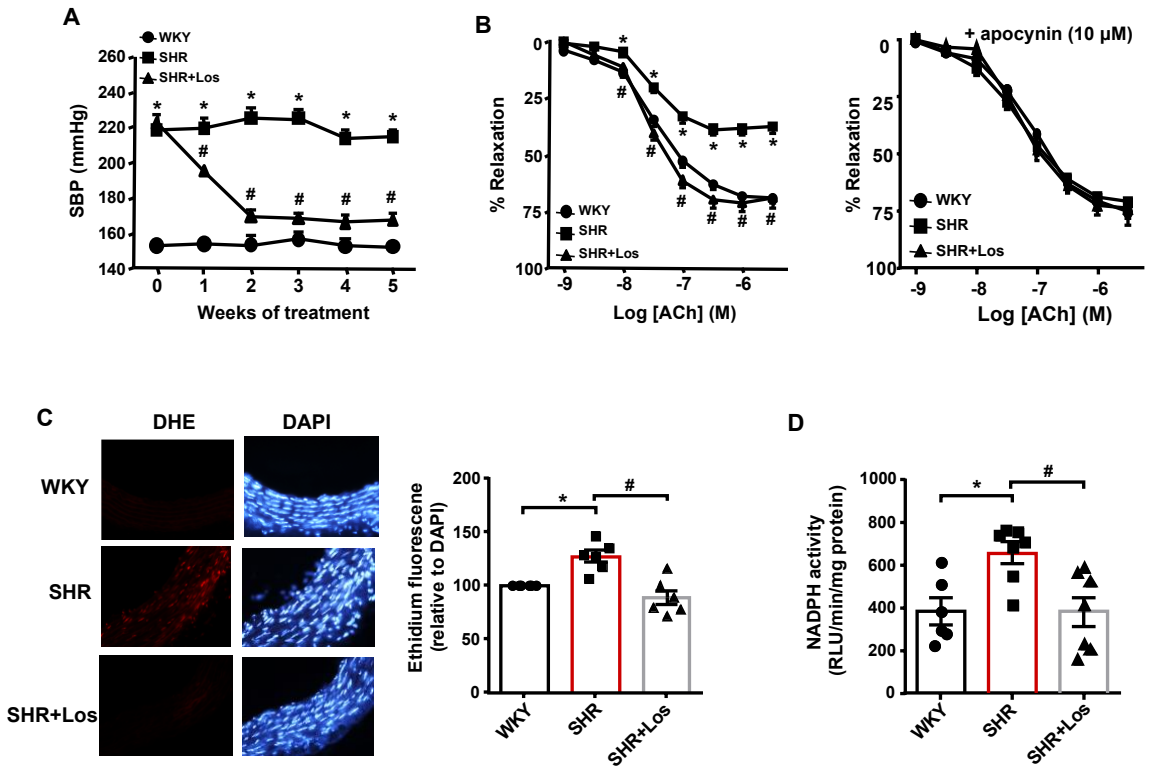


Figure S6. Protective effects of Losartan (Los) in vascular function and oxidative stress in spontaneously hypertensive rats (SHR). *Systolic blood pressure (SBP, **A**) was measured by tail-cuff plethysmography in all experimental groups. Vascular relaxation responses induced by acetylcholine (ACh) in endothelium-intact aortas pre-contracted by phenylephrine in the absence (**B**) and in the presence of the NADPH oxidase inhibitor apocynin (10 µM). Pictures on the left show aortic rings incubated in the presence of dihydroethidium (DHE), which produces a red fluorescence when oxidized to ethidium by reactive oxygen species. Pictures on the right show blue fluorescence of the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI; x 400 magnification). Red ethidium fluorescence values normalized to blue DAPI fluorescence, averaged and expressed as mean ± SEM (**C**). Lucigenin-enhanced chemiluminescence measurement of NADPH oxidase activity (**D**) in Wistar Kyoto rats (WKY), SHR and SHR treated with Los (SHR-Los). Values are expressed as mean ± SEM. *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with untreated SHR.*

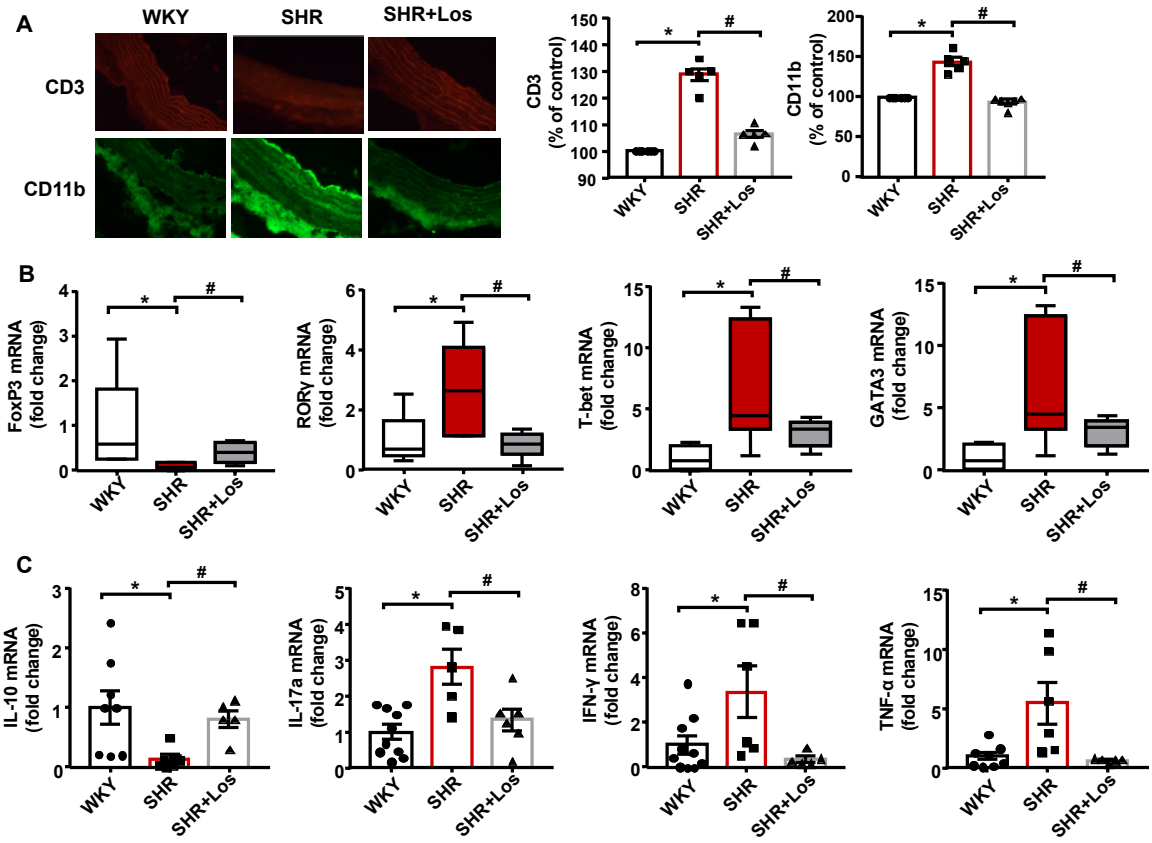


Figure 5. Losartan (Los) induces improvement of macrophage and T cell infiltration in vascular wall from spontaneously hypertensive rats (SHR). *Pictures on the top show aortic T cell infiltration measured by immunostaining of CD3 (red fluorescence). Pictures on the bottom show aortic macrophage infiltration measured by immunostaining of CD11b (green fluorescence) (x 400 magnification). Data are represented as mean ± SEM of the red or green fluorescence (**A**). T-cells infiltration in aortas from all experimental groups measured by mRNA levels of regulatory T cells (Treg; FoxP3), T helper (Th)17; RORγ), Th1 (T-bet), and Th2 (GATA-3) cells (**B**). mRNA levels of interleukin (IL)-10, IL-17a, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (**C**) in aortas from Wistar Kyoto rats (WKY), SHR and SHR treated with losartan (SHR-Los). Values are expressed as mean ± SEM. *P< 0.05 significant differences compared with WKY. #P< 0.05 significant differences compared with untreated SHR.*

Hydralazine reduced blood pressure, cardiac hypertrophy and improved endothelial function, but did not restore gut microbiota and gut integrity.

To differentiate between the effects of blocking AT1 receptors *vs.* lowering arterial pressure, the effect of hydralazine, another vasodilator-acting antihypertensive drug, on gut microbiota was tested. This drug also reduced SBP, being significant at the first week of treatment and reaching a decrease of 73.4 ± 7.6 mm Hg after 5 weeks, similar to that induced by losartan (Figure 6A). In contrast, hydralazine significantly increased heart rate (Figure 6B), suggesting increased sympathetic activity. Hydralazine treatment also reduced left ventricular hypertrophy (Figure 6C), improved the endothelium-dependent relaxation to acetylcholine (Figure 6D) and reduced the vascular NADPH oxidase activity (Figure 6E). The composition of bacterial community was evaluated. The major ecological parameters were not altered by hydralazine treatment. Only a reduced Shannon diversity was found in SHR-hydralazine group compared to SHR (Figure 7A). An unclear separation was observed in the PCA between the 2 clusters representing the microbial compositions of SHR and SHR-hydrazine groups, indicating 2 similar gut environments (Figure 7B). The key bacterial populations that are responsible for discriminating among groups was the phylum *Bacteroidetes* (loading 0.74). The KMO test was 0,71, indicating a middling sampling. The Barlett's test of sphericity was < 0.05. In addition, the proportion of bacteria among phyla (Figure 7C), the F/B ratio (Figure 7D), the proportion SCFAs-producing bacteria (Figure 7E), and the percentage of strict anaerobic bacteria (Figure 7F) were similar between both groups, showing that hydralazine did not alter gut dysbiosis, despite its antihypertensive effect. In the gut, hydralazine was unable to change the mRNA levels of the RAS components (data not shown), but it increased the TH expression and the NA content (Figure 8A), suggesting higher colonic sympathetic drive. In addition, no significant modifications were induced by hydralazine in mRNA levels of barrier-forming junction proteins and mucins (Figure 8B), showing that this drug did no improve gut integrity. Furthermore, colonic α-defensins expression was not restored by hydralazine (Figure 8C).

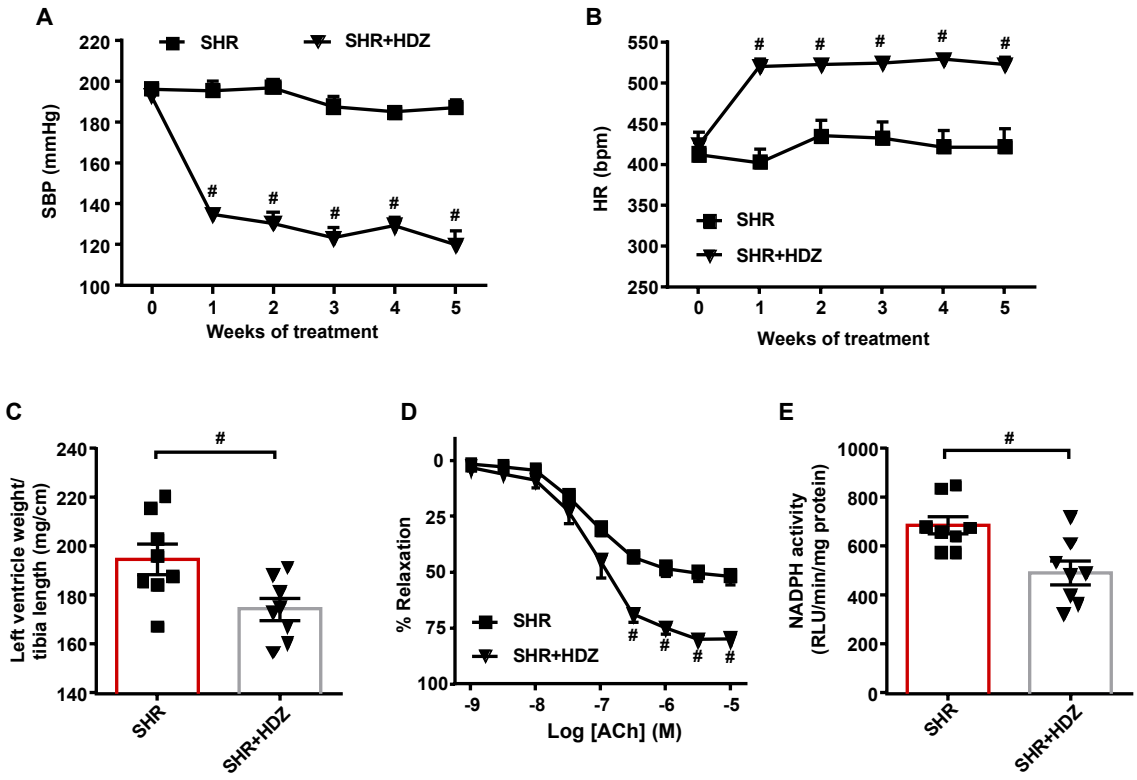


Figure 6. Protective effects of hydralazine (HDZ) in endothelial dysfunction and hypertension in spontaneously hypertensive rats (SHR). *Time course of systolic blood pressure (SBP, **A**) and heart rate (HR, **B**) measured by tail-cuff plethysmography in SHR treated with the hydralazine or saline. Morphological data from the left ventricle (**C**). Endothelium dependent relaxation induced by acetylcholine (ACh) in aortas precontracted by phenylephrine (**D**) and NADPH oxidase activity measured by lucigenin enhanced chemiluminescence (**E**) in aorta from SHR and SHR+HDZ groups. Values are represented as means ± SEM (n = 8). #P< 0.05 significant differences compared with untreated SHR.*

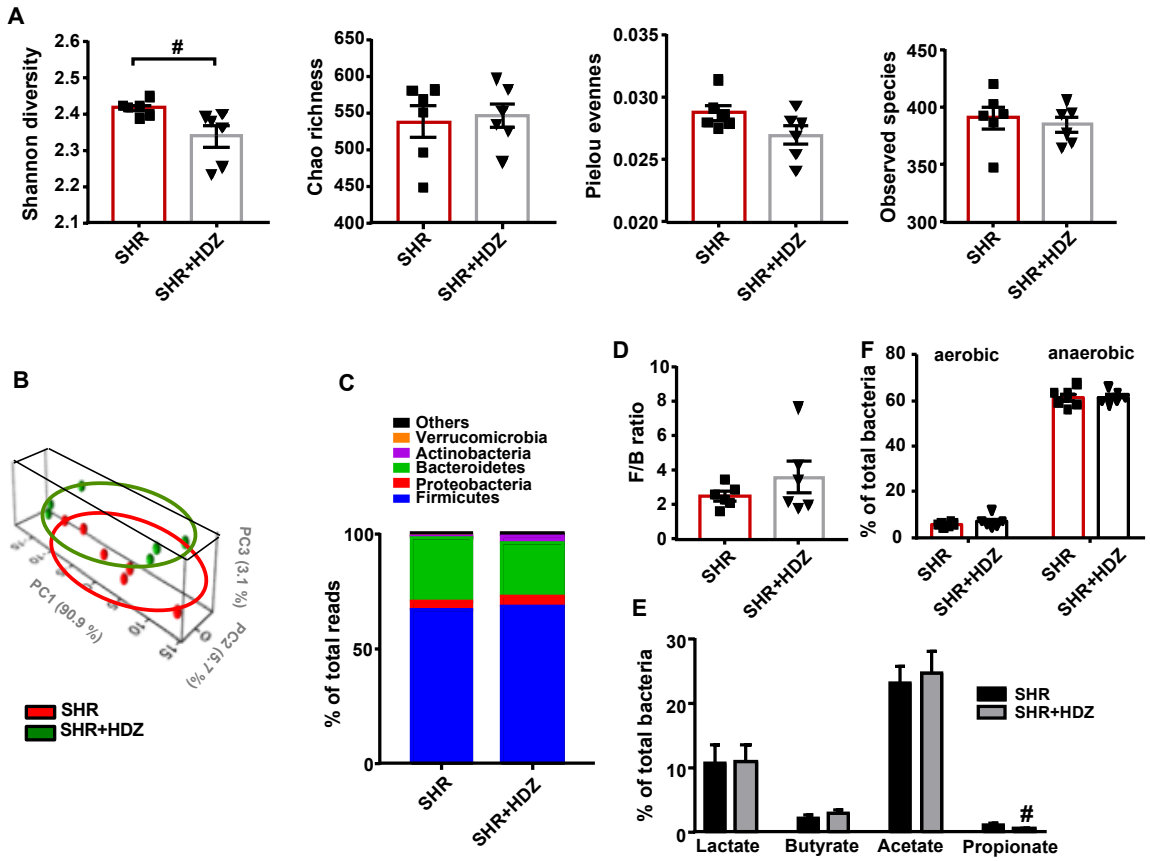


Figure 7. Hydralazine (HDZ) does not induce changes in the gut microbiota composition in spontaneously hypertensive rat (SHR). *The microbial DNA from faecal samples was analysed by 16S rRNA gene sequencing. To evaluate general differences of microbial composition amongst all experimental groups, diversity, richness, evenness, and observed species (**A**) were examined. Principal coordinate analysis in the gut microbiota from all experimental groups (**B**). Phylum breakdown of the 5 most abundant bacterial communities in samples from all groups (**C**). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis (**D**). Relative proportions of anaerobic and aerobic bacteria (**F**), and lactate-, butyrate-, acetate- and propionate-producing bacteria expressed as relative proportions (**E**) in the gut microbiota in all experimental groups are shown. Values are represented as mean ± SEM. #P< 0.05 significant differences compared with untreated SHR.*

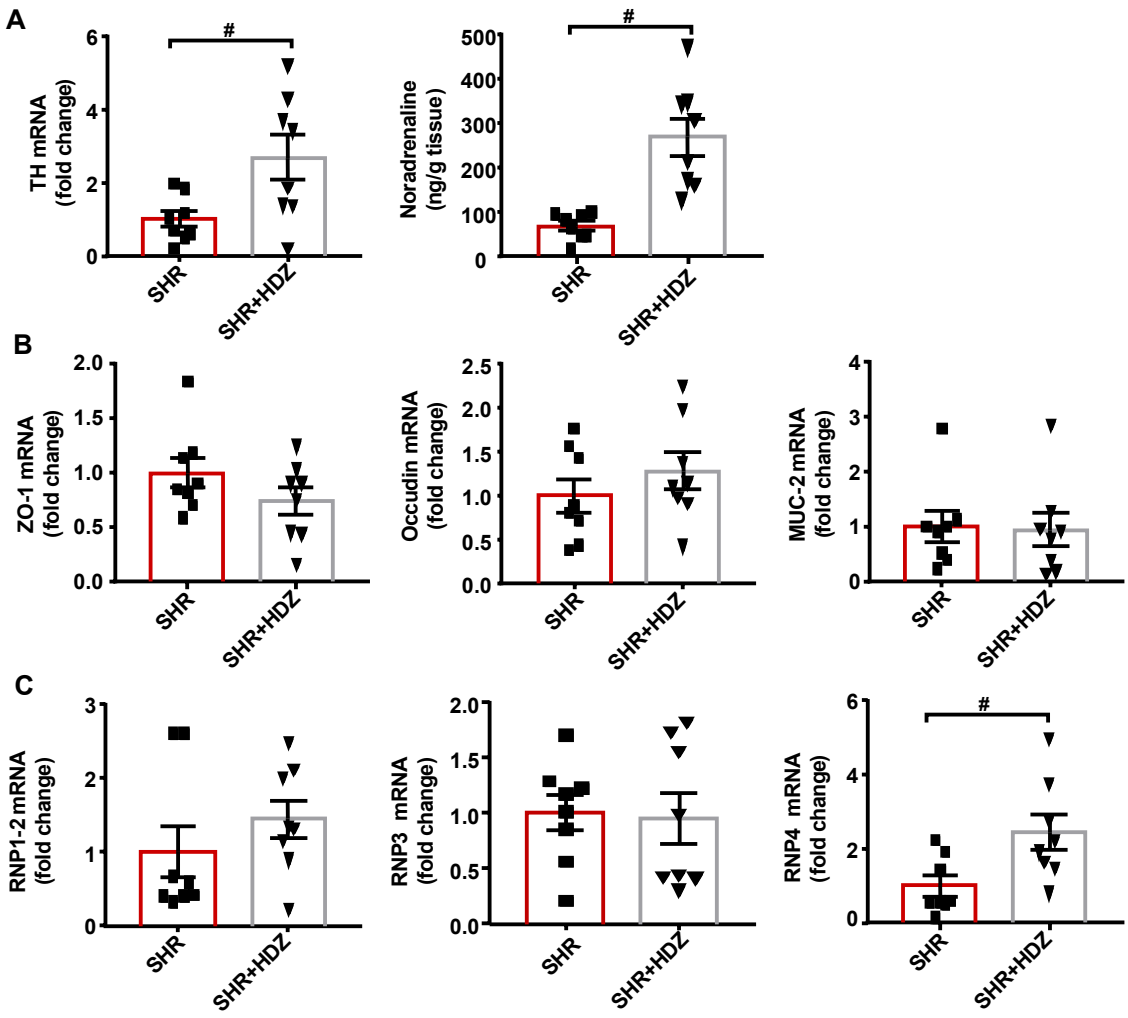


Figure 8. Effects of hydralazine (HDZ) on the sympathetic tone and gut integrity in spontaneously hypertensive rats (SHR). *Colonic mRNA levels of tyrosine hydroxylase (TH) and noradrenaline levels (**A**). Zonula occludens-1 (ZO-1), occluding, mucin (MUC)-2 (**B**), and α-defensins, RNP1.2, RNP3, and RNP4 (**C**) measured by RT-PCR in the colon from all experimental groups. Values are represented as mean ± SEM. #P< 0.05 significant differences compared with untreated SHR.*

Faecal microbiota transplantation (FMT) from SHR-losartan reduces blood pressure and improves endothelial function in SHR

To test if changes in gut microbiota composition induced by a chronic treatment with losartan were involved in their antihypertensive effects, a FMT from SHR treated with losartan to SHR was performed, and compared to FMT from an untreated SHR to another untreated SHR. We found a significant decrease of 16.6 ± 7.0 mm Hg in SBP after 2 weeks of FMT from SHR-Losartan to SHR, whereas no changes in SBP were

found when FMT was performed from SHR to SHR (Figure 9A). In addition, an improvement of the endothelium-dependent relaxation to acetylcholine (Figure 9B) and a reduced NADPH oxidase activity (Figure 9C) were also induced by stool transplantation from SHR treated with losartan. These vascular changes were linked to both increased Treg and decreased Th17 cells population in the vascular wall (Figure 9D).

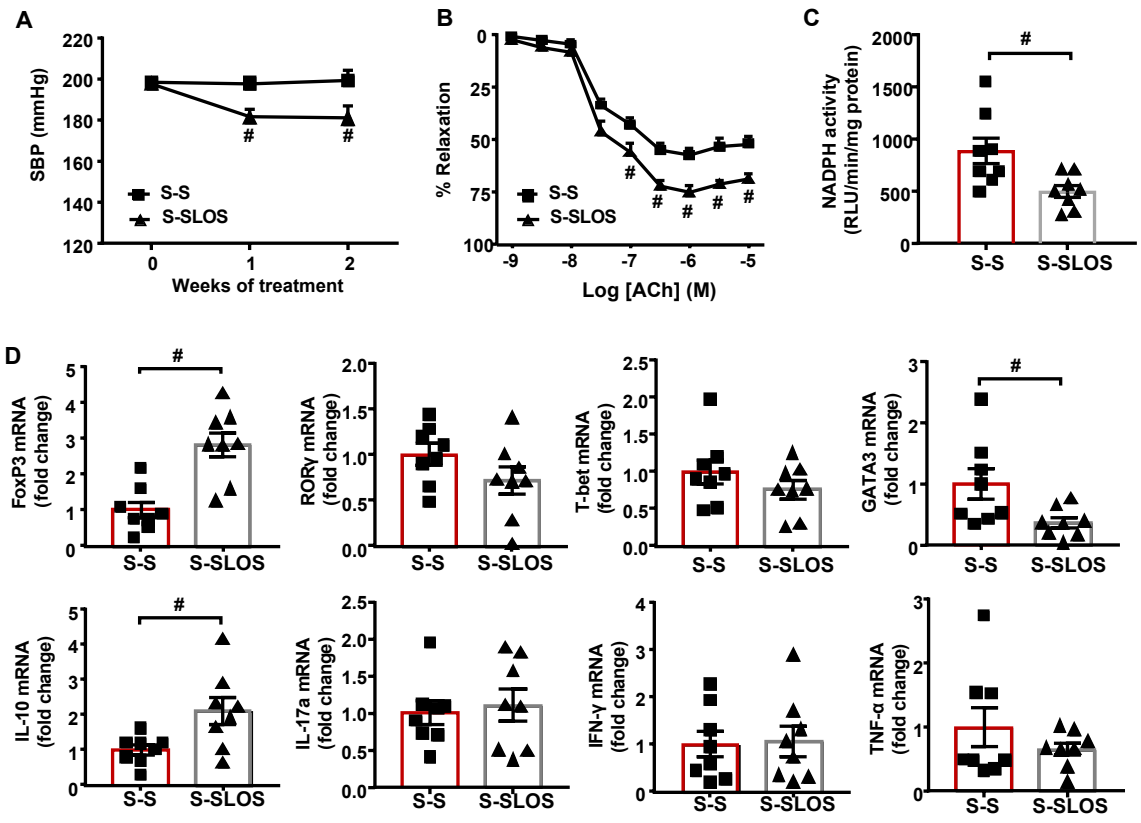


Figure 9. Effects of faecal microbiota transplantation from spontaneously hypertensive rat (SHR)-losartan to SHR on vascular function in SHR. *Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in SHR with stool transplant from SHR (S-S) or from SHR treated with losartan (S-SLOS, **A**). Endothelium-dependent relaxation induced by acetylcholine (ACh) in aortas pre-contracted by phenylephrine (**B**) and NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta from S-S, S-SLOS (**C**). mRNA levels of regulatory T cells (Treg; FoxP3), T helper (Th)-17; RORγ), Th1 (T-bet), and Th2 (GATA-3) cells and mRNA levels of interleukin (IL)-10, IL-17a, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (**D**) in aortas from S-S and S-SLOS groups. Values are represented as mean ± SEM. #P< 0.05 significant differences compared with S-S group.*

In addition, FMT from SHR-losartan to SHR increased colonic ZO-1, and MUC-2 expression (Figure 10A) and reduced colonic TH mRNA level (Figure 10B), suggesting improvement of gut integrity and gut sympathetic tone. In addition, increased Tregs cells in MLNs were detected in S-SLOS group compared to S-S (Figure 10C), indicating that microbiota from SHR treated with losartan induced positive changes in this secondary lymph organ in the gut. It has been reported that 60% losartan can be found in faeces and the donor samples could contain the drug. However, a low concentration of 0.60 µg/mL of losartan was detected in faeces from SHR-Losartan group, which rendered a losartan dose of 0.15 µg Kg^{-1} excluding that the observed effects in S-SLOS group were related to direct actions of the drug. As expected, losartan were not detected in faecal samples from untreated SHR.

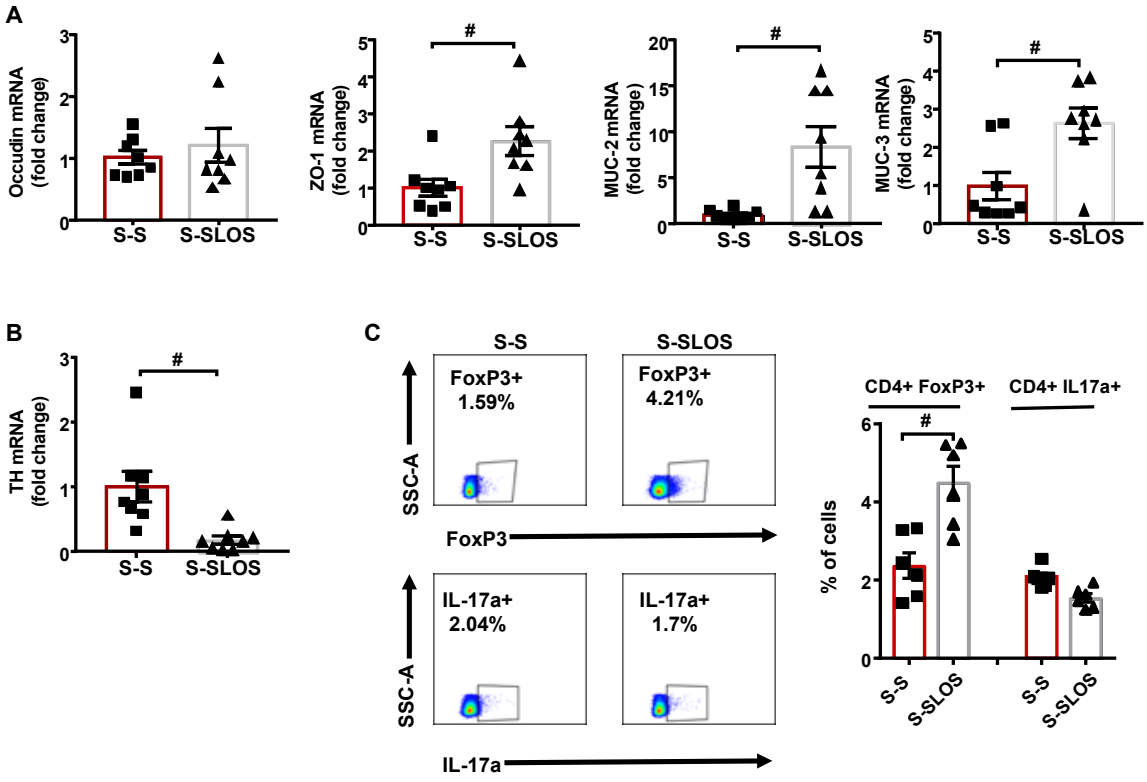


Figure 10. Effects induced by faecal microbiota transplantation from spontaneously hypertensive rats (SHR)-losartan on gut integrity and gut sympathetic activity. *mRNA levels of occludin, zonula occludens-1 (ZO-1), mucin (MUC)-2, MUC-3 (**A**), and tyrosine hydroxylase (TH, **B**) measured by RT-PCR in colon from all experimental groups. Regulatory T cells (Treg) and T helper (Th)-17 measured in mesenteric lymph nodes from all S-S and S-SLOS groups by flow cytometry. Values are represented as mean ± SEM. #P< 0.05 significant differences compared with S-S group.*

At the end of the experiment the composition of faecal microbiota was analysed. The bacterial communities evaluated by calculating major ecological parameters (Chao richness, Pielou evenness, Shannon diversity and the number of observed species), were similar between both groups (data not shown). The proportion of bacteria from the *Fimicutes* and *Verrucomicrobia* phyla was significantly lower, whereas the proportions of *Bacteroidetes* were higher in S-SLOS than in S-S group (Figure S7A). The F/B ratio was also lower in S-SLOS group (Figure S7B). In addition, significant lower percentages of lactate- and propionate-producing bacteria, and a higher percentage of acetate-producing bacteria, were found in S-SLOS compared to S-S (Figure S7C). Moreover, the percentage of strict anaerobic bacteria was significantly higher in S-SLOS compared to S-S (Figure S7D). Figure S8 shows the bacterial taxa that prominent changes in bacterial taxa occurred, where the relative abundance of 30 taxa was increased (green) and 64 taxa were decreased (red) in S-SLOS compared to S-S. A clear separation was observed in the PCA between the 2 clusters representing the microbial compositions of S-S and S-SLOS groups (Figure S9A). The key bacterial populations that is responsible for discriminating among groups the genus *Bacteroides* (loading 0.56). The KMO test was 0,84, indicating a meritorious sampling. The Barlett's test of sphericity was < 0.05. Bacterial genera were associated with changes in the composition of microbiota (Figure S9B). A significant reduction of *Oscillospira*, with a higher abundance of *Bacteroides, Prevotella and Clostridium* was found in the microbiota of S-SLOS group compared to S-S (Figure S9C). Interestingly, increased proportion of *Bacteroides acidifaciens* was detected as main strain in S-SLOS group (Figure S9D).

A

Phylum	S-S (n=7)	S-SLOS (n=6)
Firmicutes	51.20± 3.3	36.9 ± 5.2#
Bacteroidetes	42.8 ± 3.5	59.2 ± 5.2#
Proteobacteria	3.1 ± 0.7	2.8 ± 0.5
Verrucomicrobia	1.6 ± 0.7	0.4 ± 0.0#
Actinobacteria	0.6 ± 0.2	0.6 ± 0.3

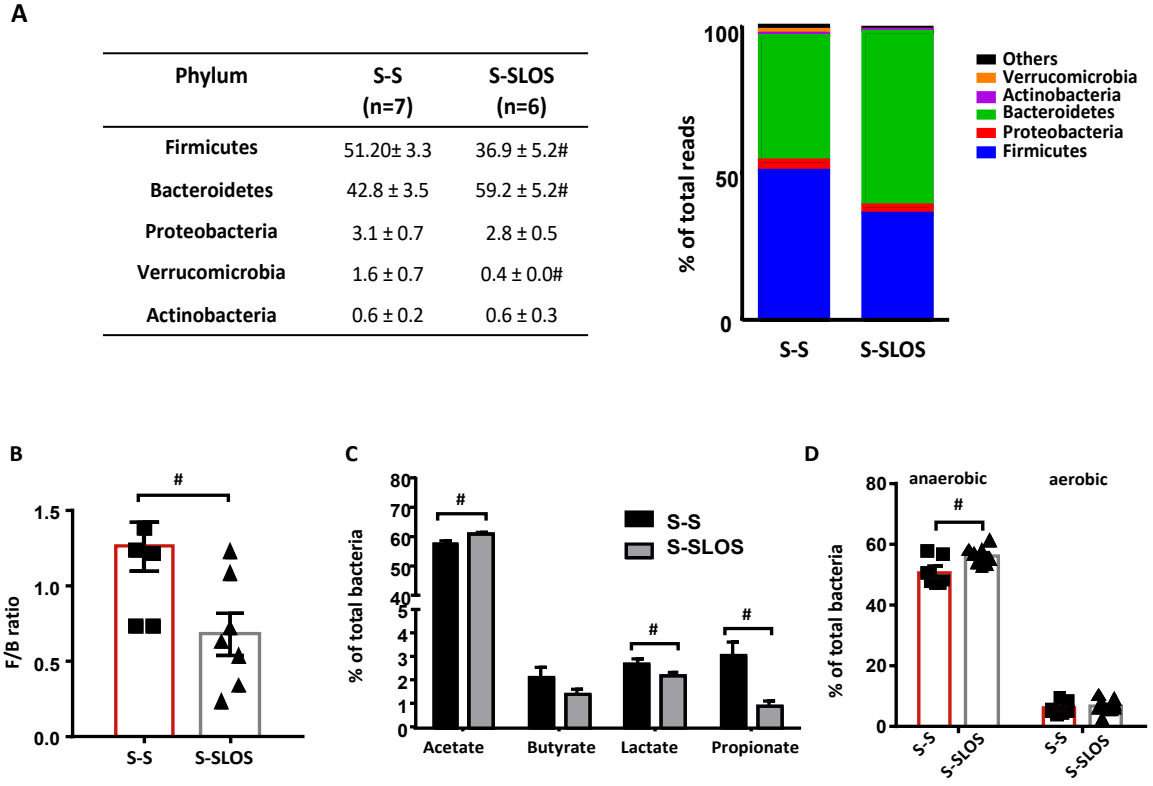
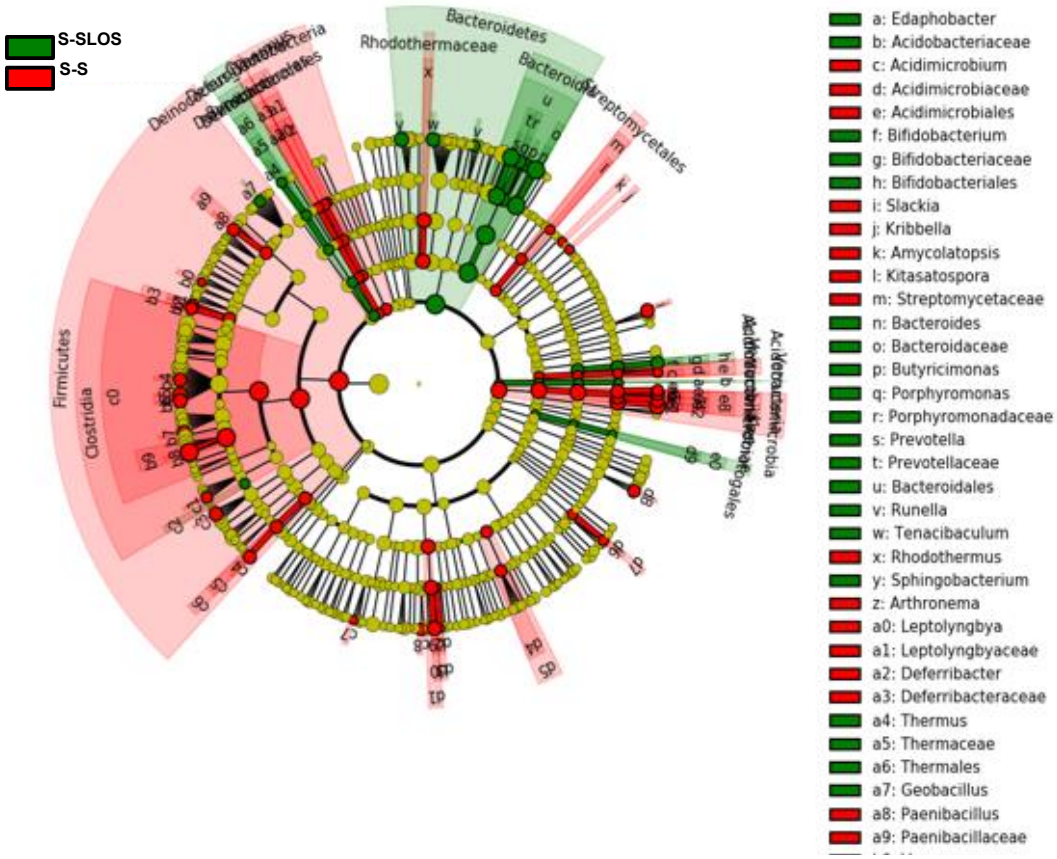


Figure S7. Effects of faecal microbiota transplantation from spontaneously hypertensive rat (SHR)-losartan to SHR on different behaviours of the gut microbiota of SHR. *Faecal samples were collected at the end of FMT experiment (SHR with stool transplant from SHR (S-S) or from SHR treated with losartan (S-SLOS)) and bacterial 16S ribosomal DNA was amplified and sequenced to analyse the composition of microbial communities. Phylum breakdown of the 5 most abundant bacterial communities in the faecal samples obtained from all experimental groups (**A**). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis (**B**). Relative proportions of acetate-, butyrate-, lactate-, and propionate-producing bacteria (**C**). Anaerobic and aerobic bacteria expressed as relative proportions (**D**) in the gut microbiota. Values are expressed as mean ± SEM. #P< 0.05 significant differences compared with S-S group.*

A


S-SLOS
S-S
Bacteroidetes
Rhodothermaceae
Bacteroidia
Streptomycetales
Firmicutes
Clostridia
a: Edaphobacter
b: Acidobacteriaceae
c: Acidimicrobium
d: Acidimicrobiaceae
e: Acidimicrobiales
f: Bifidobacterium
g: Bifidobacteriaceae
h: Bifidobacteriales
i: Slackia
j: Kribbella
k: Amycolatopsis
l: Kitasatospora
m: Streptomycetaceae
n: Bacteroides
o: Bacteroidaceae
p: Butyricimonas
q: Porphyromonas
r: Porphyromonadaceae
s: Prevotella
t: Prevotellaceae
u: Bacteroidales
v: Runella
w: Tenacibaculum
x: Rhodothermus
y: Sphingobacterium
z: Arthronema
a0: Leptolyngbya
a1: Leptolyngbyaceae
a2: Deferribacter
a3: Deferribacteraceae
a4: Thermus
a5: Thermaceae
a6: Thermales
a7: Geobacillus
a8: Paenibacillus
a9: Paenibacillaceae

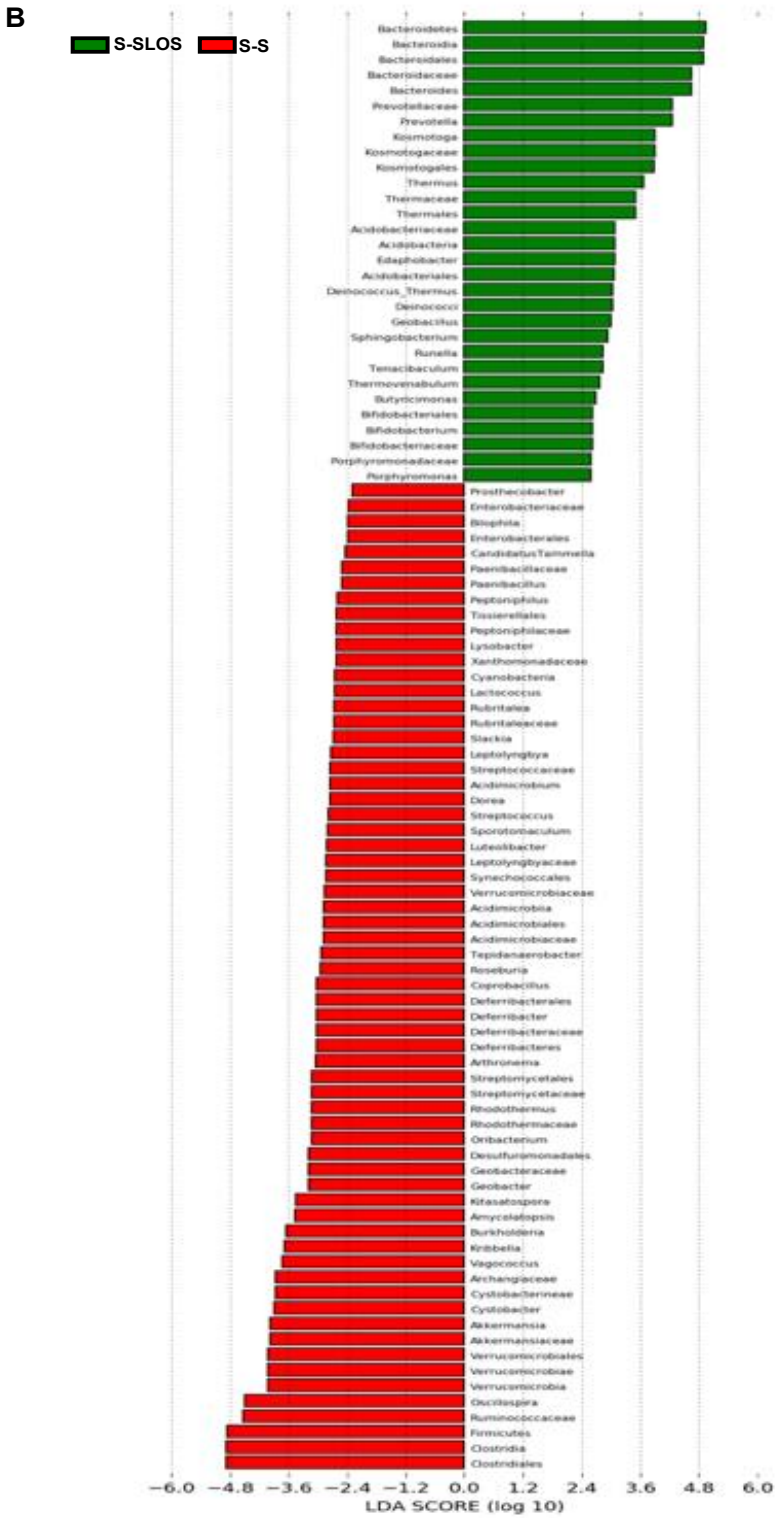


Figure S8. Comparisons of gut microbiome changes in SHR with stool transplant from SHR (S-S) versus from SHR treated with losartan (S-SLOS). *Cladograms show the significantly enriched taxa, the taxa are identified on the key to the right of each panel. Larger circles represent greater differences in abundance between groups (**A**). Linear discriminant analysis effect size (LEfSe) identified significantly different bacterial taxa enriched in each cohort at LDA Score > 2, $p<0.05$ (red bars S-S enriched, green bars S-LOS enriched) (**B**). n = 7-6 rats per experimental group in each comparison.*

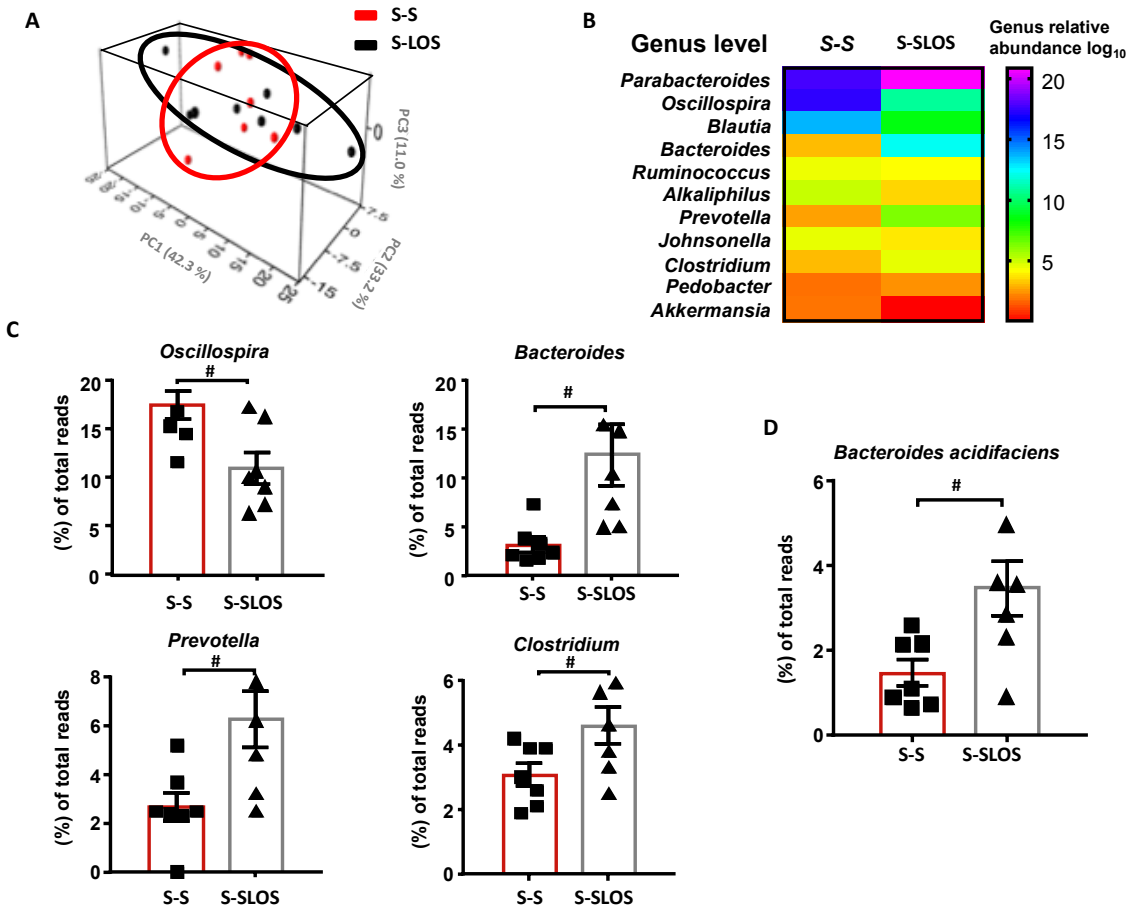


Figure S9. Effects of faecal microbiota transplantation from spontaneously hypertensive rat (SHR)-losartan at the genus level in the gut microbiota of SHR. *Faecal samples were collected at the end of FMT experiment (SHR with stool transplant from SHR (S-S) or from SHR treated with losartan (S-SLOS)). Principal coordinate analysis of genera (**A**) in the gut microbiota from all experimental groups. Samples clustered by experimental group demonstrated that the experimental conditions resulted in distinct populations of bacteria at the genus level. (**B**) Heat map showing differences in the relative abundance of the bacterial genera between the three groups studied. The colours of the heat map represent the relative percentage of microbial genus assigned within each sample. The most significantly modified bacterial genera (**C**) and species (**D**) in the gut microbiota Values are expressed as mean ± SEM. #P< 0.05 significant differences compared with S-S group.*

Discussion and conclusions

The main new findings of this study are the following: 1) chronic losartan treatment reduces gut dysbiosis in SHR; 2) changes in gut microbiota composition induced by losartan are independent of its BP reducing effects, and seem to be related to improvement of gut integrity and the normalisation of colonic α-defensins production, as a result of reduced gut sympathetic drive; 3) microbiota SHR-losartan is able to improve gut integrity, aortic endothelial dysfunction and reduce BP in rats with genetic hypertension. Abundant evidence has demonstrated the association between gut dysbiosis and hypertension (Mell et al*.,* 2015; Yang et al*.,* 2015, Li et al 2017, Toral et al., 2019; Sun et al., 2019). Our results are consistent with the main features of dysbiotic microbiota described in SHR (Yang et al., 2015; Toral et al., 2019, Yang et al., 2019): a) a reduced richness and diversity, b) an increased F/B ratio, and c) a reduction in acetate- and propionate-producing bacteria, with higher proportion of lactate-producing bacteria. Losartan treatment tended to increase richness and diversity, whereas separated bacteria clusters from SHR towards WKY, normalised F/B ratio and SCFAs-producing bacteria. The increased abundance of *Lactobacillus ssp.* found in SHR samples was also normalised. All these changes induced by losartan in microbiota were linked to reduced BP, improvement of left ventricle hypertrophy, reduced both vascular oxidative status and accumulation of pro-inflammatory lymphocytes, and improvement of endothelial dysfunction. Losartan may elicit changes in gut microbiota by several mechanisms. It has been commonly observed that a change in the host health status has been accompanied by a shift in the gut microbiota. Therefore, microbiota could be adapted to BP reduction, shifting to a microbiota composition similar to WKY. However, our results are contrary to this hypothesis because hydralazine, which reduced BP in a similar extent to losartan, was unable to alter dysbiosis in SHR. Changes in gut microbiota composition have been associated with gut integrity (Kim et al, 2018). The mammalian digestive tract epithelial cells create a tight barrier in the gut, contributing to the hypoxic environment of the lumen. Damage to this barrier makes the environment less hypoxic, conducive to aerobic bacterial growth (Konig et al., 2016; Earley et al, 2015). In fact, intestines of angiotensin II-hypertensive mice were significantly less hypoxic and with increased aerobic bacteria in faeces (Kim et al., 2018). We also found decreased abundance of anaerobic bacteria in faeces from SHR, which was associated with lost of gut integrity. SHR treated with losartan showed increase colonic integrity and a proportion of strict anaerobic bacteria

similar to WKY, whereas hydralazine was unable to improve both gut integrity and the proportion of anaerobic bacteria. This data support the key role of gut integrity in the composition of intestinal microbiota. In addition, intestinal epithelial cells and Paneth cells secrete antimicrobial peptides, such as defensins, which selectively kill Gram-positive bacteria (Pamer, 2007; Ayabe et al., 2000; Vora et al, 2004; Vaishnava et al., 2008). Components of the microbiota, such as LPS, are recognized by Toll-like Receptors expressed by these intestinal cells and trigger production and secretion of these defensins. We found changes in mRNA levels of defensins in colonic samples from SHR compared to WKY, which might also be involved in changes in microbiota found in this hypertensive rats. Losartan restored defensins expression to become similar to that found in WKY, whereas hydralazine did not altered the expression of RNP1-2 and RPN3 and enhanced the increased expression of RNP4 found in SHR, suggesting a role of defensins in the different changes in gut microbiota induced by both drugs. Angiotensin receptor blockers have gut anti-inflammatory properties via inhibition of angiotensin II/ angiotensin II type 1 receptor (AT1) axis (Takeshita and Murohara, 2014). Inhibition of the classical RAS pathway is also involved in the upregulation of ACE2, which activates the Ang-(1-7)/Mas pathway to counteract inflammatory signaling (Yisireyili et al., 2018). In our study, no significant changes in AGT, ACE and ACE2 expression were found among colonic samples from WKY, SHR and SHR-losartan groups ruling out that changes in this pathway were involved in the protective effects of losartan in gut integrity. Our current study is consistent with Santisteban et al., (2017), demonstrating an increased gut sympathetic drive (increased TH expression and NA accumulation) associated with lost of gut integrity and microbial dysbiosis in SHR. Recently, the hypothesis posing the presence of a brain-gut communication driven by sympathetic system has been reinforced. Central administration of a modified tetracycline inhibited microglial activation, normalised sympathetic activity, attenuated pathological alterations in gut wall, restored certain gut microbial communities altered by angiotensin II and reduced BP (Sharma et al., 2019). Our data also support that gut sympathetic drive is a key regulator of gut integrity and microbiota composition. It is pertinent to note that sympathetic tone in SHR is attenuated by RAS-inhibition (Tsuda et al., 1988, Demirci et al., 2005; Santisteban et al., 2017), and increased by hydralazine (Tsoporis and Leenen, 1988). In fact, losartan treatment reduced sympathetic activity in the colon, improving gut integrity and reducing gut dysbiosis, whereas hydralazine (that increased gut sympathetic drive) was unable to restore gut integrity and microbiota composition. Normal

microbiota could regulate immune system and/or gut-brain communication reducing BP. In fact, Karbach et al (2016), showed that angiotensin II infusion to germ-free mice reduced ROS formation in the vasculature, attenuated vascular expression of NADPH oxidase subunit Nox2, and reduced upregulation of RORγ, the signature transcription factor for IL-17 synthesis in the aortic vessel wall. These vascular changes exerted protection from endothelial dysfunction and attenuation of BP increase in response to angiotensin II, involving immune system in the effects of microbiota in vascular function and BP regulation. We have recently described that FMT from WKY to SHR reduced neuroinflammation, sympathetic nervous system activity and BP (Toral et al., 2019a). In addition, the antihypertensive effect of the ACE inhibitor captopril has sustained influence on the brain-gut axis even after the withdrawal of captopril (Yang et al., 2019). When we explore the effects of FMT from donors SHR-losartan to receptors SHR, we found decreased F/B ratio, thus improving gut dysbiosis, and increased the prevalence of acetate-producing bacteria and those of the genus *Bacteroides acidifaciens*. This bacterium was recently associated to reduce SBP in DOCA-salt hypertension (Marques et al., 2017). In addition, it shown to prevent obesity and to improve insulin sensitivity in mice (Yang et al., 2017), but whether it can prevent the development of high BP in monocolonized animal is yet to be determined. These changes in gut microbiota were accompanied by a reduced BP, an improvement of endothelial dysfunction, a reduction of aortic NADPH oxidase activity linked to increased aortic Tregs infiltration without changes in Th17. 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 (Kassan et al., 2011). The changes in T cell infiltration in the vascular wall were similar to that found in MLNs, suggesting that a regulation in T cell population in this gut secondary lymph organ by microbiota is involved in the antihypertensive effects of losartan. In fact, microbial metabolites, such as acetate, derived from acetate-producing bacteria in S-SLOS, might promote T-cell differentiation into Tregs cells (Park et al., 2015), leading to reduced BP. In conclusion, we have found for the first time that losartan treatment reduced gut dysbiosis in SHR. This effect seems to be related to its capacity to reduce sympathetic drive in the gut, improving gut integrity and defensins production. The changes induced by losartan in gut microbiota contributed, at least in part, to protect the vasculature and reduce BP, possibly by modulating gut immune system (Figure S10). No sex-differences have been described in the antihypertensive effects of angiotensin

receptor antagonists in humans. However, if the effects of losartan are sex-independent deserves further investigation in female SHR.

Methods

Animals and experimental groups

This study was conducted in accordance with the regulations and requirements of the European Union concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethics Committee of Laboratory Animals of the University of Granada (Spain; permit number 03-CEEA-OH-2013). Animal studies are reported in compliance with the ARRIVE guidelines (McGrath and Lilley, 2015) and with the recommendations made by the British Journal of Pharmacology. Twenty weeks old male SHR/Kyo@Rj and WKY/Kyo@Rj from Envigo (RRID:RGD_5508396, Barcelona, Spain) were used in the present study. We selected SHR as an animal model of genetic hypertension. The SHR is a model with similar characteristics to essential hypertension in non-obese humans and one in which males have higher BP than females as young adults, but both sexes are hypertensive compared with normotensive Sprague-Dawley or WKY rats. Rats were kept in specific pathogen-free facilities at University of Granada Biological Services Unit. All animals were housed under standard laboratory conditions (12 hr light/dark cycle, temperature 21-22ºC, 50-70% humidity). Rats were housed in Makrolom cages (Ehret, Emmerdingen, Germany), with dust-free laboratory bedding and enrichment. In order to avoid horizontal transmission of the microbiota among animals, each rat was housed in a separate cage. Rats were provided with water and standard laboratory diet (SAFE A04, Augy, France) *ad libitum*. Water was changed every day, and both water and food intake was recorded daily for all groups. Studies were designed to generate groups of equal size. Animals were randomly assigned to treatment groups and the experimenter was blinded to drug treatment until data analysis has been performed.

Experiment 1: Rats were randomly assigned to three different experimental groups (n = 8): a) untreated WKY (WKY, 1 mL of tap water once daily), b) untreated SHR (SHR), and c) SHR treated with losartan (SHR-losartan, 20 mg kg^{-1} day^{-1} by oral gavage).

Experiment 2: SHR were randomly assigned to two groups (n = 8): a) SHR, and b) SHR treated with hydralazine (SHR-hydralazine, 25 mg kg^{-1} day^{-1} by oral gavage).

In both experiments, rats were treated for 5 weeks. Body weight was measured every week.

Experiment 3: To explore the role of microbiota from SHR-losartan group in the antihypertensive effects of losartan, a faecal microbiota transplantation (FMT) experiment was performed. For this purpose, faecal contents were collected fresh and pooled from individual rats from SHR and SHR-losartan groups at the end of the Experiment 1, twenty-four hours after the last dose of losartan. Twenty-week-old recipient SHR were orally gavaged with donor faecal contents for 3 consecutive days, and once every 3 days for a total period of 2 weeks. Animals were randomly assigned to two different groups of 8 animals each: SHR with SHR microbiota (S-S), and SHR with SHR-losartan microbiota (S-SLOS).

Faecal microbiota transplantation

The FMT to recipient rats was performed as previously reported (Toral et al., 2019). Faecal contents were diluted 1:20 in sterile PBS and centrifuged at 800 rpm for 5 minutes. The supernatant was aliquoted and stored at -80ºC. One week prior to transplantation, 1 mL ceftriaxone sodium (400 mg kg^{-1} day^{-1}) was administered daily to recipient rats for 5 consecutive days by oral gavage. Forty-eight hours after the last antibiotic treatment, recipient rats were orally gavaged with donor faecal contents (1 mL) as explained above. Losartan concentration in the supernatant used for FMT was measured by mass spectroscopy using previously published techniques (Okawada et al., 2011).

Blood pressure measurements

SBP and heart rate (HR) were measured weekly at room temperature using tail-cuff plethysmography as described previously (Vera et al., 2007). All experiments were performed in a quiet room by the same-blinded experimenter.

Cardiac and renal weight indices

When the experimental period was completed, 18 h fasting animals were anaesthetised with 2.5 mL kg^{-1} equitensin (i.p.) and blood was collected from the abdominal aorta. Finally, the rats were killed by exsanguination. The kidneys and the heart were then removed and weighed. The heart was divided into right ventricle and left ventricle plus septum. All tissue samples were frozen in liquid nitrogen and then stored at -80ºC.

Plasma and colonic parameters

Blood samples were cooled on ice and centrifuged for 10 min at 3,500 rpm at 4ºC, and plasma was frozen at -80°C. Plasma lipopolysaccharide (LPS) concentration was measured using the Limulus Amebocyte Lysate chromogenic endotoxin quantitation Kit (Lonza, Valais, Switzerland), according to the manufacturer's instructions. We used enzyme-linked immunosorbent assay kits (IBL International, Hamburg, Germany) to measure colonic noradrenaline (NA) concentrations following the manufacturer's protocol. Colon samples were collected and placed in the appropriate conservation solution. EDTA 1 mM and sodium metabisulfite 4 mM were added to prevent the catecholamine degradation. Then tissue samples were stored at -80ºC for later use.

Vascular reactivity studies

Segments of thoracic aortic rings (3 mm) were dissected from animals and were mounted in organ chambers filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 and glucose 11) as previously described (Gómez-Guzman et al., 2011). The concentration-relaxation response curves to acetylcholine (10^{-9} -10^{-5} M) were studied in aorta pre-contracted using phenylephrine (1 μM) in the absence or the presence of N_G-nitro-L-arginine methyl ester (L-NAME, 100 μM) or apocynin (10 μM). The concentration-relaxation response curves to nitroprusside (10^{-9} -10^{-6} M) were performed in the dark in aortic rings without endothelium pre-contracted using 1 μM phenylephrine. Relaxation responses were expressed as a percentage of precontraction.

Measurement of *ex vivo* vascular reactive oxygen species (ROS) levels

We used dihydroethidium (DHE), an oxidative fluorescent dye, to localise ROS in aortic segments *in situ*, as previously described (Zarzuelo et al., 2011). Briefly, the aorta segments were included in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), quickly frozen, and cut into 10 μm thick sections in a cryostat (Microm International Model HM500 OM). Sections were incubated at room temperature for 30 min with 10 μM DHE in the dark, counterstained with the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI, 300 nM) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS production was estimated from the ratio of ethidium/DAPI fluorescence.

Immunofluorescent detection of macrophages and lymphocytes

Immunofluorescence microscopy was performed on 10-µm-thick cryostat aortic sections. Aortic sections were air-dried for 30 min, fixed for 5 min in methanol at -20˚C and washed with PBS for 15 min. Sections were blocked with PBS containing 5% non-fat dry milk for 2 h at room temperature and then incubated with a mouse anti-macrophage-specific antigen CD11b polyclonal antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), and a rabbit anti-CD3 polyclonal antibody (1:200, Abcam, Cambridge, UK) in blocking solution overnight at 4°C. The sections were washed 3 times with PBS and incubated with Alexa Fluor® 594 goat anti-rabbit, Alexa Fluor™ 488 goat anti-mouse (1:200, Molecular Probes, Oregon, USA) in the blocking solution for 1h at room temperature and then washed 3 times with PBS. Images were captured using a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). The percentage of CD11 and CD3 staining/total surface area was quantified with Image J software (version 1.32j, NIH, http://rsb.info.nih/ij/) (Barhoumi et al., 2011).

NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings as previously described (Zarzuelo et al., 2011). Aortic rings from all experimental groups were incubated for 30 minutes at 37ºC in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, $MgSO_4$ 1, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 1, $CaCl_2$ 1.2 and glucose 5.5. Then NADPH (100 µM) was added to the buffer containing the aortic rings, and lucigenin (5 µM) was injected automatically. NADPH oxidase activity was determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those found in the presence of NADPH and expressed as RLU (relative light units)/min per mg of tissue for aortic rings.

Flow Cytometry

Mesenteric lymph nodes (MLNs) were collected from all groups. The tissues were mashed with wet slides to decrease friction and then the solutions were filtered through a 70μM cell strainer. $1\times10_6$ cells were counted and incubated with a protein transport inhibitor (BD GolgiPlugTM) for an optimum detection of intracellular cytokines by flow cytometry. For intracellular staining, cells were stimulated with 50 ng mL^{-1} phorbol 12-myristate 13-acetate plus 1 μg mL^{-1} ionomycin. After 4.5 hours, aliquot cells, of each sample, were blocked with anti-CD32 (clone D34-485) for 30 minutes at 4°C to avoid non-specific binding to Fc-gamma receptors. After that, cells were transferred to polystyrene tubes for the surface staining with mAbs anti-CD4 (PerCP-Vio700, clone REA482, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD45 (APC, clone RA3-6B2 BD PharmigenTM, New Jersey, USA), and viability dye (LIVE/DEAD® Fixable Aqua Dead cell Sain Kit, Molecular Probes, Oregon, USA) for 20 min at 4°C in the dark. The lymphocytes were then fixed, permeabilised with the Fix/Perm Fixation/Permeabilisation kit (eBioscience, San Diego, USA) and intracellular staining was achieved with mAbs anti-forkhead box P3 (FoxP3) (PE,clone FJK-16s, eBioscience, San Diego, USA), anti-interleukin (IL)-17A (PE-Cy7, clone eBio17B7, eBioscience, San Diego, USA) and anti-IFNγ (Alexa Fluor® 647, DB-1, 6B2 BD Pharmigen™, New Jersey, USA) for 30 min at 4°C in the dark. All samples were analysed using a flow cytometer CANTO II (BD Biosciences) and data were analysed with FlowJo software (Tree Star, Ashland, OR, USA) (Romero et al., 2017).

Gene expression analysis

The analysis of gene expression was performed by reverse transcription polymerase chain reaction (RT-PCR), as previously described (Zarzuelo et al., 2011). For this purpose, total RNA was extracted by homogenisation using TRI Reagent® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 2 μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). The sequences of the forward and

reverse primers used for amplification are described in Table S1. The efficiency of the PCR reaction was determined using a dilution series of standard vascular samples. To normalise mRNA expression, the expression of the housekeeping gene β-actin was used. The mRNA relative quantification was calculated using the ΔΔCt method.

DNA Extraction, 16S rRNA Gene Amplification, Bioinformatics

For analysis of the bacterial population present in the gut, faecal samples were collected from six individual animals at the end of the experimental period. DNA was extracted from faecal samples using G-spin columns (INTRON Biotechnology) and starting from 30 mg of samples resuspended in PBS and treated with proteinase K and RNAses. DNA concentration was determined in the samples using Quant-IT PicoGreen reagent (Thermo Fischer), and DNA samples (about 3 ng) were used to amplify the V3-V4 region of 16S rRNA gene (Caporaso et al., 2011). PCR products (approx. 450 bp) included extension tails, which allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent) and samples were pooled in equimolar amounts. The pool was further cleaned, quantified and the exact concentration was estimated by real time PCR (Kapa Biosystems). Finally, DNA samples were sequenced on an Illumina MiSeq instrument with 2 x 300 bp paired-end sequencing reads at the Genomics Unit (Madrid Science Park, Spain). We used the BIPES pipeline to process the raw sequences (Zhou et al., 2011). First, the barcode primers were trimmed and filtered if they contained ambiguous bases or mismatches in the primer regions according to the BIPES protocol. Second, we removed any sequences with more than one mismatch within the 40–70 bp region at each end. Third, we used 30 Ns to concentrate the two single-ended sequences for the downstream sequence analyses. A detailed description of these methods was previously reported (Liu et al., 2017). Third, we performed UCHIME (implemented in USEARCH, version 6.1) to screen out and remove chimeras in the *de novo* mode (using-minchunk 20-xn 7-noskipgaps 2) (Edgar and Flyvbjerg, 2015). Between 90,000 and 220,000 sequences were identified in each sample. All subsequent analyses were performed using 16S Metagenomics (Version: 1.0.1.0) from Illumina. The sequences were then clustered to an operational taxonomic unit (OTU) using USEARCH with default parameters (USERACH61). The threshold distance was set to 0.03. Hence,

when the similarity between two 16S rRNA sequences was 97%, the sequences were classified as the same OTU. QIIME-based alignments of representative sequences were performed using PyNAST, and the Greengenes 13_8 database was used as the template file. The Ribosome Database project (RDP) algorithm was applied to classify the representative sequences into specific taxa using the default database (Edgar and Flyvbjerg, 2015). The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature. Bacteria were classified based on the short chain fatty acids (SCFAs) end-product as previously described (Wang et al., 2007; Antharam et al., 2013). Bacteria were classified based on the oxygen requirement using Genomes OnLine Database (GOLD) (Mukherjee et al., 2019).

Chemicals

All chemicals were obtained from Sigma-Aldrich (Barcelona, Spain), unless otherwise stated.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was undertaken only for studies where each group size was at least n=5. Group size is the number of independent values, and statistical analysis was done using these independent values. The Shannon, Chao, Pielou and observed species indexes were calculated using QIIME (PAST 3x). Reads in each OTU were normalised to total reads in each sample. Only taxa with a percentage of reads > 0.001% were used for the analysis. Principal component analysis (PCA) was also applied to these data to identify significant differences between groups, using PAST 3x, and SSPS. Linear Discriminant Analysis (LDA) scores greater than 2 were displayed. Taxonomy was summarised at the genus level within QIIME-1.9.0 and uploaded to the Galaxy platform (Segata et al., 2011) to generate the LDA effect size (LEfSe)/cladogram enrichment plots considering significant enrichment at a $P<0.05$, LDA score > 2. All data were analysed using GraphPad Prism 7

(RRID:SCR_000306). Results are expressed as means ± SEM of measurements. The evolution of tail SBP and the concentration-response curves to acetylcholine were analysed by two-way repeated-measures analysis of variance (ANOVA) with the Bonferroni *post hoc* test. The remaining variables were tested on normal distribution using Shapiro-Wilk normality test and compared using an unpaired t test or 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. $P < 0.05$ was considered statistically significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Conflict of interest

The authors declare no conflict of interest

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Author contributions

J.D. conceived and designed the research; I.R.V, M.T., N.V.P., M.S., M.G.G., R.M., F.A. and M.R. performed the experiments and analysed the data; R.J., J.G., J.M.R. and J.D. interpreted the results; I.R.V, M.T. and R.J. prepared figures; J.D. drafted this manuscript;

I.R.V, M.T., R.J., J.G., J.M.R. and J.D. edited and revised the manuscript; All authors approved the final version of manuscript.

Abbreviations

ACE, angiotensin-converting enzyme; AGT, angiotensinogen; AT1, angiotensin II type 1 receptor; BP, blood pressure; DAPI, 4,6-diamidino-2-phenylindole dichlorohydrate; DHE, dihydroethidium; F/B, *Firmicutes*/*Bacteroidetes*; FMT, faecal microbiota transplantation; FoxP3, forkhead box P3; GOLD; Genomes OnLine Database; HR, heart rate; IBD, inflammatory bowel diseases; IL, interleukin; KMO, Kaiser-Meyer-Olkin; L-NAME, NG-nitro-L-arginine methyl ester; LDA, Linear Discriminant Analysis; LPS, lipopolysaccharide; MLNs, mesenteric lymph nodes; MUC, mucin; NA, noradrenaline; NO, nitric oxide; OTU, operational taxonomic unit; PCA, three-dimensional principal component analysis; PLS, Partial Least Square; RAS, renin-angiotensin system; RDP, Ribosome Database project; RORγ, retinoid-related orphan receptor-γ; ROS, reactive oxygen species; SBP, systolic blood pressure; SCFAs, short chain fatty acids; SHR, spontaneously hypertensive rats; Th, T helper; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-alpha; Treg, regulatory T cells; WKY, Wistar Kyoto rats; ZO-1, zonula occludens-1.

Bullet point summary:

'What is already known'

Gut microbiota is involved in the control of BP. RAS inhibition reduces BP, reverses gut pathology, and shifts the gut microbiota composition in SHR.

'What this study adds'

RAS inhibition corrects gut dysbiosis due to its capacity to inhibit sympathetic drive, leading to improvement of gut integrity. These changes in gut microbiota contributed to the reduction of BP.

'Clinical significance'

Gut microbiota represent a new target for losartan in the BP control. The gut dysbiosis inhibition by antihypertensive drugs might be involved in lower BP in genetic hypertension.

Table S1. *Primers for real-time RT-PCR.*

mRNA targets	Descriptions (Gene ID)	Forward	
TNF-α	tumor necrosis factor-alpha (24835)	ACGATGCTCAGAAACACACG	CA
IL-6	interleukin-6 (24498)	GATGGATGCTTCCAAACTGG	AC
IL-10	interleukin-10 (25325)	GAATTCCCTGGGAGAGAAGC	GC
IL-17a	interleukin-17a (301289)	CTTCACCTTGGACTCTGAGC	TC
IFNϒ	interferon gamma (29197)	GCCCTCTCTGGCTGTTACTG	CC
T-bet	T-bet (303496)	CCACCAGCACCAGACAGAGA	AA
GATA-3	GATA-3 (85471)	GCCTGCGGACTCTACCATAA	GT
FOXP3	forkhead box P3 (317382)	AGGCACTTCTCCAGGACAGA	CT
RORϒ	ROR-gamma 1(9885)	GCCTACAATGCCAACAACCACACA	TG
Occludin	Occludin (83497)	AGCCTGGGCAGTCGGGTTGA	AC
Muc2	mucin-2 (24572)	CGATCACCACCATTGCCACTG	AC
MUC3	mucin-3 (687030)	CACAAAGGCAAGAGTCCAGA	AC
ZO-1	zonula occludens-1 (292994)	GCCAGCCAGTTCCGCCTCTG	AC
Th	tyrosine hydroxylase (25085)	GATTGCTACCTGGAAGGAGGT	AC
RNP1-2	alpha defensin RNP1-2 (613220)	GGACGCTCACTCTGCTTACC	TC
RNP3	alpha defensin RNP3 (498659)	AAGAGCGCTGTGTCTCTTGC	CA
RNP4	alpha defensin RNP4 (286958)	TCTGCTCATCACCCTTCTCC	AA
RNP5	alpha defensin RNP5 (28699)	ACCAGGCTTCAGTCATGAGG	CA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (28383)	GTCGGTGTGAACGGATTT	AT

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ARTICLE 4

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

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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 seems 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 (Yang et al., 2015; Mell et al., 2015;Gomez Arango et al., 2016; Li et al., 2017;Marques et al., 2017;Sun et al., 2019). 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 (Yang et al., 2015; Gomez Arango et al., 2016; Li et al., 2017). On the other hand, no significant changes in F/B were present in renin- independent hypertensive mice (Marques et al., 2017). Angiotensin II-infused germ-free mice showed that changes in gut microbiota are involved in angiotensin II-induced vascular dysfunction and hypertension (Karbach et al., 2016). In addition, fecal transplantation from spontaneously hypertensive-stroke prone rats (Adnan et al., 2017) or SHR (Toral et al., 2019a,b) to Wistar Kyoto rats (WKY) or from hypertensive subjects (Li et al., 2017) 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 (Toral et al., 2019b). 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 (Marques et al., 2018). The signalling molecules are bacterial metabolic products, including short chain fatty acids (SCFAs) (Pluznick et al., 2013), and bacterial wall components such as lipopolysaccharide (LPS) (Caroff et al., 2003). 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 (Yang et al., 2015;Toral et al., 2019b). SCFAs can impact renin secretion and blood pressure regulation stimulating host G- protein–coupled receptor pathways (Pluznick et al., 2013). In fact, butyrate has been shown to attenuate angiotensin II-induced hypertension in mice (Kim et al., 2016; Kim et al., 2018), 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 (Marques et al., 2017). Increased bacterial production of SCFAs is associated with reduced circulating CD4+ immune cells (Kim et al., 2018). 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 (Bomfim et al., 2012). 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 (Khalesi et al., 2014). Furthermore, a beneficial role of Lactobacillus probiotics and kefir in blood pressure regulation and vascular protection have been described in SHR with stablished hypertension (Gomez-Guzman et al., 2015;Frigues et al., 2015), 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. Results

2.1 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 ± 7.8 %, and 23.6 ± 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 ± 5.4 %, and 21.4 ± 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).

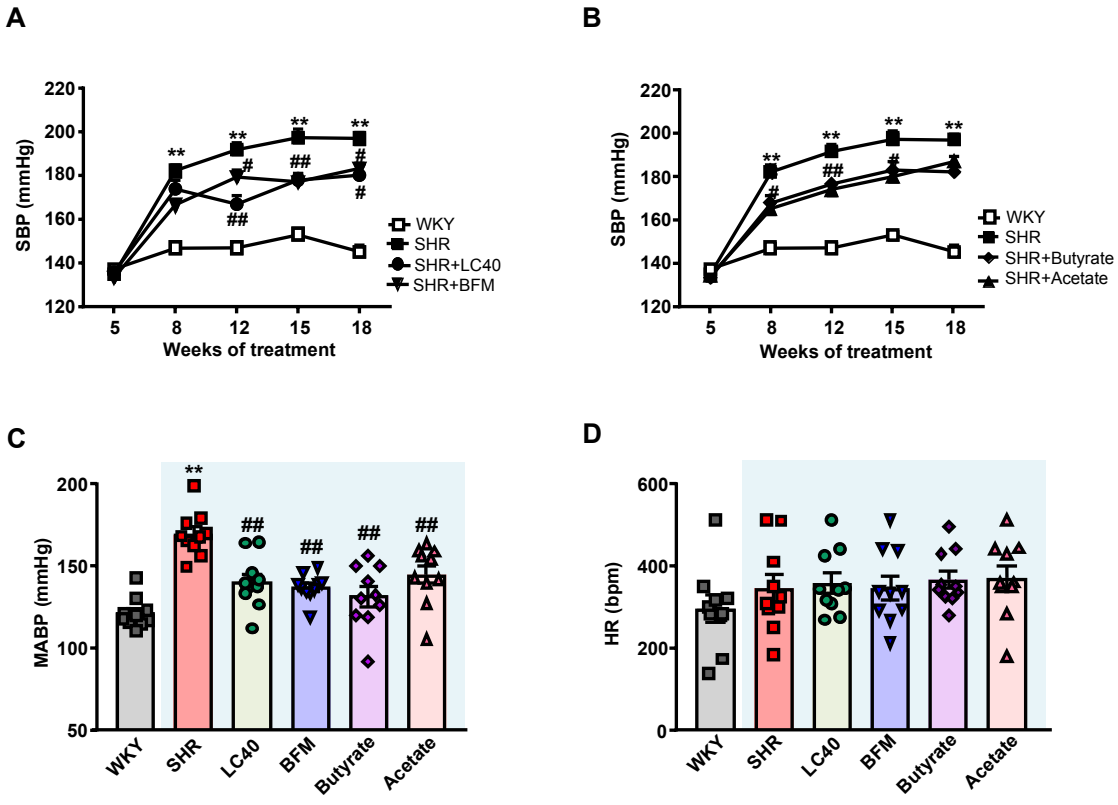

Figure 1. Probiotic treatments prevent elevated blood pressure in spontaneously hypertensive rats (SHR). *Time course of systolic blood pressure (SBP), measured by tail- cuff plethysmography in all experimental groups (**A, B**). Mean arterial blood pressure (MABP), measured by intra-arterial recording into left carotid*

*artery, at the end of the experimental period (**C**) and heart rate (HR) (**D**). Results are shown as mean ± 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.*

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).

Table S2. Body and organ weights and cardiac and renal indices.

	WKY (n = 10)	SHR (n = 10)	LC40 (n = 10)	BFM (n = 10)	I
BW (g)	402.0 ± 9.4	405.7 ± 8.9	387.9 ± 17.2	373.0 ± 15.3	39
HW (g)	1.12 ± 0.03	1.26 ± 0.04**	1.25 ± 0.04	1.17 ± 0.03	1.
LVW (g)	0.76 ± 0.02	0.94 ± 0.02**	0.90 ± 0.03	0.87 ± 0.02#	0.
KW (g)	1.26 ± 0.05	1.21 ± 0.03	1.22 ± 0.07	1.16 ± 0.04	1.
HW/TL (mg/cm)	206 ± 5	244 ± 5**	235 ± 6	222 ± 4#	
LVW/TL (mg/cm)	143 ± 4	180 ± 4*	167 ± 4#	165 ± 4##	
KW/TL (mg/cm)	238 ± 8	228 ± 6	232 ± 11	220 ± 7	

BW, body weight; HW, heart weight; LVW, left ventricle weight; KW, kidney weight, TL, Tibia length. V
of n rats. # P<0,05 y ##P<0,01 vs WKY y *P<0,05 vs SHR-control.

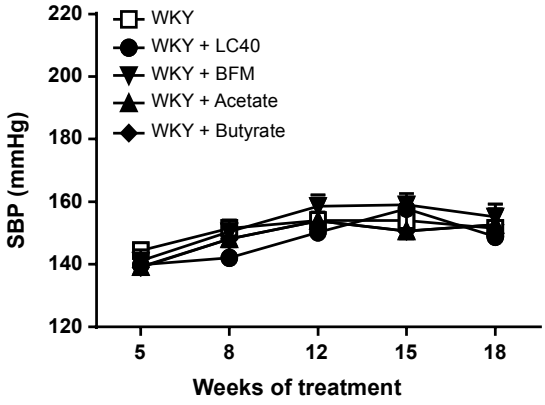

Figure S1. *Effects Probiotic treatments in blood pressure in Wistar Kyoto rats (WKY). Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography in all experimental groups. Results are shown as mean ± SEM (n = 6). LC40, Lactobacillus fermentum CECT5716; BFM, Bifidobacterium breve CECT7263.*

2.2. 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 (≈ 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 (≈ -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 (Yang et al.,2015), was ≈ 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 (≈ -50 and -42%, respectively, P<0.05), and a ≈ 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 ≈ 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 ≈ 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.

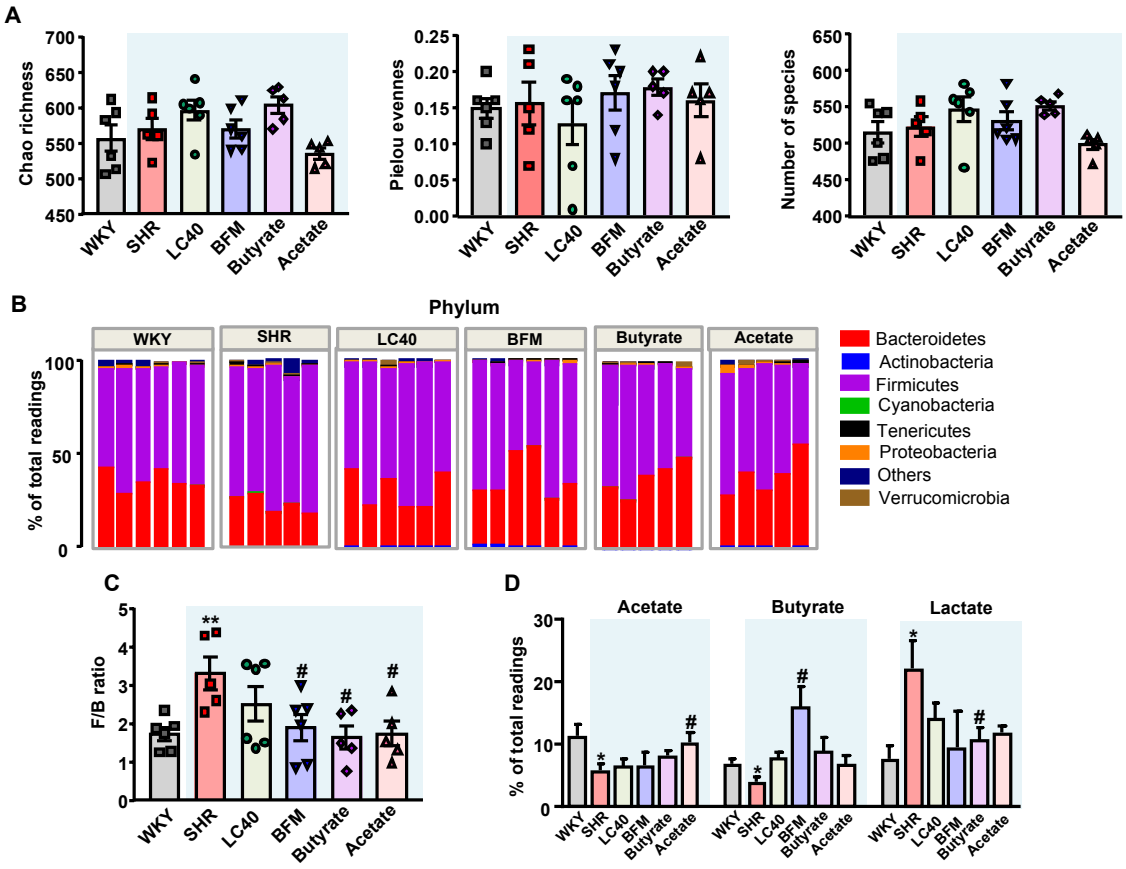

Figure 2. Probiotic treatments prevent gut dysbiosis in spontaneously hypertensive rats (SHR). *Bacterial 16S ribosomal DNA were amplified and sequenced to evaluate three major ecological parameters, Chao richness, Pielou evenness, and the number of observed species (**A**). Phylum breakdown of the 7 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 ± 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.*

Table S3. Effects of *Lactobacillus fermentum* CECT5716 (LC40), *Bifidobacterium breve* CECT7263 (BFM spontaneously hypertensive rats (SHR) on phyla changes in the gut microbiota.

Phylum	WKY (n = 6)	SHR (n = 5)	LC40 (n = 6)	BFM (n = 6)
Tenericutes	0.5 ± 0.2	0.7 ± 0.3	0.3 ± 0.2	0.3 ± 0.1
Actinobacteria	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
Verrucomicrobia	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.5	0.1 ± 0.0#
Proteobacteria	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.2
Bacteroidetes	36.1 ± 2.5	22.8 ± 2.4**	30.6 ± 4.4	36.6 ± 5.5#
Firmicutes	61.0 ± 2.5	72.3 ± 2.9*	67.7 ± 4.5	61.6 ± 5.3
Cyanobacteria	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0#	0.0 ± 0.0
Others	1.2 ± 0.6	2.7 ± 1.4	0.2 ± 0.1	0.5 ± 0.3

Values are expressed as mean ± SEM. *P< 0.05 and **P<0.01 compared with the Wistar Kyoto (WKY) grou
treated SHR group.

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 ≈ 3-fold in *Lactobacillaceae*, ≈ 2-fold *Turicibacteriaceae*, >100-fold *Peptostreptococcaceae*, and ≈ 4-fold *Anaeroplasmataceae*, and a ≈ 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 (≈ 3-, and 2-fold, respectively, $P<0.05$) and S24-7g were reduced ≈ 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 ≈ 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).

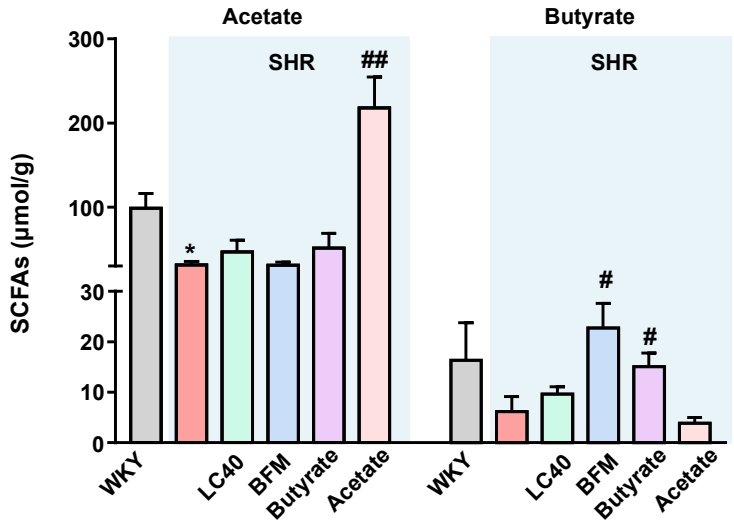

Figure S2. *Effects Probiotic treatments in short-chain fatty acids (SCFAs) level in feces of spontaneously hypertensive rats (SHR).* *Acetate and butyrate content in feces from all experimental groups measured by GC. Results are shown as mean ± SEM (n = 5 - 6). *P< 0.05 compared with WKY group. #P< 0.05 and ##P< 0.01 compared with the non-treated SHR group. LC40, Lactobacillus fermentum CECT5716; BFM, Bifidobacterium breve CECT7263.*

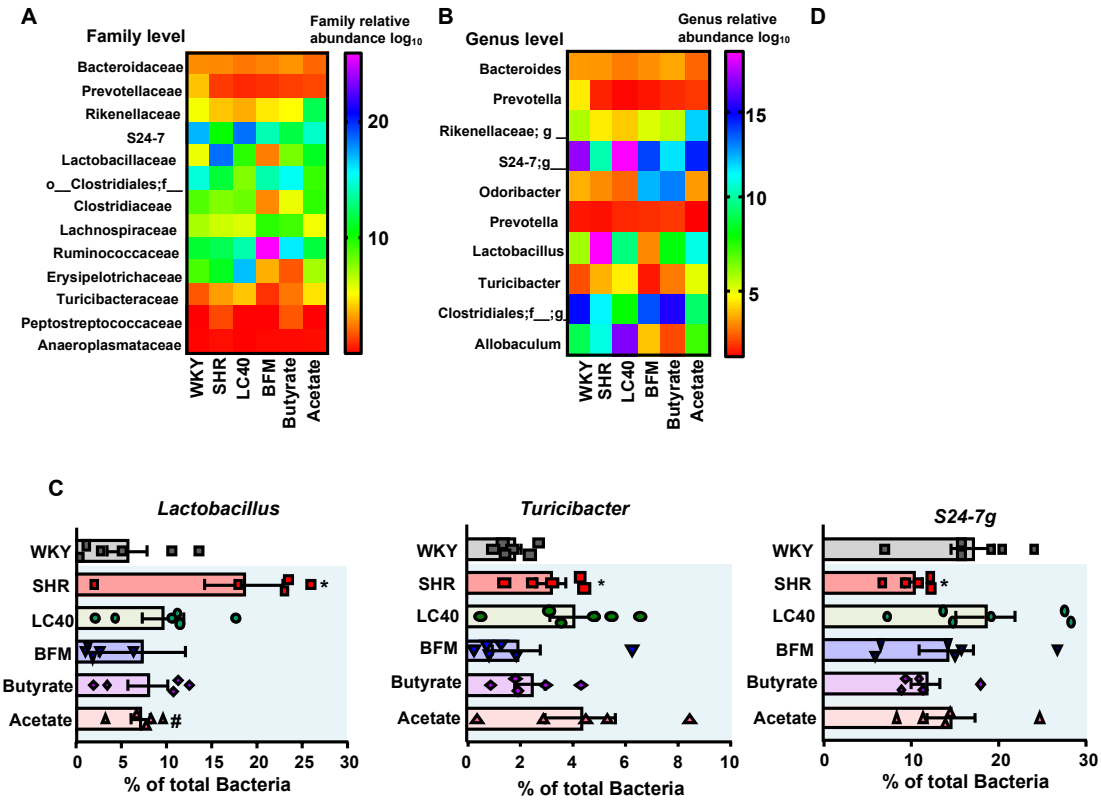

Figure 3. Probiotics treatments contribute gut microbiota homeostasis in spontaneously hypertensive rats (SHR). *Heat map showing the bacterial families and genera most differing in abundance between the experimental groups. Samples clustered by treatment group showed that the treatments resulted in distinct populations of bacteria at the family and genus level. A, B) The heat map colors represent the relative percentage of microbial family assigned within each sample as compared to SHR. C) Main significantly modified bacterial genera in the gut microbiota in Wistar Kyoto (WKY) and SHR groups. D) Ratio Lactobacillus/ S24-7 g and correlated with systolic blood pressure (SBP). Results are shown as mean ± 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.*

2.3. 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 ≈ 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 ≈ 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 ≈ 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 ≈ 4-fold MCT-1 and butyrate that increased ≈ 7-fold MCT-4. Lactate was significantly decreased in plasma (≈ - 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 (≈ 23, 64, 23 and 43%, respectively), and ≈ 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 ≈ 25% by butyrate treatment.

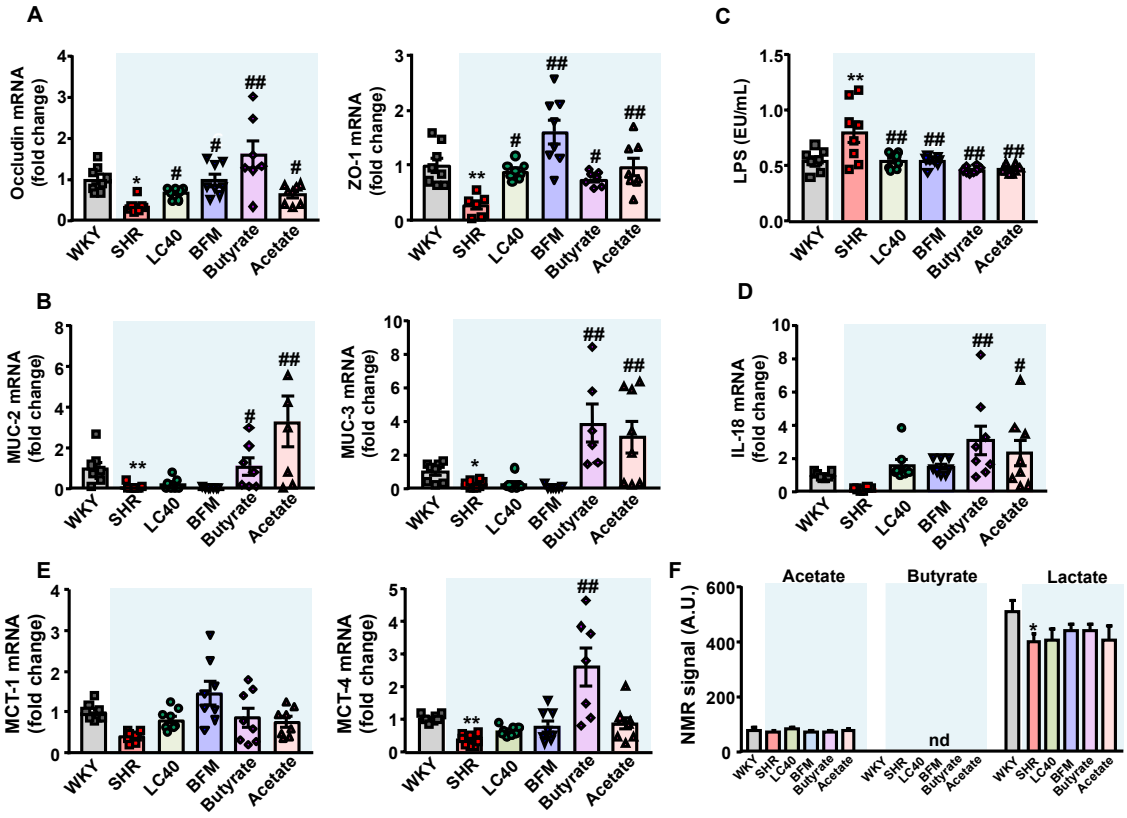

Figure 4. *Probiotic treatments improve altered gut integrity in spontaneously hypertensive rats (SHR). A) Colonic mRNA levels of occludin, and zonula occludens-1 (ZO-1). B) Mucin (MUC)-2, and MUC-3. C) Plasma endotoxin concentrations (LPS, EU mL^{-1}, endotoxin units mL^{-1}. D) Tissue repair cytokine IL-18. Expression of the E) short-chain fatty acids (SCFAs) monocarboxylate transporters, MCT-1 and MCT-4, F) the plasma levels of SCFAs from the nuclear magnetic resonance (NMR) spectra, and G) plasma butyrate levels by GC in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean ± 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.*

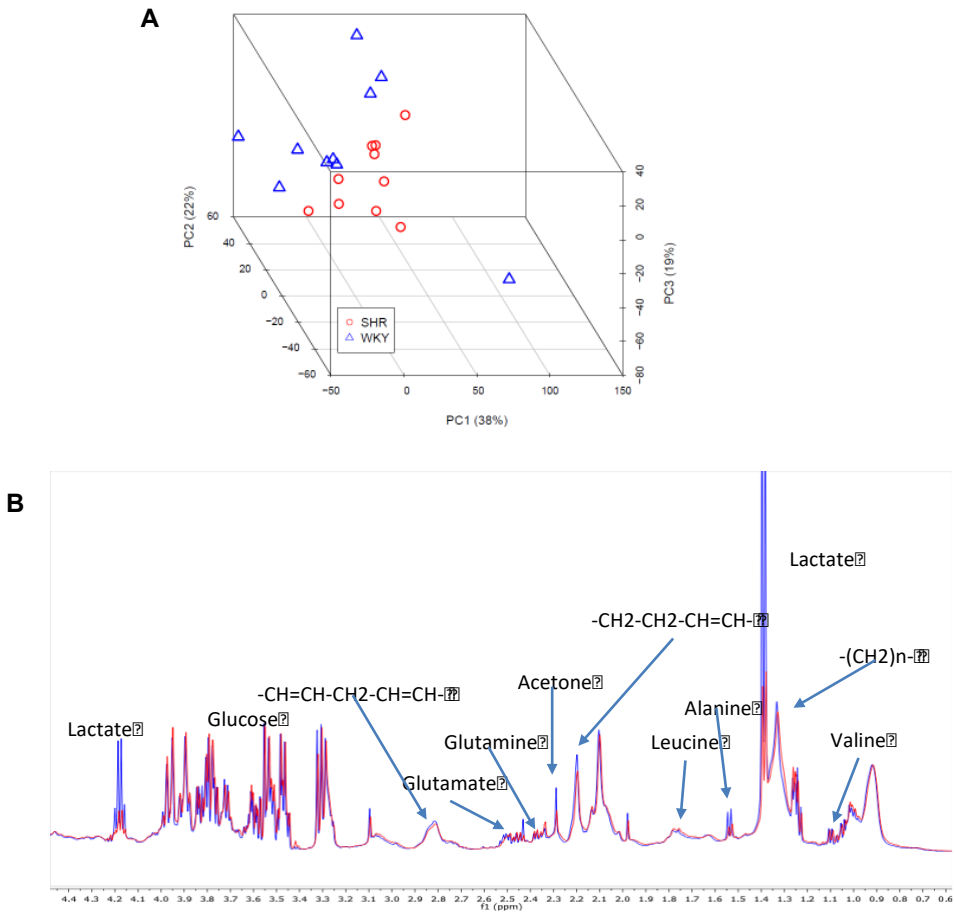

Figure S3. *Plasma metabolome of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR).* ***(A)*** *PCA scoring performed on* ^{1}H *NMR spectra of plasma samples from WKY (triangles) and SHR (open circles) rats. (B) Representative part of* ^{1}H *NMR spectra of plasma samples from WKY (blue) and SHR (red).*

Table S4. Effects of *Lactobacillus fermentum* CECT5716 (LC40), *Bifidobacterium breve* CECT7263 (BFM spontaneously hypertensive rats (SHR) on plasma metabolites expressed as relative units.

Phylum	WKY (n = 10)	SHR (n = 10)	LC40 (n = 9)	BFM (n = 10)
Valine+Propionate	64.3±6.3	79.3±3.2*	77.2±6.2	75.1±4.0
Leucine	58.9±5.4	72.7±2.4*	70.4±3.6	68.5±3.2
Creatinine	20.8±1.7	25.6±1.2*	27.9±3.2	25.8±1.1
Glucose	102.6±5.5	145.8±7.3**	143.2±10.3	137.9±4.5
Phenylalanine	2.2±0.5	3.6±0.2*	2.6±0.5#	2,4±0.6#
-(CH_2)n-	22.6±1.7	18.5±1.2*	18.5±1.0	22.0±1.2
-CH_2-CH_2-CH=CH-	51.7±4.5	39.9±2.5*	39.2±3.4	39.4±1.4
-CH=CH-CH_2-CH=CH-	23.3±2.3	17.9±1.4*	17.6±2.0	17.4±1.0

Values are expressed as mean ± SEM. *P< 0.05 and **P<0.01 compared with the Wistar Kyoto (WKY) grou treated SHR group.

2.4. 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 ≈ 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 ≈ 65%, whereas Th17 (CD4+/IL-17+) lymphocytes were ≈ 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.

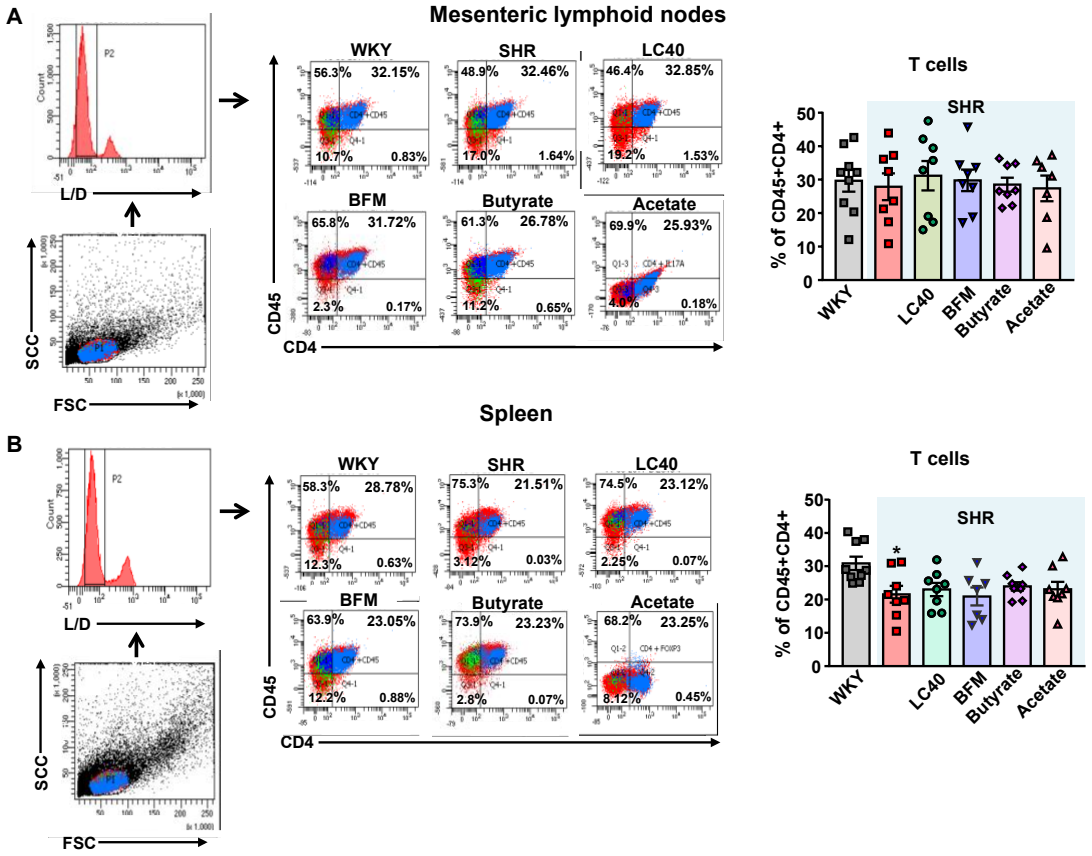

Figure S4. *Effects of probiotics treatments on percentage of total T cells in mesenteric lymph nodes and spleen of spontaneously hypertensive rats (SHR). Total T cells in mesenteric lymphoid nodes (A) and spleen (B) in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean ± SEM (n = 7-10). *P< 0.05 compared with WKY group. LC40, Lactobacillus fermentum CECT5716 (LC40); BFM, Bifidobacterium breve CECT7263.*

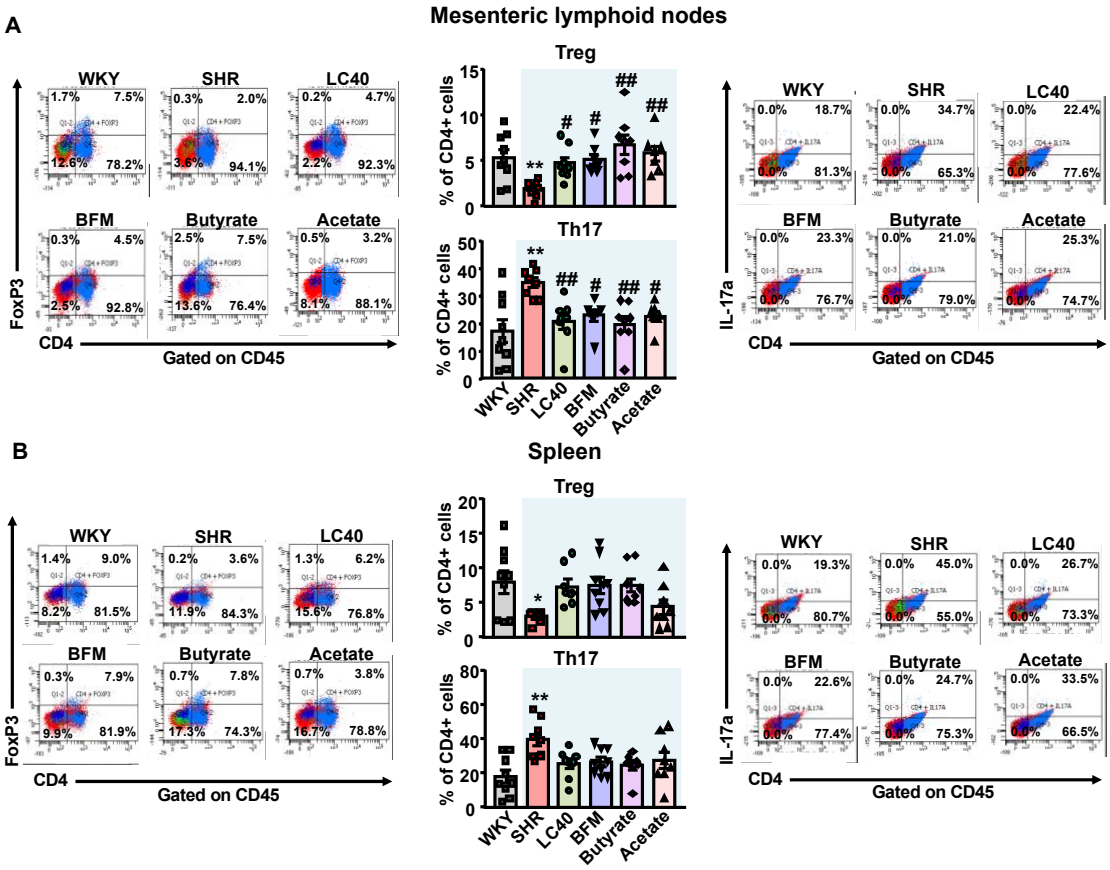

Figure 5. *Probiotic treatments restore T cell imbalance in spontaneously hypertensive rats (SHR). Regulatory T (Treg) cells, and T-helper (Th) 17 cells measured in A) mesenteric lymphoid nodes and B) spleen in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean ± SEM (n = 7–10). *p < 0.05 and **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*

2.5. 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 (Emax = 59 ± 4 % vs 84 ± 3 %, respectively). Both LC40 and BFM treatments increased the relaxation induced by acetylcholine in SHR rats (Emax = 70 ± 5 %, and 69 ± 2 %, respectively, P<0.05 vs SHR control) (Fig. 6A). Similarly, chronic butyrate and acetate also improved the relaxation to acetylcholine (Emax = 74 ± 5 %, and 63 ± 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 (Emax = 80 ± 4 %), reaching similar relaxation percentages to those found in WKY (Emax = 85 ± 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 ≈ 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, p47phox and p22phox (≈ 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 ≈ 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).

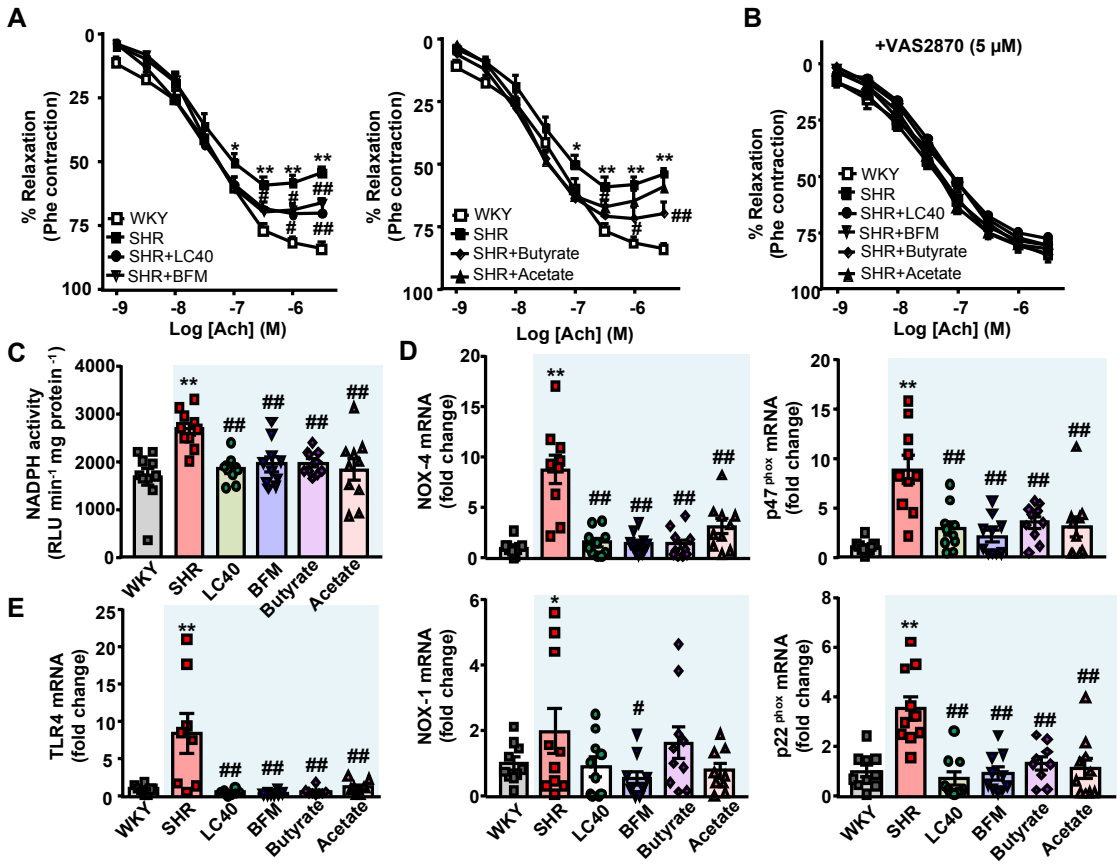

Figure 6. *Protective effects of probiotic treatments in endothelial function and oxidative stress in spontaneously hypertensive rats (SHR). Vascular relaxant responses induced by acetylcholine (Ach), in endothelium-intact aortae pre-contracted by phenylephrine (Phe) in the A) absence and in the B) presence of the NADPH oxidase inhibitor VAS2870 (5 µm) in Wistar Kyoto (WKY) and SHR groups. NADPH oxidase activity measured by C) lucigeninenhanced chemiluminescence, and aortic mRNA levels expression of NADPH oxidase subunits D) NOX-4, p47$_{phox}$, NOX-1, and p22$_{phox}$, and E) toll-like receptor (TLR)-4 in Wistar Kyoto (WKY) and SHR groups. Results are shown as mean ± 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.*

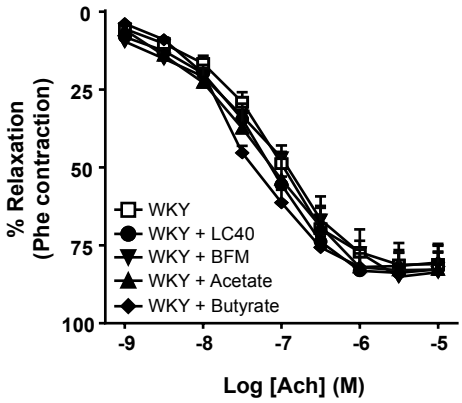

Figure S5. *Effects of probiotic treatments in endothelial function in Wistar Kyoto rats (WKY). Vascular relaxant responses induced by acetylcholine (Ach), in endothelium-intact aortae pre-contracted by phenylephrine (Phe) in all experimental groups. Results are shown as mean ± SEM (n = 6). LC40, Lactobacillus fermentum CECT5716; BFM, Bifidobacterium breve CECT7263.*

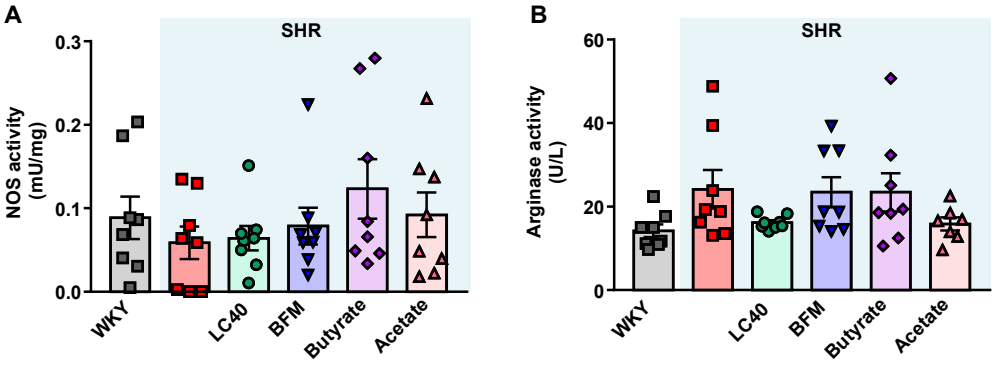

Figure S6. *Effects of probiotics treatments on aortic nitric oxide synthase (NOS) activity (A) and arginase activity (B) in spontaneously hypertensive rats (SHR). Results are shown as mean ± SEM. LC40, Lactobacillus fermentum CECT5716 (LC40); BFM, Bifidobacterium breve CECT7263*

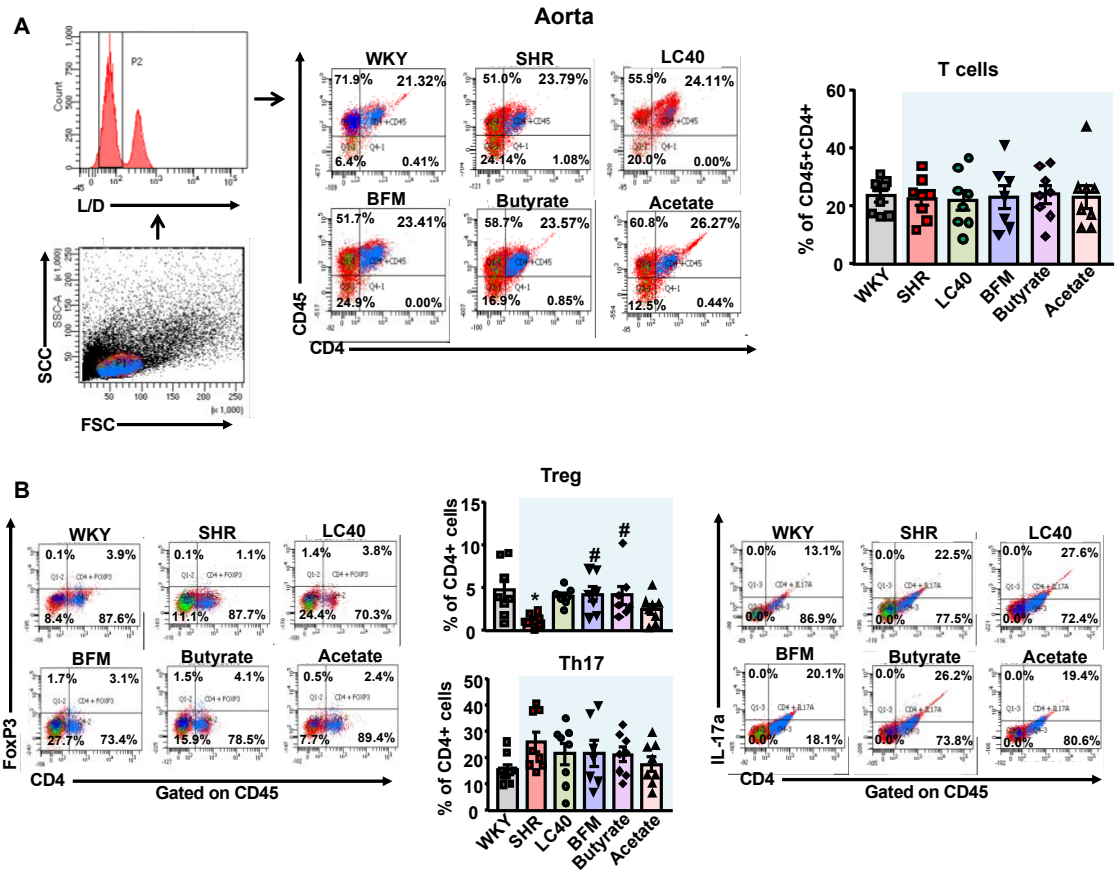

Figure 7. *Probiotic treatments improve T cell infiltration in spontaneously hypertensive rats (SHR). A) Total T cells, regulatory T (Treg) cells, and B) T-helper (Th) 17 cells measured in aortae of Wistar Kyoto (WKY) and SHR groups. Results are shown as mean ± SEM (n = 7–10). *p < 0.05 compared with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

3. 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 (Yang et al., 2015; Mell et al., 2015; Li et al., 2017; Sun et al., 2019; Toral et al., 2019b). Our results are consistent with the main features of dysbiotic microbiota described in SHR (Yang et al., 2015; Yang et al., 2018; Toral et al., 2019b): 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 (Gomez-Guzman et al.,2015). These cardiovascular protective effects induced by Lactobacillus strains were associated with changes in some bacterial genera (increased *Lactobacillus spp* and reduced *Bacteroides* and *Clostridium ssp*) 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 (Toral et al., 2019). 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 (Krych et al., 2015). 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 (Toral et al., 2019a). 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 (Arpaia et al., 2013; Furusawa et al., 2013;Smith et al.,2013). 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 (Sugiyama et al., 2010); 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 (Halestrap et al., 2013). 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 (Kim et al., 2018). 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 (Chen et al., 2017; Zhang et al., 2019). 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 (Aguilar et al., 2016). Hypertension is associated with the altered expression of gut tight junction proteins, increased permeability and gut pathology (Santisteban et al., 2017). SCFAs have been demonstrated to exert many beneficial effects on intestinal epithelium, including inhibition of inflammation (Huang et al., 1997), and modulation of oxidative stress (Hammer et al., 2009). Furthermore, an improved barrier function by SCFAs has been reported in vitro (Peng et al., 2007; Van Deun et al., 2008; Elammin et al., 2013; Elinav et al., 2013), ex vivo (Suzuki et al., 2008) and in animal studies studies (Suzuki et al., 2008; Kim et al., 2018). 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 (Santisteban et al., 2017). These cells produce mucins, which protect the gut from pathogen invasion, thereby regulating the gut immune response (Mowat et al., 2014). 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 (Elinav et al., 2013), 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 (Niess et al., 2005). 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 (Harrison et al., 2015). 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 (Bomfim et al., 2012). Actually, TLR4-/- mice demonstrated a full blood pressure protection against L-NAME-induced hypertension (Sollinger et al.,2014). 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 (Liang et al.,2013;Toral et al.,2014). 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 (Ryan et al.,2012). 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 (Toral et al.,2019b). 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 (Nguyen et al.,2013). By contrast, in agreement with Katsuki et al. 2015, 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 (Kassan et al.,2011). 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 (d'Uscio et al.,2011). 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.

4. 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).

4.1. 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 37ºC in anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, Hants, UK). For the probiotics treatment, the lyophilized bacteria were aliquoted and stored at -20ºC until usage, at that moment, they were suspended in tap water and administered.

4.2. 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 mg kg-1 day-1 in the drinking water). The doses of probiotics selected were similar to that previously used to reduce BP in SHR (Gomez-Guzman et al., 2015). We used a dose of acetate similar to that used to prevent hypertension in DOCA-salt mice (Marques et al., 2017), and a dose of butyrate that prevents the rise of blood pressure in angiotensin II- infused mice (Kim et al., 2016). Oral gavage of probiotics was 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.

4.3. 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 (Toral et al., 2019b). At the end of the experimental period, animals were put under isofluorane 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) (Toral et al., 2019a).

4.4. 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.

4.5. 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 ºC. 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, Pyrosperse, a dispersing agent, was added to

remove fat from the samples. After those two pre-treatments, samples were incubated at 70 ºC 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 (Izquierdo-Garcia et al., 2009). 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 4ºC (Juanola et al., 2019). 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 (Algieri et al.,2014). Briefly, the samples were homogenized

with 150 mM $NaHCO_3$ (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.

4.6. Vascular reactivity studies

Thoracic aortic rings were incubated in organ baths filled with Krebs solution (composition in mmol/L: $CaCl_2$ 2, glucose 11, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118 and $NaHCO_3$ 25) at 37 ºC and infused with carbogen (95% O2 and 5% CO_2) 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 (Toral et al., 2019a). 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 NG-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 µmol/L phenylephrine. Relaxant responses were expressed as a percentage of precontraction.

4.7. 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 (Toral et al., 2019a). 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 intructions. 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 intructions.

4.8. 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 70μM cell strainer. 1×106 cells were counted and incubated with a protein transport inhibitor (BD GolgiPlugTM) and a 50 ng/mL phorbol 12-myristate 13-acetate plus 1 μg/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 (Galkina et al.,2006). 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).

4.9. 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 (Toral et al., 2019a). The sense and antisense primer sequences used for amplification are described in Table S1.

Table S1. Oligonucleotides for real-time RT-PCR.

mRNA targets	Descriptions	Sense
NOX-1	NOX-1 subunit of NADPH oxidase	TCTTGCTGGTTGACACTTGC
NOX-4	NOX-4 subunit of NADPH oxidase	ACAGTCCTGGCTTACCTTCG
p22phox	p22phox subunit of NADPH oxidase	GCGGTGTGGACAGAAGTACC
p47phox	p47phox subunit of NADPH oxidase	CCCAGCGACAGATTAGAAGC
Occludin	occludin	AGCCTGGGCAGTCGGGTTGA
Muc2	mucin-2	CGATCACCACCATTGCCACTG
MUC3	mucin-3	CACAAAGGCAAGAGTCCAGA
ZO-1	zonula occludens-1	GCCAGCCAGTTCCGCCTCTG
MUC-2	Mucin 2	CGATCACCACCATTGCCACTG
MUC-3	Mucin 3	CACAAAGGCAAGAGTCCAGA
IL18	Interleukin 18	ACCGCAGTAATACGGAGCAT
MCT1	Monocarboxylate transporter 1	GAAAAACTCAAGTCCAAAGAGTCT
MCT4	Monocarboxylate transporter 4	TCAGGAGGCAAGCTGCTGGACGCAA
TLR-4	toll-like receptor-4	GCCTTTCAGGGAATTAAGCTCC
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	GTCGGTGTGAACGGATTT

4.10. 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 (Yang et al., 2015). The PCR amplicons were purified by QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified by Qubit (thermos 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 ≥ 93.5% and 97.17 ± 0.34% of total cluster passes the filter.

4.11. 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 (Bokulich et al., 2013). 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 phylogenic tree-based unifrac distance metric were generated using scripts from QIIME package.

4.12. Chemicals

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

4.13. 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 (Segata et al., 2011). 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 ± 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 $P<0.05$.

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Authors contribution

IRV, MT and JD participated in the research design. IR-V, MT, N.d.l.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.

ARTICLE 5

The probiotic *Lactobacillus fermentum* prevents dysbiosis and vascular oxidative stress in rats with hypertension induced by chronic nitric oxide blockade

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Authors contribution

I. R.-V., M. T., N. d. V., M. S., and M. R., performed experiments. R. J., and M. O. performed experiments and contributed to discussion. J. D. designed experiments and wrote the manuscript.

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Abbreviations: APC, antigen presenting cells; AUC, area under curve; BW, body weight; BP, blood pressure; CECT, Collection of Type Cultures; CFU, colony-forming units; DHE, dihydroethidium; DAPI, 4,6-diamidino-2-phenylindole dichlorohydrate; DSMZ, German Collection of Microorganisms and Cell Cultures; eNOS, endothelial nitric oxide synthase; HR, heart rate; HW, heart weight; IFN-γ, interferon-γ; IL, interleukin; KW, kidney weight; L-NAME, N^G-nitro-L-arginine methyl ester; LVW, left ventricular weight; MLN, mesenteric lymph nodes, NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; RLU, relative luminescence units; RORγ, orphan receptor-γ; SBP, systolic blood pressure; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; Th17, T helper-17 cells; Treg, regulatory T cells; TNF-α, tumor necrosis factor α.

Keywords: Probiotic bacteria, endothelial dysfunction, L-NAME- hypertension, immune system, nitric oxide, reactive oxygen species.

Abstract

Scope: We tested whether the probiotic *Lactobacillus fermentum* CECT5716 (LC40) ameliorates hypertension in rats with chronic nitric oxide (NO) synthase inhibition.

Methods and results: Rats were randomly divided into four different groups and treated for 4 weeks: a) vehicle (control), b) vehicle plus L-NAME (50 mg 100 mL^{-1} in drinking water), c) LC40 (10^9 colony-forming units/day by gavage), and d) LC40 plus L-NAME. L-NAME induced gut dysbiosis, characterized mainly by an increased *Fimicutes/Bacteroidetes* (F/B) ratio and reduced *Bifidobacterium* content, increased Th17 cells and reduced Treg in mesenteric lymph nodes (MLN), increased aortic Th17 infiltration and reactive oxygen species, reduced aortic endothelium-dependent relaxant response to acetylcholine, and hypertension. LC40 prevented gut dysbiosis, altered the Th17/Treg balance in MLN, vascular oxidative stress and inflammation, improved slightly endothelial dysfuncion but did not inhibit the development of L-NAME-induced hypertension.

Conclusion: Chronic LC40 treatment, in this model of chronic inhibition of NO synthesis, reduced early events involved in atherosclerosis development, such as vascular oxidative stress and pro-inflammatory status, as a result of prevention of gut dysbiosis and immune changes in MLN, but not hypertension, confirming the critical role of NO in the antihypertensive effects of LC40 in genetic hypertension.

1. Introduction

Hypertension is an important risk factor for cardiovascular events as stroke and myocardial infarction. All available antihypertensive regimens are able to decrease the risk of incident major cardiovascular events (Turnbull et al., 2003). Recent hypertension guidelines recommend lifestyle changes, including dietary measures and exercise, whenever possible, for all patients, including those undergoing drug treatments. The goal of these lifestyle modifications consists of lowering blood pressure (BP) and reduces other cardiovascular risk factors as well as doses and the number of antihypertensive drugs (Mancia et al., 2014).

The pathogenesis of hypertension is complex, but a growing body of evidence supports the role of the gut microbiota in the regulation of BP, and the promising therapeutic potential of gut microbiota modification to prevent or treat hypertension (Marques et al., 2017). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". The expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) recommends that the term probiotic be used only on products that deliver live microorganisms with a suitable viable count of well-defined strains with a reasonable expectation of delivering benefits for the wellbeing of the host (Hill et al., 2014). Probiotics can be selectively used to introduce and expand new types of microorganisms. Probiotics exert antihypertensive effects in human hypertensive patients (Khalesi et al., 2014) and rat with genetic hypertension (genetic hypertensive rats) (Lollo et al., 2015; Robles-Vera et al., 2018). In fact, chronic consumption of *Lactobacillus* strains not only reduced BP, but also inhibited organ target damage and improved endothelial dysfunction in spontaneously hypertensive rats (SHR) (Gomez-Guzman et al., 2015). This antihypertensive effect seems to be related to increased nitric oxide (NO) bioavailability, as a result of reduced vascular pro-inflammatory and pro-oxidative status. However, there is no information about the antihypertensive effects of probiotic in other non-genetic forms of hypertension, and whether NO plays a key role.

The immune system is involved in the exaggerated inflammatory responses in the development of hypertension (Wenzel et al., 2016). The intestinal microbiota composition has a marked role in host immune functions (Gensollen et al., 2016), and its modulation can result in beneficial effects. *Lactobacillus fermentum* CECT5716 (LC40) can modulate the human immune system, increasing regulatory T cells (Treg) (Arribas et al., 2009;

Pérez-Cano et al., 2010). Tregs produce interleukin (IL)-10, which inhibits NADPH oxidase activity, the main source of reactive oxygen species (ROS) in the vasculature and improves endothelial function (Kassan et al., 2011).

We hypothesized that antihypertensive effects of LC40 might be related to changes in gut microbiota, linked to changes in T cells population in secondary immune organs. These immune changes could reduce vascular T cells infiltration lending to reduction of vascular oxidative stress and vascular inflammation, thus protecting NO from ROS inactivation. To explore this hypothesis we reduced NO production in rats by chronic blockade of NO synthase (NOS) with NG-Nitro-L-Arginine Methyl Ester (L-NAME). Thus, the aim of this study was to analyze the effects of the probiotic LC40 in the hypertension and endothelial dysfunction induced by L-NAME (Baylis et al., 1992). This experimental model of hypertension is characterized by minor changes in gut microbiota (Xu et al., 2013) and T cells activation (Shah et al.,2015). We have also analyzed if the protective effects of LC40 are linked to changes in T cell populations in mesenteric lymph nodes (MLN).

2. Results

2.1 LC40 treatment prevented gut dysbiosis induced by chronic NOS inhibition

Microbiota composition was characterized in relation to the three major ecological parameters, Shannon diversity, Pielou evenness and Chao richness, as well as the number of observed species. However, no relevant changes were detected in any of the aforementioned parameters for all groups (**Fig. 1A**). When phyla composition was examined, *Firmicutes* and *Bacteroidetes* and, in lower proportions, *Proteobacteria* and *Actinobacteria* dominated in fecal microbial communities of all groups (**Fig. 1B**). *Firmicutes:Bacteroidetes* (F/B) ratio, a marker of gut dysbiosis in hypertension, was significantly higher in L-NAME-treated group compared to control group. This effect was attributed to an increase in *Firmicutes* and a decrease (≈ 4.6 times) in *Bacteroidetes* populations. Besides, probiotic treatment normalized this ratio in L-NAME-treated group (**Fig. 1C**). No other significant changes were observed at phylum level (**Fig. 1C**). About SCFA producing bacteria, an increase in the production of propionate while did not

change the acetate, butyrate and lactate production, were also found in L-NAME groups as compared to control, which was abolished by LC40 consumption (**Fig. 1D**).

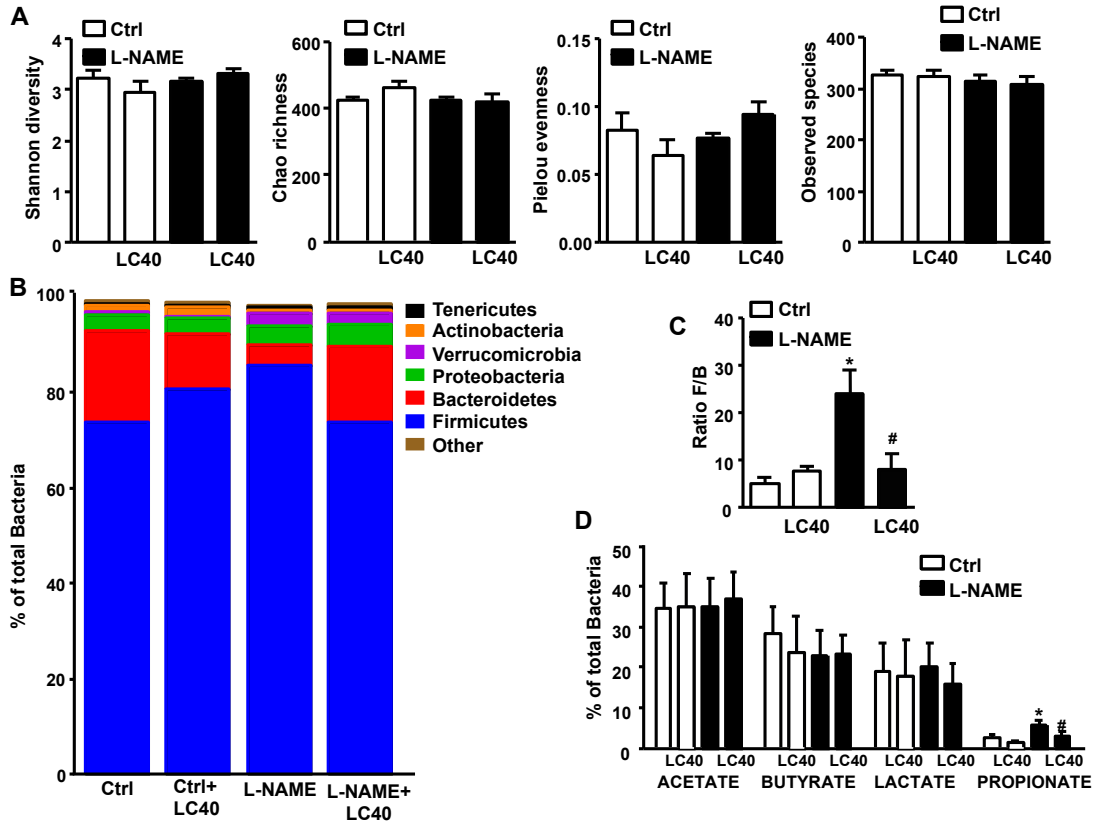
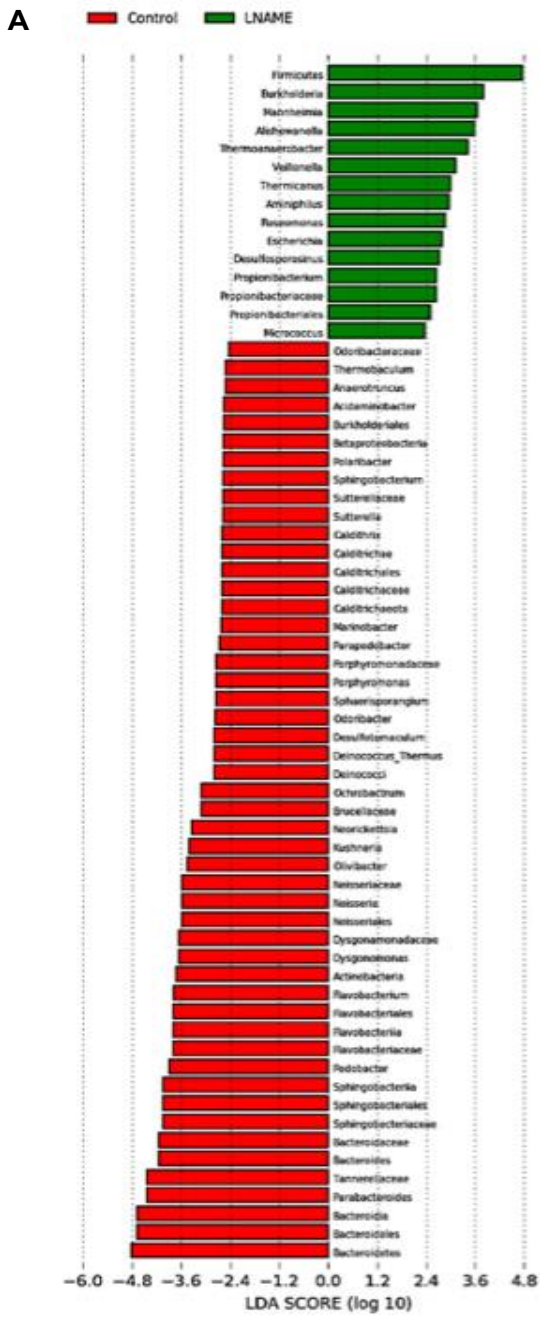


Figure 1. Lactobacillus fermentum CECT5716 (LC40) treatment induces phyla changes in the microecological parameters and gut microbiota composition in Nɢ-nitro-L-arginine methyl ester (L-NAME) group. *The microbial DNA from fecal samples was analyzed by 16S rRNA gene sequencing. To evaluate general differences of microbial composition amongst all experimental groups diversity, richness, evenness, and observed species (**A**) were examined. Phylum breakdown of the 6 most abundant bacterial communities in samples from all groups (**B**). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker for gut dysbiosis (**C**). Lactate-, butyrate-, acetate- and propionate-producing bacteria expressed as relative proportions (**D**) in the gut microbiota in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6). *P< 0.05 compared with the Ctrl group. #P< 0.05 compared with the non-treated L-NAME group.*

Fig. S1 shows the bacterial taxa (class, order, family, and genus) that were altered by L-NAME administration, according to LEfSe analysis. Prominent changes in bacterial taxa occurred after four weeks of L-NAME administration, where the relative abundance of 15 taxa was increased (green) and 50 taxa were decreased (red), when compared with control rats. LC40 treatment of L-NAME rats also induced several changes in the

microbiota taxa as compared to L-NAME group, where the relative abundance of 25 taxa was increased (green) and 6 taxa was decreased (red) (**Fig. S2**). Our group also identified what bacterial genera was associated to the change in the composition of microbiota (**Fig. 2A**) significant depletion of *Parabacteroides, Bifidobacterium, Olivibacter, Dysgonomonas, Pedobacter, Flavobacterium,* and *Desulfotomaculum*, with a higher abundance of *Propionibacterium* was found in the microbiota of L-NAME group as compared to control. LC40 treatment significantly restored changes in *Olivibacter* and *Propionibacterium* (**Fig. 2B**)



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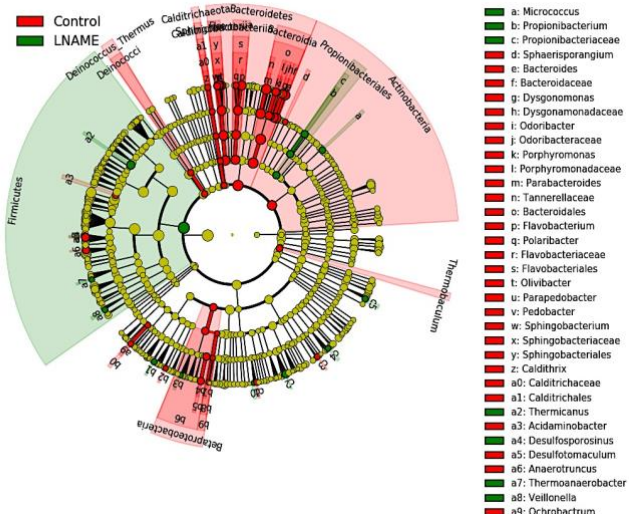

Figure S1. Changes of the gut microbiome of NG-nitro-L-arginine methyl ester (L-NAME) group. *Com*
control versus L-NAME rats. Linear discriminant analysis effects size (LEfSe) identified significantly differ
*cohort at LDA Score > 2, p<0.05 (red bars Control enriched, green bars L-NAME enriched) (**A**). Cladogr*
taxa, the taxa are identified in the key to the right of each pane. Larger the circles greater the difference in a
= 6 rats per treatment group in each comparison.

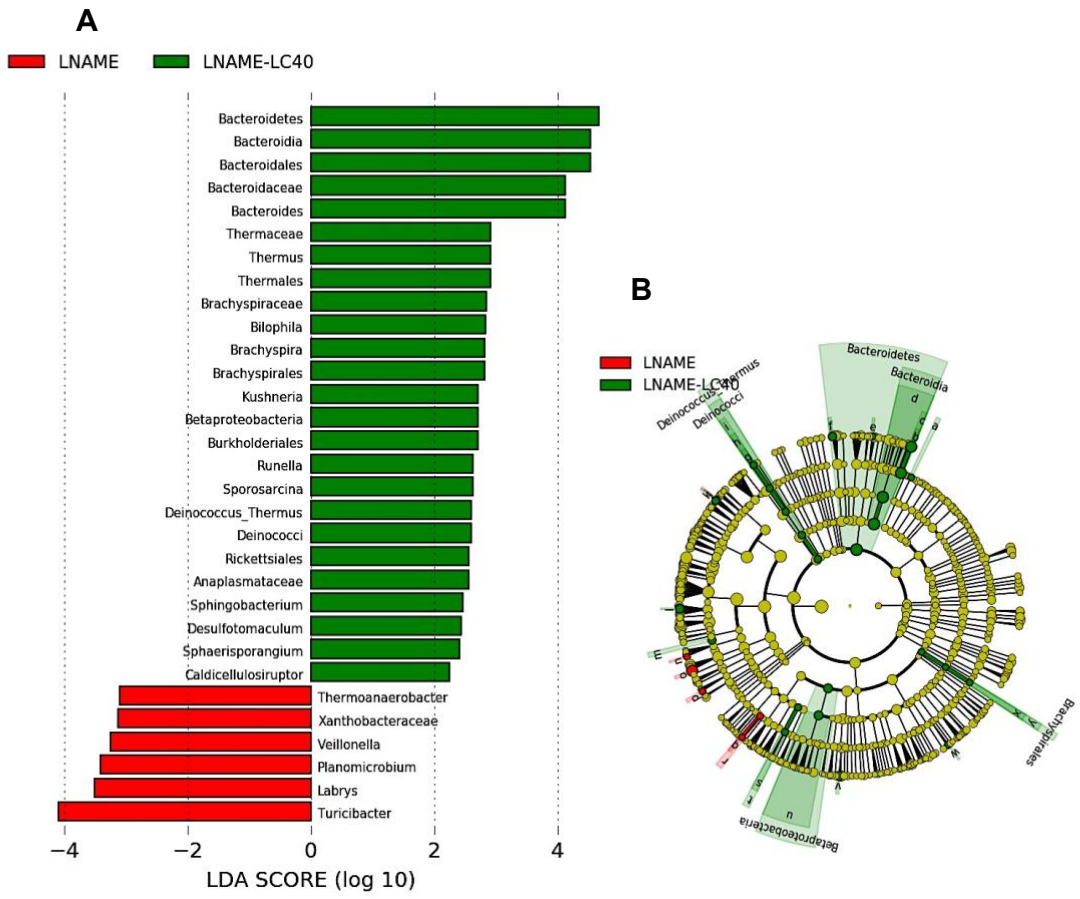

Figure S2. Lactobacillus fermentum CECT5716 (LC40) treatment induces changes of the gut microbio ester (L-NAME) group. *Comparisons of microbiome changes in L-NAME versus L-NAME+LC40 rats. Line (LEfSe) identified significantly different bacterial taxa enriched in each cohort at LDA Score > 2, p<0.05 bars L-NAME enriched) (**A**). Cladograms show the significantly enriched taxa, the taxa are identified in the the circles greater the difference in abundance between the groups **(B)**. n = 6 rats per treatment group in eac*

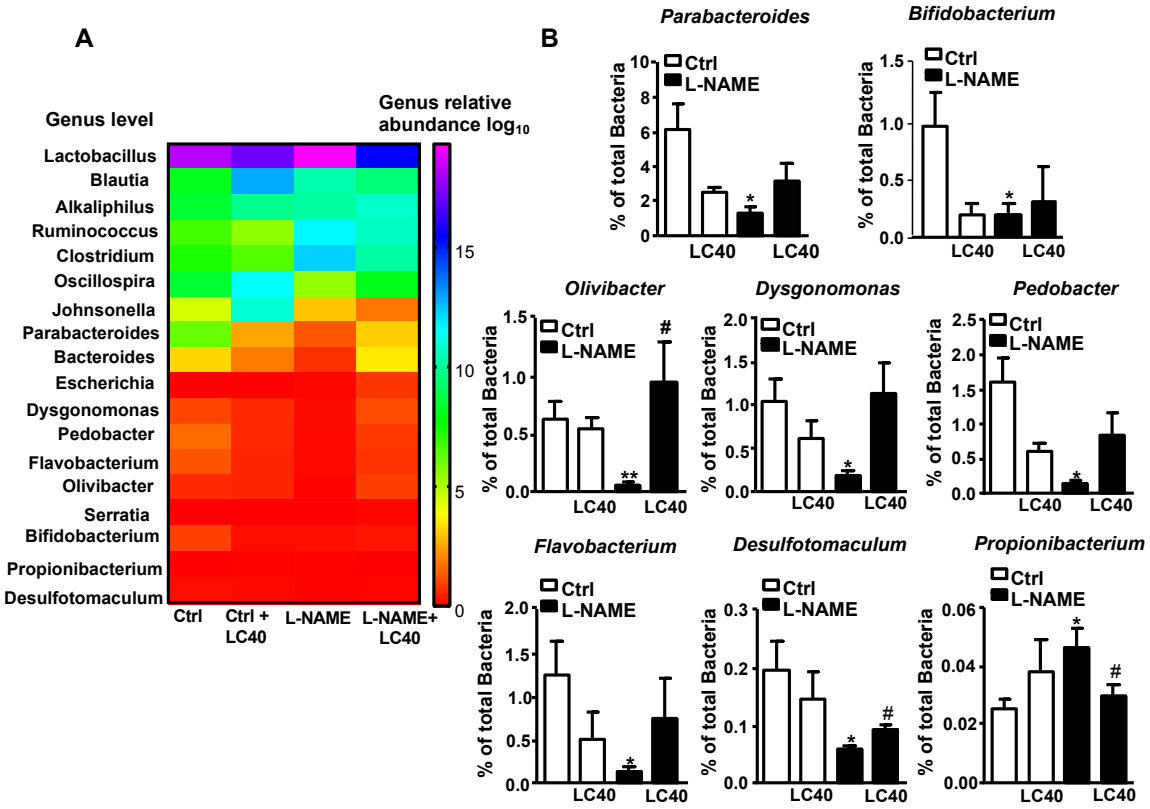

Figure 2. Lactobacillus fermentum CECT5716 (LC40) treatment induces genera changes in the gut microbiota composition in Nᴳ-nitro-L-arginine methyl ester (L-NAME) group. *Heat map showing the bacterial genera most differing in abundance between different groups. Samples clustered by treatment group demonstrated that the treatments resulted in distinct populations of bacteria at the genus level. The heatmap colors represent the relative percentage of microbial genus assigned within each sample (**A**). Main significantly modified bacterial genera (**B**) in the gut microbiota in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6). *P< 0.05 compared with the Ctrl group. #P< 0.05 compared with the non-treated L-NAME group.*

2.2 Effects in blood pressure and organ damage

Rats receiving chronic L-NAME treatment showed a progressive increase in SBP (**Fig. 3A**), and DBP (**Fig. 3B**), which were already significant after the first week. HR was significantly reduced in L-NAME group as compared to control at the first week, but was unchanged at the end of the experiment (**Fig. 3C**). Concomitant treatment with LC40 did not significantly prevent the raise in both SBP and DBP, and did not change HR. LC40 treatment did not affect HR At the end of the study period, L-NAME significantly increased the left ventricular weight index (≈ 18 %) and proteinuria (≈ 90 %), as compared with the control group (**Table S2**). These changes in target organs were unaffected by

LC40 consumption. Body weight (BW) increased in both control and L-NAME groups after 4 weeks (17.6 ± 2.6 % and 14.0 ± 6.6 %, respectively), which was unaltered by LC40 administration. No statistical differences among all experimental groups in both food intake and water intake were observed (**Table S2**).

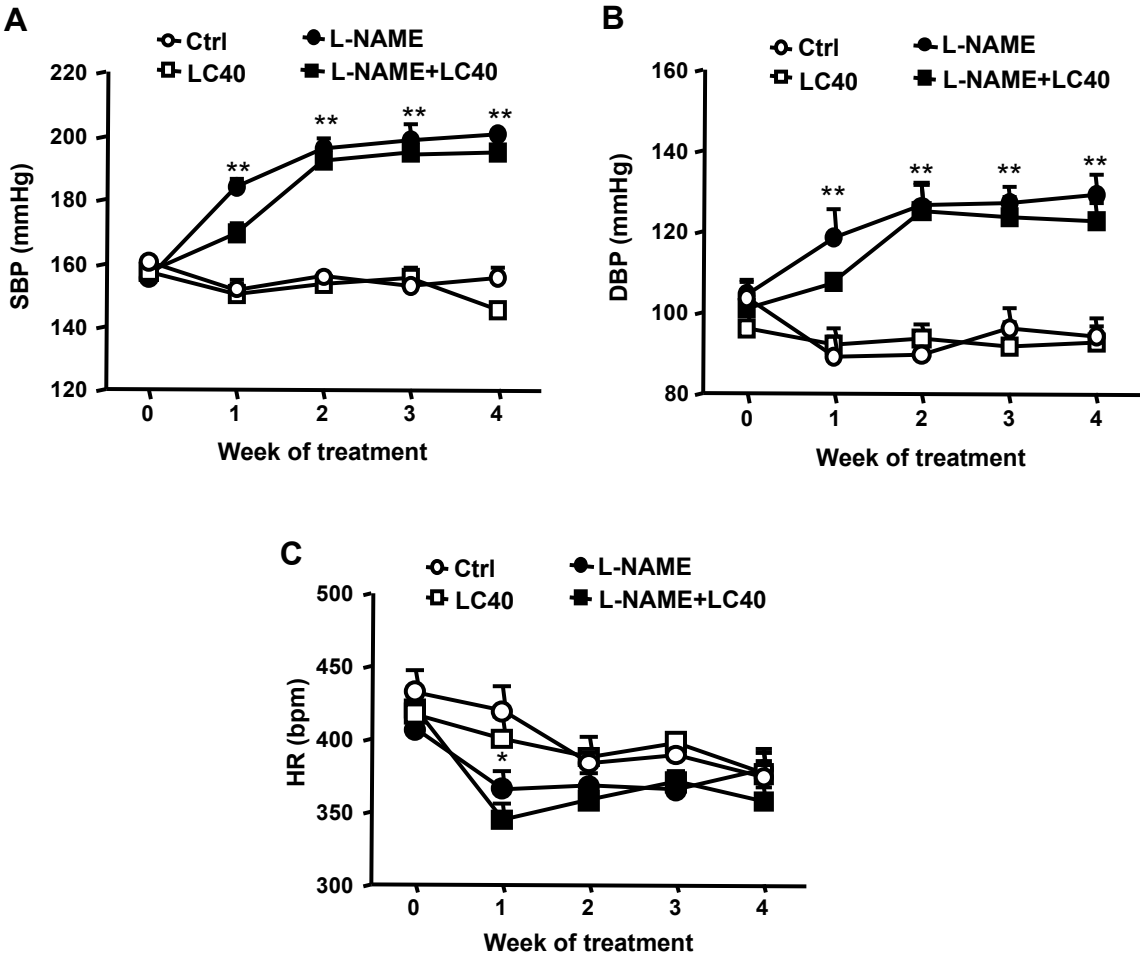

Figure 3. Lactobacillus fermentum CECT5716 (LC40) treatment not prevents hypertension induced by NG-nitro-L-arginine methyl ester (L-NAME) group. *Systolic blood pressure (SBP) (A), diastolic blood pressure (DBP) (B), and heart rate (HR) (C), were measured by tail-cuff plethysmography in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6-8). *P< 0.05 and **P< 0.01 compared with the Ctrl group.*

Table S2. General determinations in all experimental groups

	Ctrl (n = 8)	Ctrl+LC40 (n = 6)	L-NAME (n = 7)	L-NAME +LC40 (n = 7)
Initial BW (g)	300 ± 8	305 ± 7	298 ± 5	299 ± 6
Final BW (g)	353 ± 14	361 ± 11	342 ± 20	353 ± 6
Food intake (g/day)	17.3 ± 1.1	18.4 ± 1.6	17.5 ± 0.9	18.0 ± 0.9
Water intake (ml/day)	25.0 ± 1.6	27.7 ± 2.2	28.0 ± 1.8	27.8 ± 1.5
HW/TL (mg/cm)	192 ± 6	194 ± 6	240 ± 27	219 ± 7
LVW/TL (mg/cm)	133 ± 8	134 ± 5	157 ± 7*	154 ± 6
KW/TL (mg/cm)	197 ± 9	190 ± 8	186 ± 4	183 ± 10
Proteinuria (mg/24h/100g BW)	13.7 ± 3.2	16.0 ± 4.4	26.0 ± 3.4*	27.6 ± 5.1

*BW, Body weight; HW, Heart weight; LVW, Left ventricular weight; KW, Kidney weight; TL, Tibia length. Results are shown as mean ± SEM. All parameters were assessed in control (Ctrl) and NG-nitro-L-arginine methyl ester (L-NAME) rats treated with vehicle or Lactobacillus fermentum CECT5716 (LC40). *P < 0.05 vs control (Ctrl) group.*

2.3 Effects in vascular NO pathway

As expected, aortae from L-NAME treated rats showed strongly reduced endothelium-dependent vasodilator responses to acetylcholine (**Fig. 4A**). The aortae from L-NAME plus LC40 showed a small, but significant, increase in the vasodilation induced by acetylcholine as compared to that obtained in aorta from the L-NAME group. The incubation with L-NAME in the organ bath abolished the relaxant response to acetylcholine in all experimental groups (**Fig. 4B**), involving NO in this relaxation. The presence in the organ bath of the non-selective NADPH oxidase inhibitor apocynin increased the relaxant response to acetylcholine in L-NAME group as the level found in L-NAME-LC40 group (**Fig. 4C**), suggesting that an increased NADPH oxidase activity is involved, at least in part, in the impaired relaxation to acetylcholine in aorta from L-NAME group. To determine whether a defect in the signaling of NO in vascular smooth

muscle could be the cause of the impaired response to endothelial-derived NO, we analyzed the effects of sodium nitroprusside, a direct activator of soluble guanylate cyclase in vascular smooth muscle cells, mimicking the actions of endogenously synthesized NO. However, no differences were observed among groups in the endothelium-independent vasodilator responses to the NO donor sodium nitroprusside in aortic rings, excluding changes in NO pathway in smooth muscle (**Fig. 4D**).

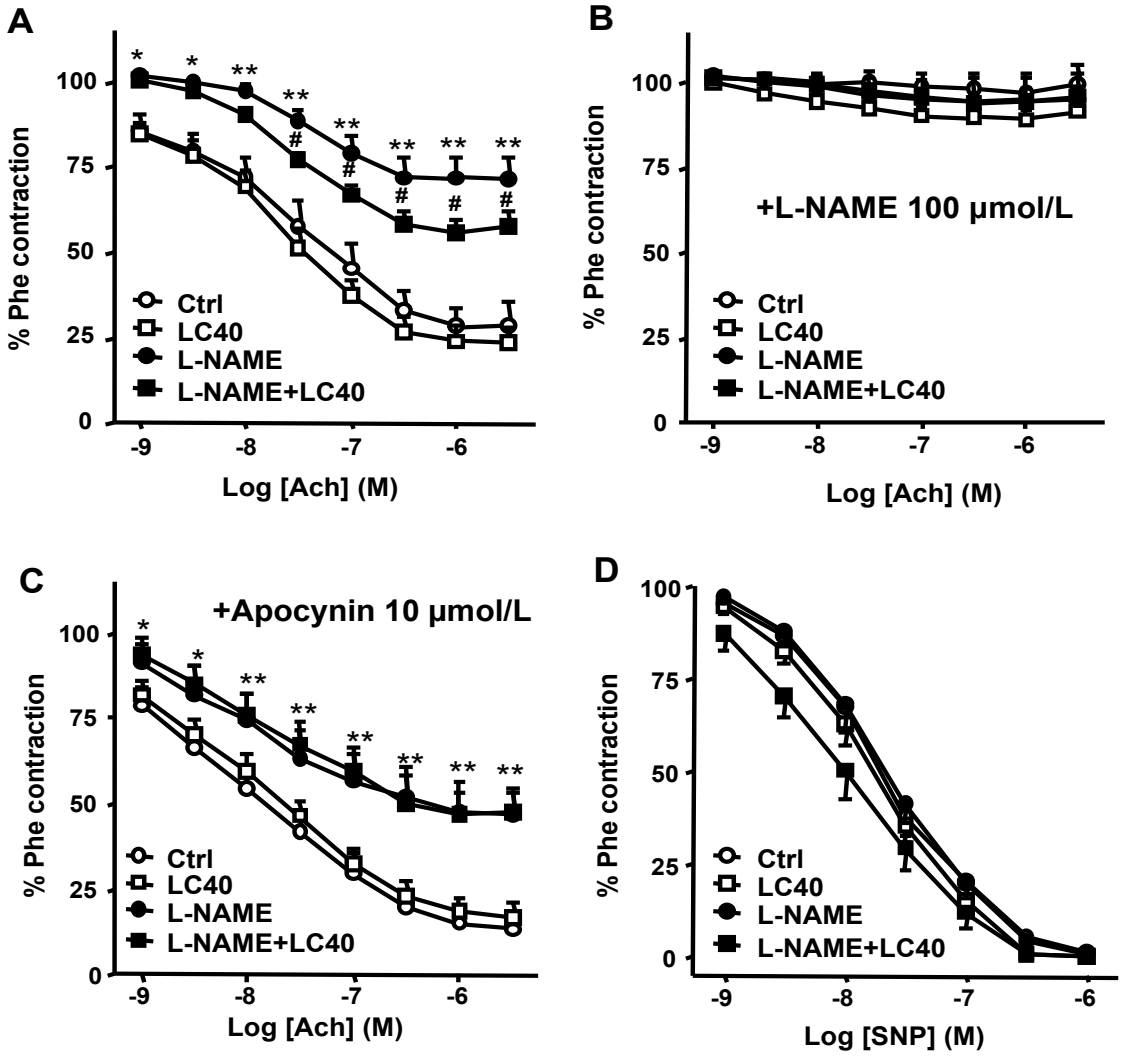

Figure 4. Control of endothelial dysfunction by Lactobacillus fermentum CECT5716 (LC40) treatment in NG-nitro-L-arginine methyl ester (L-NAME) group. *Vascular relaxant responses induced by acetylcholine (Ach), in endothelium-intact aortae pre-contracted by phenylephrine (Phe) (1 µmol/L) in the absence (**A**) and in the presence of L-NAME (**B**) or the NADPH oxidase inhibitor apocynin (10 µmol/L) (**C**). Endothelium-independent vasodilator responses to sodium nitroprusside (SNP; **D**) in endothelium-denuded aortae pre-contracted by Phe (1 µmol/L) in control (Ctrl) and, L-NAME rats. Values are expressed as mean ± SEM (n = 6-8 from different rats). *P< 0.05 and **P< 0.01 compared with the Ctrl group. #P< 0.05 compared with the non-treated L-NAME group.*

2.4 Effects in vascular ROS production, NADPH oxidase pathway, and inflammation

To characterize and localize vascular ROS production, sections of aorta were incubated with DHE, which is oxidized by superoxide (O_2^-) to yield the red fluorescent DNA stain ethidium. Nuclear red ethidium fluorescence was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from L-NAME rats showed marked increased staining in vascular wall (≈ 30 %), which was suppressed by LC40 consumption (**Fig. 5A**). VAS2870, a selective NADPH oxidase inhibitor, abolished the increased ROS level found in rings from L-NAME group (**Fig. 5A**). NADPH oxidase activity was increased in aortic rings from L-NAME rats as compared to control rats (**Fig. 5B**). Long-term LC40 consumption abolished the increase in NADPH oxidase activity observed in L-NAME-treated rats (**Fig. 5B**). The mRNA expression of pro-inflammatory cytokines TNF-α and IL-1β in aortic homogenates was higher in aortae from L-NAME groups as compared to control rats. LC40 treatment significantly suppressed this increase (**Fig. 5C**). In addition, we also analyzed the transcript level of transcription factor retinoid-related orphan receptor-γ (RORγ), a marker of infiltration Th17 in aorta. RORγ mRNA levels were increased in L-NAME group and reduced by LC40 administration **(Fig. 5D).**

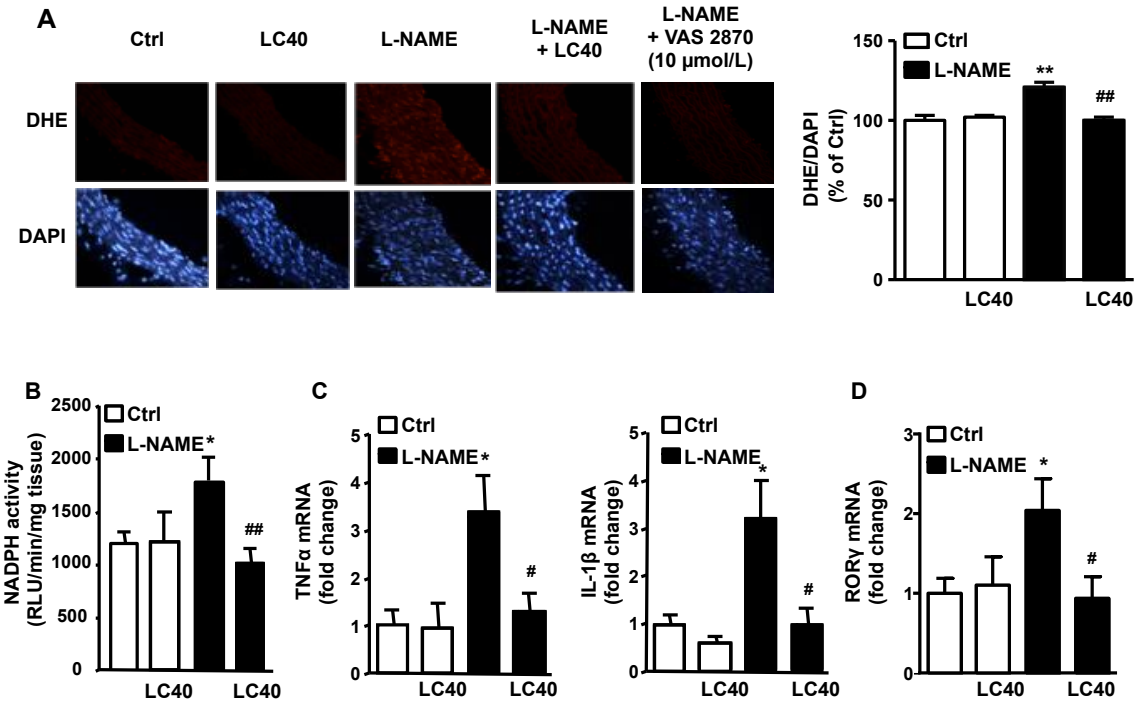

Figure 5. Control of vascular oxidative stress and inflammation by Lactobacillus fermentum CECT5716 (LC40) treatment in NG-nitro-L-arginine methyl ester (L-NAME) group. *Top pictures show aortic rings incubated in the presence of dihydroethidium (DHE), which produces a red fluorescence when oxidized to ethidium by ROS. Bottom pictures show blue fluorescence of the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI; x 400 magnification). Red ethidium fluorescence values normalized to blue DAPI fluorescence, averaged and expressed as mean ± SEM (n= 6-8 rings from different rats) (A). Lucigenin-enhanced chemiluminescence measurement of NADPH oxidase activity (**B**) and expression of pro-inflammatory cytokines (**C**) and RORγ (**D**) at the level of mRNA by RT-PCR in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6-8). *P< 0.05 and **P< 0.01 compared with the Ctrl group. #P< 0.05 and ##P< 0.01 compared with the non-treated L-NAME group.*

2.5 Effects in MLN

Under altered gut mucosal integrity, bacteria are able to translocate across the intestinal epithelium leading to activation and migration of CX3CR1+ cells, such as macrophages and dendritic cells, to draining lymph nodes of the lower intestinal tract (Niess et al., 2015). They also present soluble antigen to naïve CD4+ T cells, lending to T cells activation. We found reduced expression of barrier-forming junction transcripts (occludin and zonula occludens-1 (ZO-1)) in the colon from L-NAME group (**Fig. 6A**), linked to significantly increased levels of CX3CR1 in MLN from L-NAME group as compared to control group (**Fig. 6B**). LC40 treatment increased occludin and ZO-1 in the

colon and reduced CX3CR1 mRNA levels in MLN. Upon activation, MLN T cells upregulate integrin α4β7 and chemokine receptor CCR9 (Mora et al.,2008). We found that both CCR9 (**Fig. 6C**) and Itga4 (**Fig. 6D**) expression were increased in MLN from L-NAME group as compared to control and LC40 treatment inhibited these higher levels suggesting decreased activation of T cells. No significant changes in Itgb7 mRNA were detected among groups (**Fig. 6D**). Among many pro-inflammatory cytokines produced by activated T cells, IL-6 is known to promote Th17 proliferation and Treg suppression (Kimura et al.,2010). We measured both the transcript level of IL-6 in MLN (**Fig. 6E**) and the IL-6 level in conditionated medium from MLN (**Fig. S3A**) and found that they were significantly increased in MLN from L-NAME group and reduced by LC40 treatment.

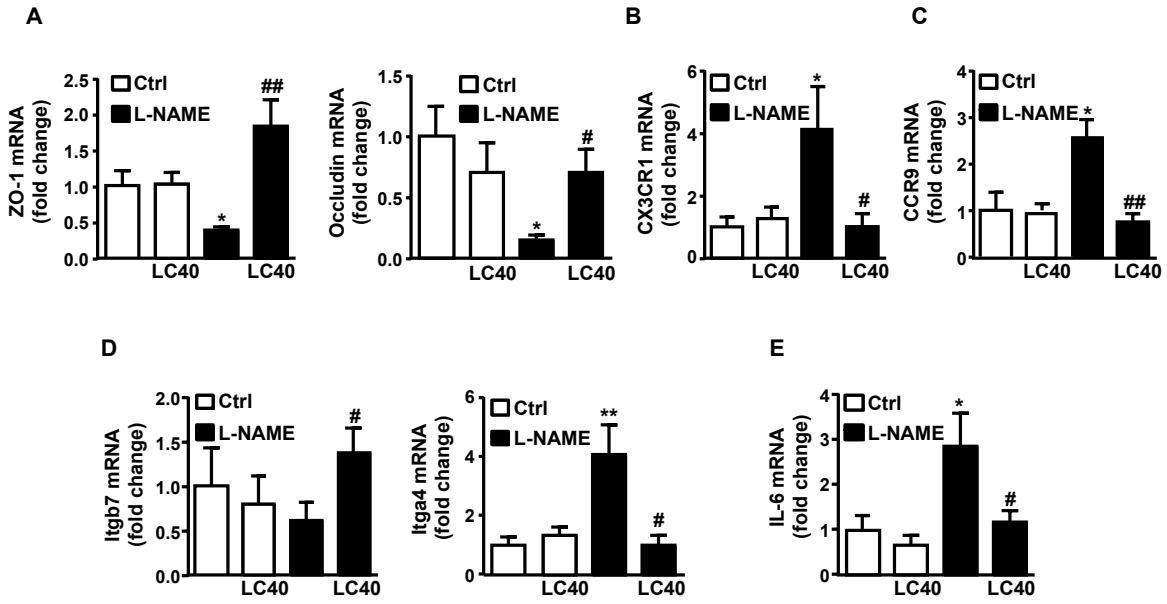

Figure 6. Control of gut integrity and inflammation by Lactobacillus fermentum CECT5716 (LC40) treatment in the dysbiosis of NG-nitro-L-arginine methyl ester (L-NAME) group. *Colonic mRNA levels of occludin, and zonula occludens-1 (ZO-1) (**A**). mRNA levels of CX3CR1 (**B**), CCR9 (**C**), α4β7 (Itgb7 and Itga4) (**D**), and interleukin (IL)-6 (**E**) in lymphoid tissues in control (Ctrl) and, L-NAME rats. Values are expressed as mean ± SEM (n = 6-8). *P< 0.05 and **P< 0.01 compared with the Ctrl group. #P< 0.05 and ##P< 0.01 compared with the non-treated L-NAME group.*

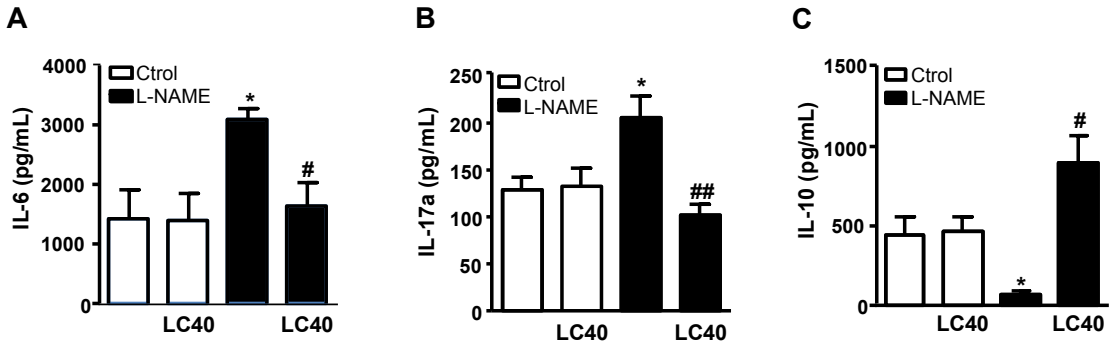

Figure S3. Control of cytokine profile by Lactobacillus fermentum CECT5716 (LC40) treatment in lymphocyte conditioned media from mesenteric lymph nodes of NG-nitro-L-arginine methyl ester (L-NAME) group. *Concentrations of interleukin (IL)-6 (**A**), IL-17a (**B**), and IL-10 (**C**) measured by ELISA in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6-8). *P< 0.05 compared with the Ctrl group. #P< 0.05 and ##P< 0.01 compared with the non-treated L-NAME group.*

In addition, the number of total lymphocytes was higher in L-NAME group as compared to control (**Fig. 7A**), but was unchanged by LC40 consumption. We also analyzed the transcript levels of transcription factors, such as RORγ and forkhead box P3 (FoxP3), which induce Th17 and regulatory T cell (Tregs) populations, respectively (Bakheet et al., 2017). The mRNA levels of these transcription factors in lymphocytes were measured as indirect markers of Treg and Th17 cells. FoxP3 and RORγ mRNA levels were reduced and increased, respectively, in L-NAME group, and LC40 administration restored the levels found in control group (**Fig. 7B**), normalizing the increased Th17/Treg ratio. In correlation with this, the mRNA levels of the cytokines IL-10, mainly produced by Treg, and IL-17a, produced by Th17, were also changed in the same way in L-NAME rats (**Fig. 7C**). Again, long-term LC40 administration normalized these cytokines. Moreover, levels of IL-10 and IL-17a levels in conditionated medium from MLN were significantly increased in MLN from L-NAME group and reduced by LC40 treatment (**Fig. S3B and S3C**). In addition, CD19 and GATA-3 mRNA, markers of B and Th2 cells, respectively, were unchanged among all experimental groups (**Fig. 7B**). The level of Th1, measured by T-bet mRNA, tended to increase (**Fig. 7B**), whereas the main cytokine produced by these cells interferon-γ (IFN-γ) increased significantly (**Fig. 7C**) in L-NAME rats. However, both mRNA levels of T-bet and IFN-γ were unaffected by LC40 administration.

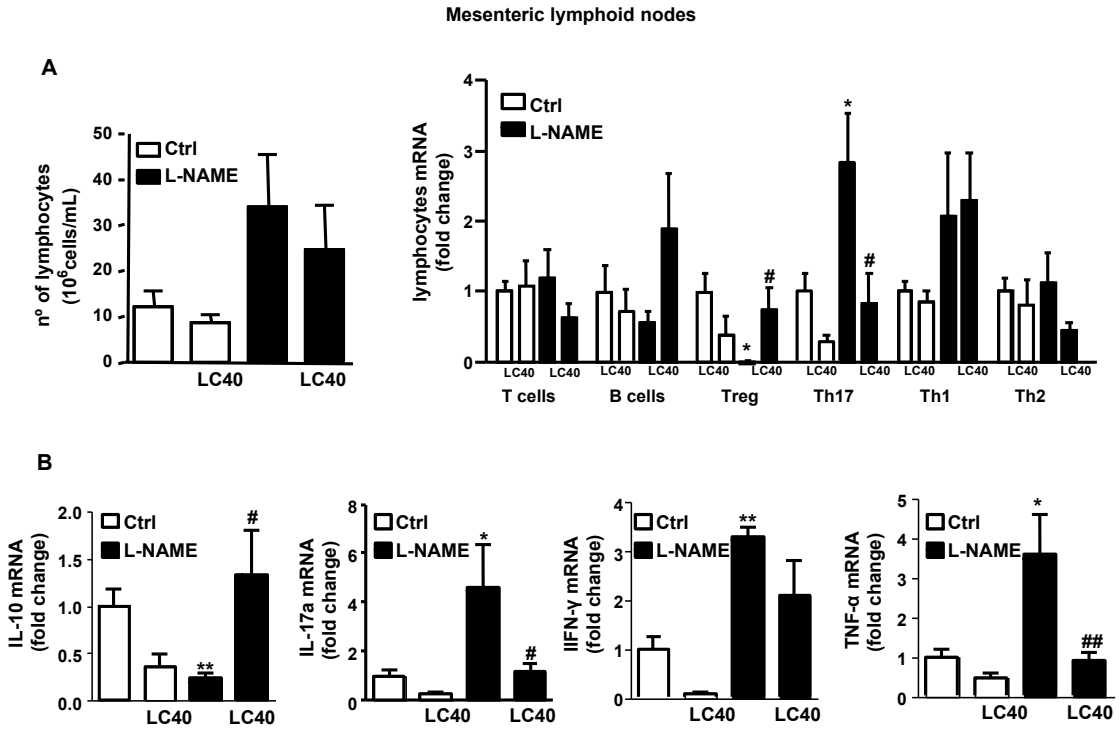

Figure 7. Control of T-cell polarization by Lactobacillus fermentum CECT5716 (LC40) treatment in mesenteric lymph nodes of Nᴳ-nitro-L-arginine methyl ester (L-NAME) group. *Total numbers of lymphocytes (**A**). T (CD4), and B cells (CD19), regulatory T rats (Treg) (FoxP3), T helper (Th)17 (RORγ), Th1 (T-bet), and Th2 (GATA-3) cells (**A**) measured RT-PCR in lymphocytes from all experimental groups. mRNA levels of interleukin (IL)-10, IL-17a, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (**B**), in lymphocytes from mesenteric lymph nodes in control (Ctrl) and L-NAME rats. Values are expressed as mean ± SEM (n = 6-8). *P< 0.05 compared with the Ctrl group. #P< 0.05 compared with the non-treated L-NAME group.*

We also investigated whether there was a link between the endothelial dysfunction and the changes in T-cell polarization in MLN. Thus, we tested the acetylcholine-induced relaxation in aortic rings incubated previously with conditioned media of lymphocytes from all groups for 4 hours. The pre-incubation with conditioned media of lymphocytes from L-NAME group impaired the endothelium-dependent relaxation to acetylcholine in aortic rings compared with those incubated with lymphocyte-conditioned medium from control group (**Fig. 8A**). In the presence of SOD mimetic tempol, no significant differences were found among all experimental groups, involving ROS in this impaired relaxant response to acetylcholine (**Fig. 8B**). In addition, this alteration was suppressed when the rings exposed to conditioned medium from lymphocytes of L-NAME group were also coincubated with the IL-17a neutralizing antibody, confirming the involvement of IL-17a produced by MLN lymphocytes from L-NAME group in this endothelial dysfunction (**Fig. 8C**). IL-17 activates RhoA/Rho-kinase leading to increased

phosphorylation of the inhibitory eNOS residue Thr495 and endothelial dysfunction (Nguyen et al., 2013). IL17 also promotes ROS generation by NOX (Dhillion et al., 2012). In RAECs from control animals, NO production induced by calcium ionophore A23187 was reduced (**Fig. 8D)** and levels of intracellular ROS were increased (**Fig. 8E**) in the presence of lymphocyte conditioned media from L-NAME group. However, incubation with conditioned media from L-NAME-LC40 group normalized both NO and ROS levels. These alterations in NO and ROS production induced by L-NAME lymphocyte conditioned media were inhibited by coincubation with IL-17a neutralizing antibody, the Rho kinase inhibitor Y27630, the selective NADPH oxidase inhibitor VAS2780, and tempol (**Fig 8F y 8G**). Furthermore, NADPH oxidase activity in RAECs was also increased by lymphocyte conditioned medium from the L-NAME-treated rats as compared to the control group, an effect that was again reduced by treatment with LC40 (**Fig. 7H**). This increased NADPH oxidase activity was suppressed by coincubation with IL-17 neutralizing antibody, Y27630, and VAS2780 (**Fig. 8I**). Overall, these results suggest that chronic blockade of NO production increased Th17 cells in MLN, and higher IL-17 levels in lymphocytes conditioned medium. This pro-inflammatory cytokine inhibited eNOS activity and increased ROS production in endothelial cells, through Rho kinase activation, inducing endothelial dysfunction. The reduction in Th17 cells in MLN induced by LC40 treatment seems to be involved in their protective effect in endothelial function.

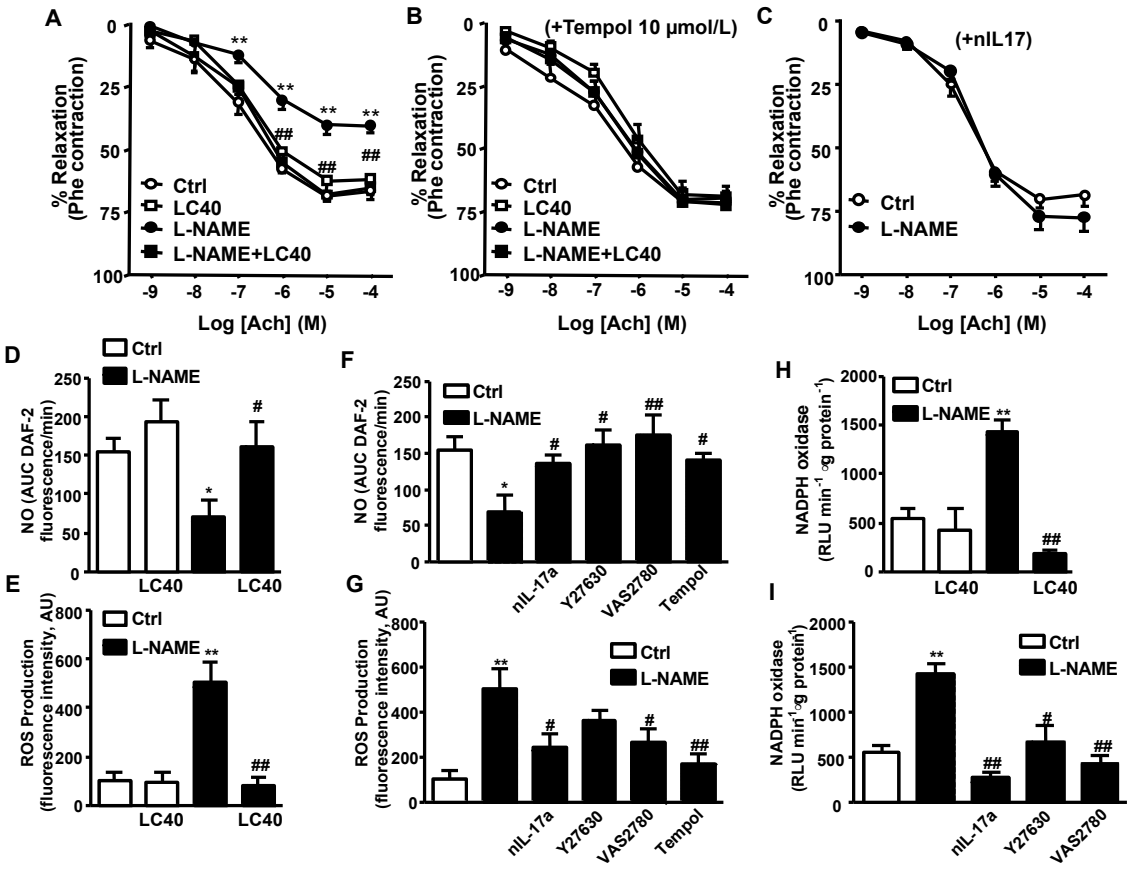

Figure 8. Control of T-cell polarization by Lactobacillus fermentum CECT5716 (LC40) treatment improves endothelial dysfunction induced by NG-nitro-L-arginine methyl ester (L-NAME). *Vascular relaxant responses induced by acetylcholine (Ach) (**A**) in aortae pre-contracted by phenylephrine (Phe, 1 µmol/L) in the absence and presence in the bath of the SOD mimetic tempol (10 µmol/L; **B**), or anti IL-17A receptor (10 µg/mL; **C**) 30 min, after incubation during 4 hours of control rings with lymphocyte conditioned media from the all experimental groups. Values are expressed as mean ± SEM (n = 6–8 rings from different rats). A23187-stimulated NO production (**D, F**), CM-H2DCFDA-detected intracellular ROS (**E, G**), and NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (**H, I**) in RAECs. RAECs were incubated during 4 hours with lymphocyte conditioned media from the experimental groups in the absence and presence of the antiIL-17a receptor (10 µg/mL), the Rho kinase inhibitor Y27632 (50 µmol/mL), the pan-NOX inhibitor VAS2870 (10 µg/mL), and the SOD mimetic tempol (10 µmol/L). Results were obtained in 3 different determinations. Values are means ± SEM (n= 5-8). *P< 0.05 and **P< 0.01 compared with the control (Ctrl) group. #P< 0.05 and ##P< 0.01 compared with L-NAME group.*

3. Discussion

The administration in drinking water of L-NAME, commonly used model of chronic hypertension (Zatz et al., 2012), induces a gradual increase in arterial BP via the inhibition of NO synthase activity reducing the vasodilating agent NO. This is the first study to show that a chronic LC40 treatment, at a dose that reduced BP in SHR, was unable to prevent the raise in BP and the hypertrophic effects in heart and kidney induced by blockade of NO synthesis, despite the improvement of vascular oxidative stress and inflammation. In addition, LC40 administration prevented the gut dysbiosis induced by L-NAME, characterized by increased F/B ratio, restored the Th17/Treg polarization in MLN and reduced Th17 infiltration in aorta. Prior works have demonstrated that dietary nitrate or exogenous NO exerts antimicrobial effects and can alter oral microbiome composition (Backlund et al., 2014; Hyde et al., 2014). However, dietary nitrates did not induced significant differences in fecal microbioma composition or diversity (Conley et al., 2017). In contrast to previous data showing no significant changes in gut microbiota after L-NAME treatment (Xu et al., 2013), we found that chronic inhibition of NO synthesis induced important changes in gut microbiota linked to hypertension. Our results are consistent with data found in both hypertensive humans (Yang et al., 2015; Li et al., 2017; Kim et al., 2018) and animals (spontaneously hypertensive rats, angiotensin II–infused mice, DOCA-salt treated mice) (Marques et al., 2017; Yang et al., 2015; Kim et al., 2018) showing increased F/B ratio, as a key marker of gut dysbiosis. However, in these hypertensive humans and animals gut microbiome is less diverse, less rich, and shows a decrease in acetate- and butyrate-producing bacteria and increase in lactate-producing bacterial populations. However, in hypertensive rats induced by L-NAME no significant changes in these parameters were observed, indicating that gut dysbiosis in hypertension varies according to the model of hypertension. In agreement to this lack of consensus in the changes of gut microbiota in hypertension, rats with hypertension induced by DOCA-salt showed a decrease in acetate-producing bacteria without changes in butyrate-producing bacteria (Marques et al., 2017). A significant depletion of *Bifidobacterium* in L-NAME-treated rat was also noted, similarly to that found in SHR (Yang et al., 2015). *Bifidobacterium* is commonly considered a beneficial bacterial genus that plays a critical role in the maturation and regulation of the immune system (Grangette et al., 2012). Consequently, *Bifidobacterium* depletion might contribute to the dysregulation of the immune system found in hypertensive L-NAME-treated rats. In addition, increased

Propionibacterium was also detected in gut microbiota from L-NAME group, which also influence intestinal immunity increasing Th17 population in MLN (Colliou et al., 2017). However, our study did not clarify either the mechanisms involved in dysbiosis induced by NO synthesis inhibition or if changes in gut microbiota induced by L-NAME are involved in the raise of BP. Interestingly, in L-NAME rats treated with LC40 showed both no significant depletion of *Bifidobacterium* and restoration of *Propionibacterium* content to control level, suggesting that these bacteria might induce changes in immune cells in MLN. *Lactobacillus* treatment significantly increased intestinal integrity in L-NAME treated rats and likely prevented detrimental bacteria and their antigens from penetrating the intestinal epithelium. LC40 consumption also decreased CX3CR1 expression in the MLN. CX3CR1-expressing cells are primarily antigen presenting cells (APC) (Medina-Contreras et al., 2011; Diehl et al., 2013) that can capture bacteria from the gut lumen and transport them to the MLN, where they present antigens and activate $CD4^+$ T cells to produce IL-6 that suppresses Treg, and increases Th17 cells.By preventing barrier compromise LC40 treatment may reduce activation and migration of APC to the MLN, hence suppressing IL-6 production and allowing for Treg to produce IL-10, and reducing Th17 to produce IL-17a. In addition, dihydrolipoamide acetyltransferase, one of the mayor proteins found in bacterial surface layer of *Propionibacterium,* has been described to stimulate intestinal APC, leading to T cell activation and Th17 differentiation (Colliou et al., 2017). The reduction of *Propionibacterium* count in the gut microbiota induced by LC40 treatment might be involved in the lesser Th17 population found in MLN from L-NAME-LC40 group, as a result of inhibition of this pathway. Additionally, loss of NO after chronic exposure to L-NAME might promote formation of isoketal-protein adducts, which have been previously shown to be immunogenic when formed in dendritic cells (Kirabo et al., 2014). LC40 consumption by increasing NO bioavailability, which rapidly reacts with lipid-peroxy-radicals, and in doing so can limit formation of lipid oxidation products, such as isoprostanes and isoketal, might reduce T cells expansion in MLN. Endothelial dysfunction is involved in the development of hypertension induced by chronic NO synthesis blockade. In addition to reduce endothelial NO production, a key mechanism of endothelial dysfunction involves the vascular production of ROS, particularly O_2^-, which reacts rapidly with NO and inactivates it (Tschudi et al., 1996). We have analyzed the effects of conditioned media of cultured lymphocytes from MLN from all experimental groups in aorta and RAEC from control rats. The incubation with the conditioned media of cultured MLN lymphocytes from L-NAME group impairs aortic

endothelium-dependent relaxation to acetylcholine, reduced NO production and increased ROS and NADPH oxidase in RAECs, showing that changes in Th17/Treg balance in favour of Th17 impair endothelial function. In agreement with this, the presence of IL-17a neutralizing antibody improves this endothelial dysfunction. Moreover, both relaxation-induced by acetylcholine and NO production were preserved when aortae or RAECs, respectively, were incubated with conditioned media of lymphocytes from L-NAME-LC40 group. Our data also confirm the involvement of IL-17/RhoA kinase/NADPH oxidase pathway in the deleterious effects of conditioned media of cultured lymphocytes from MLN from the L-NAME group in endothelial function and the protection induced by LC40 treatment. However, in L-NAME treated rats, endothelium-dependent relaxation to acetylcholine was reduced, but did not affect endothelium-independent vasodilator responses to sodium nitroprusside. LC40 treatment only weakly prevented this effect, without affecting NO sensitivity, since it could not alter the relaxation to NO donor nitroprusside. The relaxant responses to acetylcholine were abolished by incubation with L-NAME in the bath, showing the absolute dependence of NO. This protective effect was similar to that induced by the non-selective NADPH oxidase inhibitor apocynin, suggesting that chronic LC40 administration improved endothelial function reducing NADPH oxidase-driver ROS production. In fact, aortic Th17 infiltration, ROS accumulation and NADPH oxidase activity were restored by LC40 treatment. However, the increased NO bioavailability induced by the reduction in ROS production evoked by LC40 was limited by the reduced NO synthesis induced by chronic L-NAME, being unable to reduced BP. This data suggest the key role of NO in the antihypertensive effects of this probiotic in SHR. It has been previously demonstrated that the blockade of renin-angiotensin system prevented L-NAME-induced hypertension (Pollock et al., 1993; Liu et al., 1998). Moreover some *Lactobacillus* strains reduce BP as a result of Angiotensin Converting Enzyme (ACE) inhibition (Jauhiainen et al., 2005; Hütt et al., 2015). Taken into account that LC40 was unable to prevent the raise of BP in L-NAME treated rats, ACE inhibition seems to be not involved in its vascular protective effects. In conclusion, this study was able to demonstrate that chronic treatment with LC40 prevented gut dysbiosis, restored the altered Th17/Treg balance in MLN, and slightly improved endothelium-dependent vasodilator responses in hypertension induced by chronic inhibition of NO synthesis by the oral administration of L-NAME. Moreover, LC40 also reduced the vascular oxidative stress and pro-inflammatory status, early steps in the atherosclerotic process. These protective effects may contribute to the improvement of

overall health of the consumer. However, future studies should be conducted to assess the effects of this probiotic in hypertensive humans.

4. Materials and methods

Regulations for the protection of animals used for scientific purposes of the European Union were followed to carry out this study. All experimental protocols were approved by the Animal Care and Ethics Committee of the University of Granada (Spain; permit no: 03-CEEA-OH-2013).

4.1 Preparation and administration of the probiotic

LC40 supplied by Bioserch S.A. (Granada, Spain) was grown at 37ºC in de Man, Rogosa and Sharpe agar and incubated anaerobically using an Anaerogen system (Oxoid, Basingstoke, Hants, UK). For experimental protocols, the probiotic was suspended using a sterile PBS solution.

4.2 Animals and experimental groups

Male Wistar rats (175-200 g) were used in the study. Previous to the start of the experimental protocols, BP measurements were recorded during a two-week adaptation period. Rats were randomly divided into four different treatment groups: a) vehicle (control, 1 mL of tap water once daily), b) vehicle plus L-NAME (50 mg 100 mL^{-1} in drinking water), c) LC40 (10^9 colony-forming units/day in 1 mL by oral gavage), and d) LC40 plus L-NAME. Rats were kept in individual ventilated cages, and were treated for 4 weeks. Rats were maintained in a specific pathogen-free environment and were provided with water and standard laboratory diet (SAFE A04, Augy, France) *ad libitum*. Water was changed every day, and both water and food consumption was documented daily for all groups. Weekly, body weight was recorded.

4.3 Blood pressure measurements and cardiac and renal weight indices

As previously described (Vera et al., 2007), tail-cuff plethysmography was performed weekly to measure systolic blood pressure (SBP), diastolic BP (DBP) and heart rate (HR) at room temperature. Once the experimental period reached its endpoint, animals fasted for 18 hours and then were anaesthetized with 2.5 mL/kg equitensin (i.p.) collecting their blood from the abdominal aorta. Finally, the animals were sacrificed by exsanguination. Kidneys and hearts were removed and weighted. Hearts were divided into right ventricle and left ventricle plus septum.

4.4 Lymphocyte conditioned media preparation

MLN were obtained from rats. The MLN were carefully mashed with wet slides, which reduced friction. The cell suspensions were then filtered through a 70 μm cell strainer. According to the previous study (Kassan et al., 2011), total lymphocytes ($2.5 \times 10_6$/mL) from lymph nodes were stimulated with 5 μg/mL Concanavaline A in RPMI 1640 with 10% fetal bovine serum, 2 mmol/L glutamine, 10 mmol/L 6-HEPES, 100 U/mL penicillin, 100 g/mL streptomycin, and 50 μM β-mercaptoethanol in 25-mL flasks for 48 h. After centrifugation, lymphocyte conditioned media was stored at -80ºC until use. We measured levels of IL-10, IL-6, and IL-17a in lymphocyte conditioned media cytokines from all experimental groups were measured by ELISA.

4.5 Vascular reactivity studies

Rings from rat thoracic aorta (3 mm in length) were dissected and mounted in individual organ chambers with Krebs buffer containing NaCl 118 mM, NaHCO3 25 mM, glucose 11 mM, KCl 4.75 mM, CaCl2 2 mM, MgSO4 1.2 mM and KH2PO4 1.2 mM (Gomez-Guzman et al., 2011). The concentration-relaxation response curves to acetylcholine (10_{-9} -10_{-5} mol/L) were studied in aorta pre-contracted by phenylephrine (1 μmol/L). The concentration-relaxation response curves to nitroprusside (10_{-9} -10_{-6} mol/L) were performed in the dark in aortic rings without endothelium pre-contracted by 1 μmol/L phenylephrine. In another experimental set, control rings were incubated during 4 hours with 100 μL of lymphocytes conditioned media from all experimental groups in the

absence or in the presence of antiIL-17a receptor (10 μg/mL, R&D Systems, Minneapolis, USA). Then, rings were mounted in the organ bath and the relaxant response to acetylcholine was tested. In some rings, responses to acetylcholine were studied after incubation with the SOD mimetic tempol (10 μmol/L) for 30 min. Relaxant responses were expressed as a percentage of precontraction.

4.6 Measurement of *ex vivo* vascular reactive oxygen species (ROS) levels and NADPH oxidase activity

The oxidative fluorescent dye dihydroethidium (DHE) was used for in situ ROS detection in aortic segments as previously described (Zarzuelo et al., 2011). Briefly, aortic rings were included in Optimum Cutting Temperature Compound Medium (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), snap frozen and cut into 10 μm thick sections using a cryostat (Microm International Model HM500 OM). These rings were incubated with DHE (10 μmol/L) for 30 min at room temperature in the dark, and then counterstained with 4,6-diamidino-2-phenylindole 4dichlorohydrate (DAPI, 300 nmol/L) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS production was estimated from the ratio of ethidium/DAPI fluorescence. In some experiments, aortae from L-NAME group were co-incubated with the pan-NOX inhibitor VAS2870 (10 μmol/L). NADPH oxidase activity was determined using a lucigenin-enhanced chemiluminescence method in intact aortic rings and cultured RAECs as previously described (Zarzuelo et al., 2011). Briefly, aortic rings from each group were incubated for 30 min at 37 ºC in HEPES buffer (in mmol/L: NaCl 119, HEPES 20, glucose 5.5, KCl 4.6, $CaCl_2$ 1.2, $NaHCO_3$ 1, $MgSO_4$ 1, KH_2PO_4 0.4 and Na_2HPO_4 0.15; pH 7.4). On the one hand, RAECs were incubated as mentioned above. After this period, cells were washed with cold PBS and homogenized in lysis buffer composed of 20 mmol/L KH_2PO_4, 1 mmol/L EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.75 μg/mL pepstatin, 0.5 mmol/L phenylmethysulfonyl fluoride (PMSF) (pH~7.4). Then NADPH (100 μmol/L) was added to the buffer containing the RAECs homogenate suspension (30 μg of protein in 500 μL) or aortic rings, and lucigenin (5 μmol/L) was injected automatically. NADPH oxidase activity was determined by measuring luminescence over

200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH and expressed as RLU (relative light units)/min per μg of protein for cells or RLU/min per mg of tissue for aortic rings.

4.7 Gene expression analysis

As previously described (Zarzuelo et al., 2011) RT-PCRs were performed for the analysis of gene expression in aorta, colon, MLN and lymphocytes. To this effect, total RNA extraction was performed with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of the isolated RNA were determined by Thermo Scientific NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 2 μg of RNA were retrotranscribed using oligo (dT) primers (Promega, Southampton, UK). Polymerase chain reaction was performed in Techgene thermal cycler (Techne, Cambridge, UK). The sequences of the sense and antisense primers used for amplification are described in **Table S1**. The efficiency of the PCR reaction was determined using a dilution series of standard vascular samples. To normalize mRNA expression, the expression of the housekeeping gene β-actin was used. The mRNA relative quantification was calculated using the ΔΔCt method.

4.8 Quantification of NO release and intracellular ROS concentration in primary culture of rat aorta endothelial cells (RAECs)

RAECs were isolated from rat thoracic aortas as described previously (Toral et al., 2015). Briefly, fat and connecting tissues were rapidly removed. A 24-gauge cannula was inserted into the proximal portion of the aorta. After ligation at the site with silk thread, the inside of the lumen is briefly washed with PBS. The other side is bound and filled with collagenase (type P) solution (2mg/mL, dissolved in serum-free Medium 199). After incubation for 40 min at 37ºC, RAECs were removed from the aorta by flushing with 1 mL of medium 199 containing 20% FBS. RAECs should be collected by centrifugation at 2,300 rpm for 5 minutes. Then, the precipitate is gently resuspended with 2 mL of 20% FBS-medium 199 and cultured in a 35mm pretreated with 0.2% gelatin disk. To remove smooth muscle cells, after 2 hours incubation at 37ºC, the medium is removed, the cells were washed with warmed PBS and culture medium was added. The cells were cultured

(Medium 199 + 20% fetal bovine serum + Penicillin/Streptomycin 2 mmol/L+ Amphotericin B 2 mmol/L + Glutamine 2 mmol/L + HEPES 10 mmol/L + endothelial cell growth supplement 30 µg/mL + Heparin 100 mg/mL) under 5% CO_2 at 37°C. RAECs were incubated with 100 µL of lymphocytes condition media from all experimental groups during 4 hours. In some experiments, cells were co-incubated in the absence or in the presence of Rho kinase inhibitor Y27632 (50 µmol/L), antiIL-17a receptor (10 µg/mL, R&D Systems, Minneapolis, USA), VAS2870 (10 µmol/L), or antioxidant tempol (10 µmol/L) during incubation.

Quantification of NO released by RAECs was performed using the NO-sensitive fluorescent probe diaminofluorescein-2 **(**DAF-2) as described previously with several modifications (Toral et al., 2015). Briefly, cells were incubated as mentioned above. After this period, cells were washed with PBS and then were pre-incubated with L-arginine (100 µmol/L in PBS). Subsequently, DAF-2 (0.1 µmol/L) was incubated for 5 min and then the calcium ionophore calimycin (A23187, 1 µmol/L) was added for 30 min. Then the fluorescence intensity (arbitrary units, AU) was measured using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany). The autofluorescence was subtracted from each value. In some experiments, L-NAME (100 µmol/L) was added. The difference between fluorescence signal with and without L-NAME was considered NO production.

Endothelial ROS production was measured using the fluorescent probe 5-(and-6-) chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Confluent RAECs culture in 96-well plates as described above were incubated with 5 µmol/L CM-H2DCFDA for 30 min at 37 ºC. The fluorescent intensity was measured using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany).

4.9 DNA Extraction, 16S rRNA Gene Amplification, Bioinformatics

For analysis of the bacterial population present in the gut, fecal samples were collected from six individual animals at the end of the experimental period. DNA was extracted from fecal samples using G-spin columns (INTRON Biotechnology) starting from 30 mg of samples resuspended in PBS and treated with proteinase K and RNAses. DNA concentration was determined in the samples using Quant-IT PicoGreen reagent (Thermo Fischer) and DNA samples (about 3 ng) were used to amplify the V3-V4 region

of 16S rRNA gene (Caporaso et al., 2011). PCR products (approx. 450 pb) included extension tails, which allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. Individual amplicon libraries were analyzed using a Bioanalyzer 2100 (Agilent) and a pool of samples was made in equimolar amounts. The pool was further cleaned, quantified and the exact concentration estimated by real time PCR (Kapa Biosystems). Finally, DNA samples were sequenced on an Illumina MiSeq instrument with 2 x 300 paired-end read sequencing at the Unidad de Genómica (Parque Científico de Madrid). We used the BIPES pipeline to process the raw sequences (Zhou et al., 2011). First, the barcode primers were trimmed and filtered if they contained ambiguous bases or mismatches in the primer regions according to the BIPES protocol. Second, we removed any sequences with more than one mismatch within the 40–70 bp region at each end. Third, we used 30 Ns to concentrate the two single-ended sequences for the downstream sequence analyzes. A detailed description of these methods was previously reported (Liu et al., 2017). Third, we performed UCHIME (implemented in USEARCH, version 6.1) to screen out and remove chimeras in the de novo mode (using-minchunk 20-xn 7-noskipgaps 2) (Edgar et al., 2015). In each sample between 90,000 and 220,000 sequences were identified. All subsequent analyzes were performed using 16S Metagenomics (Version: 1.0.1.0) from Illumina. The sequences were then clustered to an operational taxonomic unit (OTU) using USEARCH with default parameters (USERACH61). The threshold distance was set to 0.03. Hence, when the similarity between two 16S rRNA sequences was 97%, the sequences were classified as the same OTU. QIIME-based alignments of representative sequences were performed using PyNAST, and the Greengenes 13_8 database was used as the template file. The Ribosome Database project (RDP) algorithm was applied to classify the representative sequences into specific taxa using the default database (Edgar et al., 2015). The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature. Bacteria were classified based on the SCFA end-products producer as previously described (Wang et al., 2007; Antharam et al., 2013)

4.10 Chemicals

All chemicals were obtained from Sigma-Aldrich (Barcelona, Spain), unless otherwise stated.

4.11 Statistical analysis

The Shannon, Chao, Pielou and observed species whole these indexes were calculated using QIIME (PAST 3x). Reads in each OUT were normalized to total reads in each sample. Only taxa with a percentage of reads > 0.001% were used for the analysis. Partial Least Square (PLS) analysis was also applied to these data to identify significant differences between groups. Linear Discriminant Analyze (LDA) scores greater than 2 were displayed. Taxonomy was summarized at the genus level within QIIME-1.9.0 and uploaded to the Galaxy platform (Segata et al., 2011) to generate LEfSe/cladogram enrichment plots considering significant enrichment at a $P<0.05$, LDA score > 2. Results for all measurements are expressed as the mean ± SEM. 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. If the overall difference was significant, comparisons were made using Bonferroni´s test with an appropriate error. Analysis of the nested design was also carried out with groups and concentrations to compare the concentration-response curves to acetylcholine. The remaining variables were compared using a two way factor design, where group and treatment were fixed effect factors with unequal sample sizes in the different groups. If significant interactions were found, Bonferroni´s method was used for pairwise comparisons. $P < 0.05$ values were regarded as statistically significant.

Table S1. Oligonucleotides for real-time RT-PCR

mRNA targets	Descriptions	Sense	Antisense
TNF-α	Tumor necrosis factor-alpha	ACGATGCTCAGAAACACACG	CAGTCTGGGAAGCTCTGAGG
IL-1β	Interleukin-1 beta	GTCACTCATTGTGGCTGTGG	GCAGTGCAGCTGTCTAATGG
ZO-1	Zonula occludens-1	GCCAGCCAGTTCCGCCTCTG	AGGGTCCCGGGTTGGTG
Occludin	Occludin	AGCCTGGGCAGTCGGGTTGA	ACACAGACCCCAGAGCGGCA
CX3CR1	CX3C chemokine receptor 1	TGAGTGACTGGCACTTCCTG	CGAGGACCACCAACAGATTT
CCR9	C-C chemokine receptor type 9	CGAGACATGCCCACCATCGCTTCTCCC	ATGCCAGGCCACAGAGATGGCCC
Itgb7	Integrin beta-7	ACCTGAGCTACTCAATGAAGGA	CACCGTTTTGTCCACGAAGG
Itga4	Integrin alpha-4	GGAAGCCCCAGTGGAGAAC	ATTGTCACTCCCAGCCACTGA
IL-6	Interleukin-6	GATGGATGCTTCCAAACTGG	AGGAGAGCATTGGAAGTTGG
CD4	CD4	TCTTCCCAGTCCTGGATCAC	CACCACCAGGTTCACTTCT
CD19	CD19	GAAGGAGAGCTGTGAATAC	CTTGTGGGACACCATGGAATA
FoxP3	forkhead box P3	AGGCACTTCTCCAGGACAGA	CTGGACACCCATTCCAGACT
RORγ	R retinoid-related orphan receptor-γ OR-gamma	GCCTACAATGCCAACAACCACACA	TGATGAGAACCAAGGCCGTGTAGA
T-bet	T-bet	CCACCAGCACCAGACAGAGA	AACATCCTGTAATGGCTCGTG
GATA-3	GATA-3	GCCTGCGGACTCTACCATAA	GTCTGACAGTTCGCACAGGA
IL-10	Interleukin-10	GAATTCCCTGGGAGAGAAGC	GCTCCACTGCCTTGCTTTTA
IL-17a	Interleukin-17a	CTTCACCTTGGACTCTGAGC	TGGCGGACAATAGAGGAAAC
IFN-γ	Interferon-γ	GCCCTCTCTGGCTGTTACTG	CCAAGAGGAGGCTCTTTCCT
Actb	beta actin	AATCGTGCGTGACATCAAAG	ATGCCACAGGATTCCATACC

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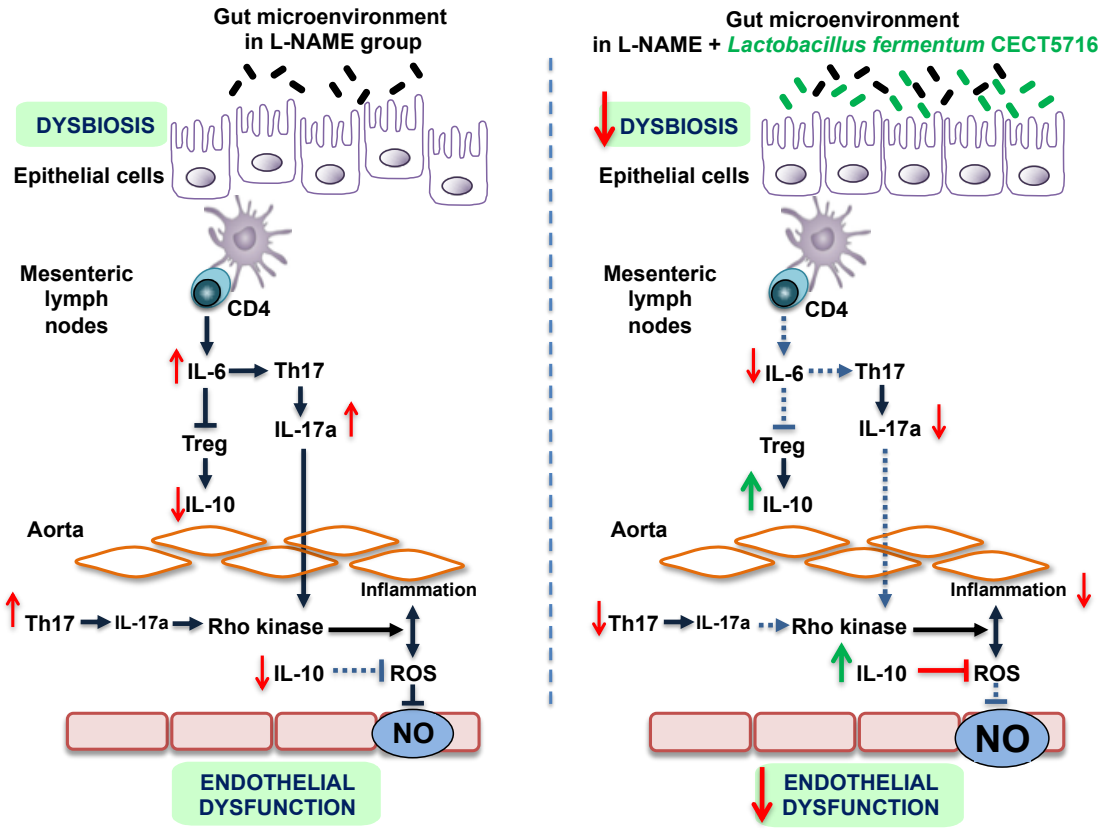
Conflict of interest

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

Abstract graphic



DISCUSSION

Discussion

6.1 Gut dysbiosis in hypertension

In the different experiments included in this Doctoral Thesis, certain characteristics in the gut microbiota of hypertensive animals compared with normotensive animals have been described. Overall, it has been found an alteration in the gut microbiota, known as dysbiosis, characterized by an increase in the F/B ratio as found in the SHR animal model, and in WKY-SHR and SHR-SHR group, and even a higher F/B ratio was found in the L-NAME hypertension model. However, this F/B ratio was unchanged in DOCA-salt hypertensive rats (Marques FZ et al., 2017), showing that the increased F/B ratio is not essential to high BP, but it is a key characteristic of gut dysbiosis in genetic hypertension. All changes in the gut microbiota composition in the different experimental models of hypertension used in this Thesis (SHR and L-NAME) are summarized in **Table 1 and Figure 4.**

Several authors have described a reduction of α-diversity, measured by Shannon and Simpson as well as a reduction in the Pielou evenness and Chao richness (Yang et al., 2015). In our experiments, reduced (article 3) or no change (article 1, 2, and 4) in richness and diversity were found in gut microbiota from SHR compared to those found in WKY, showing that reduced α-diversity is not a essential ecological characteristic of dysbiotic microbiota in genetic hypertension. Moreover, in a non-genetic model of HTN induced by L-NAME no significant changes in these parameters were observed. The alteration in the gut microbiota allowed us to find a clear separation among the different clusters of each group. When analyzing the β-diversity, a clear separation among, SHR *vs* WKY in the different experiments was found, and it was also present between CTRL *vs* L-NAME (**Table 1)**

Derived from that analysis, we found a strong positive correlation between SBP and the lactate-producing genus *Turicibacter,* which is also linked to the development of colitis in IL-10-deficient mice (Ye et al., 2008), and may have immunomodulatory or host invasion-related traits, such as internalin production (Cuív et al., 2011). By contrast, a negative correlation between SBP and the abundance of genus *S24-7g* was showed. This genus was found more abundantly in non-diabetic mice, it also correlated positively with delayed diabetes onset age, and splenic $FoxP3^{+}CD4^{+}$ Treg cells (Krych

et al., 2015). To prove the role of both bacteria in BP regulation, as pro-hypertensive or anti-hypertensive, respectively, we should carry out the monocolonization of gut microbiota in normotensive WKY and in hypertensive SHR. However, we lacked the media to perform these experiments. *S24-7_g* was also found to have a key role in the development of HTN as a ratio with *Lactobacillus*, since we were able to identify a significant direct correlation between *Lactobacillus/S24-7g* ratio and SBP. *Lactobacillus* is a lactate-producing bacteria being high lactate levels broadly linked to high BP (Toral et al., 2018). In fact, when Losartan treatment reduced BP this was associated with the normalization in the *Lactobacillus ssp.* content in faeces. However, no significant changes in *Lactobacillus* content in the gut were observed in both L-NAME-hypertension (article 5) and DOCA-salt hypertension (Marques et al., 2017), whereas were reduced in high-salt diet (8%)-induced hypertension (Wilck N, et al., 2017). In this last model, *Lactobacillus murinus* supplementation, which normalizes *Lactobacillus* content, ameliorates salt-sensitive hypertension and reduces the number of Th17 cells. This *Lactobacillus* produced intestinal indole-3-lactic acid, which reduces the polarization of proinflammatory Th17 cells (Wilck N, et al., 2017). In our experiments (article 4), LC40 treatment in SHR was unable to change *Lactobacillus ssp* content, but it reduced BP and Th17 polarization, acting as a probiotic bacteria.

In addition, it was found a significant depletion of *Bifidobacterium* in L-NAME (article 5) and SHR (Kimura et al., 2010). Commonly, *Bifidobacterium* has been considered a beneficial genus due to its role in the maturation and regulation of the immune system (Diehl et al., 2013). Consequently, *Bifidobacterium* depletion might contribute to the dysregulation of the immune system found in hypertensive L-NAME-treated rats. However, no significant changes in *Bifidobacterium* content were detected in our experiments using SHR (article 1, 2, 3, and 4), and BFM, a strain of *Bifidobacterium breve*, did not change the relative abundance of its own genera in the gut. It is interesting to note that the probiotics LC40 and BFM prevented hypertension in SHR 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.

Model	Ratio F/B	α- diversity	Phylum	Family	Genus	
SHR	↑	Chao Richness; no change o ↓ Simpsons; no change o ↓ Shannon; no change o ↓ Pielou; no change o ↓	Firmicutes ↑ Bacteroidetes ↓ Actinobacteria ↓ Proteobacteria ↓	*Lactobacillaceae* ↑ *Turicibacteriaceae* ↑ *Peptostreptococcaceae* ↑ *Anaeroplasmataceae* ↑ *Verrucomicrobiacea* ↓ *Bacteroidaceae* ↓	*Oridobacterium* ↑ *Streptococcus* ↑ *Parabacteroides* ↑ *Turicibacter* ↑ *Lactobacillus* ↑ *Blautia* ↓ *Odoribacter* ↓ *Bifidobacterium* ↓ *Allobaculum* ↓ *Alistipes* ↓ *Coprococcus* ↓ *Pseudobutyvibrio* ↓ *S24-7g* ↓ *Pedobacter* ↓ *Akkermansia* ↓	*Paraba* *Klebsie* *Anaero* *Eubact* *Prevote* *Rummi* *Bacter* *Parapre* *Parapre*
L-NAME	↑	Chao Richness; no change Simpsons; no change Shannon; no change Pielou; no change	Firmicutes ↑ Bacteroidetes ↓	*Propionobacteriaceae* ↑ *Dysgomonadaceae* ↓ *Phorphyromonadaceae* ↓ *Odoribacteriacea* ↓	*Propionibacterium* ↑ *Veillonela* ↑ *Roseomonas* ↑ *Burkholderia* ↑ *Parabacteroides* ↓ *Bifidobacterium* ↓ *Olivibacter* ↓ *Dysgomonas* ↓ *Pedobacter* ↓ *Flavobacterium* ↓ *Desulfotomaculum* ↓ *Anaerotroncus* ↓ *Termobaclulum* ↓	

Table 1. *Characteristic of gut dysbiosis in experimental models of hypertension used in this Doctoral Thesis.*

The alteration in the population of SCFAs-producing bacteria has been associated to the gut dysbiosis in HTN. The modification found in SHR was characterized by lower acetate- and butyrate-producing bacteria than those in their WKY normotensive counterparts, as was previously described by Yang et al., 2015. Both of those SCFAs are able to induce anti-inflammatory effects in the gut, mediated by GPR-43 activation (Yang et al., 2018). Butyrate also provides energy to the gut epithelium and peripheral tissues, and promotes intestinal epithelial integrity and aids in the repair of wounded epithelium, mainly through GPR-43 activation (D'Souza et al., 2017). Relative to the present Thesis, it has been found that there is not always the same SCFAs-bacteria producing profile in SHR or in L-NAME. Overall, it was found a reduction in acetate-producing bacteria and an elevation of lactate-producing bacteria. In addition, Losartan treatment induced a reduction in propionate-producing bacteria, which has been described to be able to induce a significant increase in heart rate in the wild type, but not the GPR41$^{-/-}$ mice. Also, it has been found that when the control groups (SHR vs WKY) were compared in article 4, a reduction in butyrate-producing bacteria in SHR compared to WKY is present. The differences among the different experiments with SHR may be due to the different regions used to analyze the microbiota in the different experiments (V4-V5 in experiment 1, 2 and 4, V3-V4 in experiment 3). The bacterial metabolic products profile is different between experimental model because in the L-NAME model the SCFAs-producing bacteria are characterized by an elevation in propionate-producing bacteria link to a higher level of *Propionibacterium* (**Table 1).**

The gut dysbiosis found in the increase in BP in SHR was usually associated with gut pathology that included increased intestinal permeability and decreased tight junction proteins (Santisteban et al., 2017). In agreement with that, in the present Thesis the integrity of the gut epithelial barrier was analyzed, measuring the mRNA levels for tight junction proteins and mucins, which are involved in mucus production in the proximal colon. We found that SHR and also L-NAME significantly reduced colonic ZO-1, MUC-2, MUC-3 and Occludin and increased colonic TNF-α and IL-6. All these modulations in the healthy gut might lead "leaky gut" to LPS crossing the gut epithelium, reaching systemic circulation. LPS activates TLR-4 in the vasculature (Liang et al., 2013), and by induces systemic inflammation. In addition, LPS might cross blood brain barrier to produce microglia activation, oxidative stress in cardiovascular regions of the brain, such as rostral ventrolateral medulla (Wu et al.,

2012) and PVN (Zhang et al., 2010), Interestingly, transplantation of gut microbiota from WKY to SHR reduced gut inflammation, plasma LPS levels, brain PVN inflammation, NADPH oxidase activity and sympathetic excitation, leading to a stable drop in BP. The role of LPS in the brain will be discussed in depth in the corresponding section.

The alteration in the integrity could induce lower anaerobic conditions in the gut, concretely, a reduction in the anaerobic bacteria in SHR has been found (article 3). A similar result was found by Kim et al., 2018, they described that intestines of Ang II-hypertensive mice were significantly less hypoxic and with increased aerobic bacteria in faeces.

On the other hand, the SCFAs are metabolic products of the gut bacteria, which are able to cross the epithelium gut by passive diffusion or by a monocarboxylate transporter (Bergersen et al., 2002; Vijay and Morris, 2014; Sun et al., 2016). These SCFAs are able to induce their anti-inflammatory effects in the gut or in other organs (Natarajan and Pluznick, 2014; Natarajan et al., 2016; Pluznick, 2014). In fact, it has been shown how SCFAs modulate BP through the renal and vascular Olfr and GPRs. SCFAs modulate BP through the renal and vascular olfactory receptor Olfr59 (its stimulation elevates BP), GPR-41 and GPR-43 (their stimulation both lower BP) (Pluznick et al., 2013). In addition to the vasculature (Pluznick et al., 2013; Pluznick, 2014), the SCFAs-sensing receptors Olfr78 and GPR-43, as well as GPR-41, are present on the sympathetic ganglia (Kimura et al., 2011; Nøhr et al., 2015). In the present Thesis, the SCFAs content in feces and plasma was measured just in the treatment with SCFAs and probiotics in SHR, and we found a reduction in the acetate and butyrate levels in the feces. These SCFAs modulate T cells polarization in favor of Treg population in gut secondary lymph organs. In agreement with the lower acetate and butyrate content in faeces from SHR, increased Th17/Treg was found in MLNs from SHR as compared to WKY. However, no difference was found in plasma levels of SCFAs as described by Kim et al., 2018, ruling out that functional alterations in SHR are dependent of lower plasma levels of SCFAs. 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. This data reduced the mechanistic value of pro-hypertensive plasma lactate in genetic hypertension.

Relative to the role of SCFAs in the regulation of BP by the microbiota, several works have demonstrated that a high abundance of butyrate-producing genus *Odoribacter* in the gut microbiota in pregnant women at 16 weeks of gestation is associated with decreased BP (Gomez-Arango *et al.,* 2016). As was described above, butyrate is able to cross the epithelium gut but also the blood brain barrier, hence, the circulating butyrate may induce direct effects on regions of the brain that regulate BP. The data shown in this thesis describes a significant depletion of *Odoribacter* in the WKY-SHR and SHR-SHR groups. The depletion might contribute to the dysregulation of the expression Olfr59, GPR-41, and GPR-43 found in PVN from hypertensive groups. Because Olfr59 elevates BP and GPR-41 opposes this action to lower BP, these data support the suggestion that the altered expression of SCFAs receptors in the PVN may play a role in elevated BP of the SHR. However, we have not experimental support to this question, since we did not measure plasma and brain levels of butyrate in the article 2.

The role of SCFAs could be also discussed in the FMT from donors SHR-losartan to receptors SHR because there was found an increase in the prevalence of acetate-producing bacteria and the genus *Bacteroides acidifaciens,* an important symbiont in the human gut, which was recently associated with reduced SBP in DOCA-salt hypertension (Marques et al., 2017). In addition, a high-salt diet reduced *Bacteroides acidifaciens* in gut microbiota associated with hig BP (Yan et al., 2020). The intestinal *Bacteroides fragilis* could inhibit the production of intestinal-derived corticosterone through its metabolite arachidonic acid, reducing the aldosterone pathway (Yan et al., 2020). Intestine has been reported to be an important source of bioactive corticosterone. Previous studies have shown that corticosterone produced by intestine could enter circulation (Mukherji et al, 2013), which might activate mineralcorticoid receptors and contribute to sympathetic excitation and changes in sodium handling and vascular resistance.

The systemic effect of microbiota could be also due to another metabolites as sulfate, in fact, we found in the WKY-SHR group a depletion of *Peptococcaceae,* a sulfate-reducing bacteria considered to be beneficial in the regulation of BP (Ahmad *et al.,* 2012; Yu *et al.,* 2018). Sulfate-reducing bacteria, and hydrogen sulfide released in

the colon may also contribute to the control of BP, being antihypertensive, through the suppression of sympathetic outflow (Duan et al*.,* 2015).

The key role of the gut microbiota to regulate the BP was shown mainly in article 1 and 2, because FMT of dysbiotic microbiota from SHR to recipient normotensive WKY increased BP, and FMT from WKY to recipient SHR reduced BP. In addition, changes in the composition of microbiota induced by losartan in SHR (article 3) or LC40 in tacrolimus-induced hypertension (Toral et al., 2018), when were transplanted to SHR or tacrolimus-mice, respectively, reduced BP. The data from this Thesis support that changes in gut microbiota induced by losartan are, at least in part, responsible of the antihypertensive effect of losartan in SHR. In this experiment it was found that the microbiota could be adapted to BP reduction, shifting to a microbiota composition similar to WKY. The changes in gut microbiota composition were found joined to an improvement in the gut barrier, contributing to the hypoxic environment of the lumen and thus, normalizing the amount of anaerobic bacteria. This improvement was induced by reduced gut sympathetic activity induced by losartan, because hydralazine (that increased gut sympathetic drive) was unable to restore gut integrity and microbiota composition, despite similar reduction in BP. Damage to this barrier makes the environment less hypoxic, conducive to aerobic bacterial growth (Konig et al., 2016; Earley et al, 2015). SHR treated with losartan showed increase colonic integrity and a proportion of strict anaerobic bacteria similar to WKY.

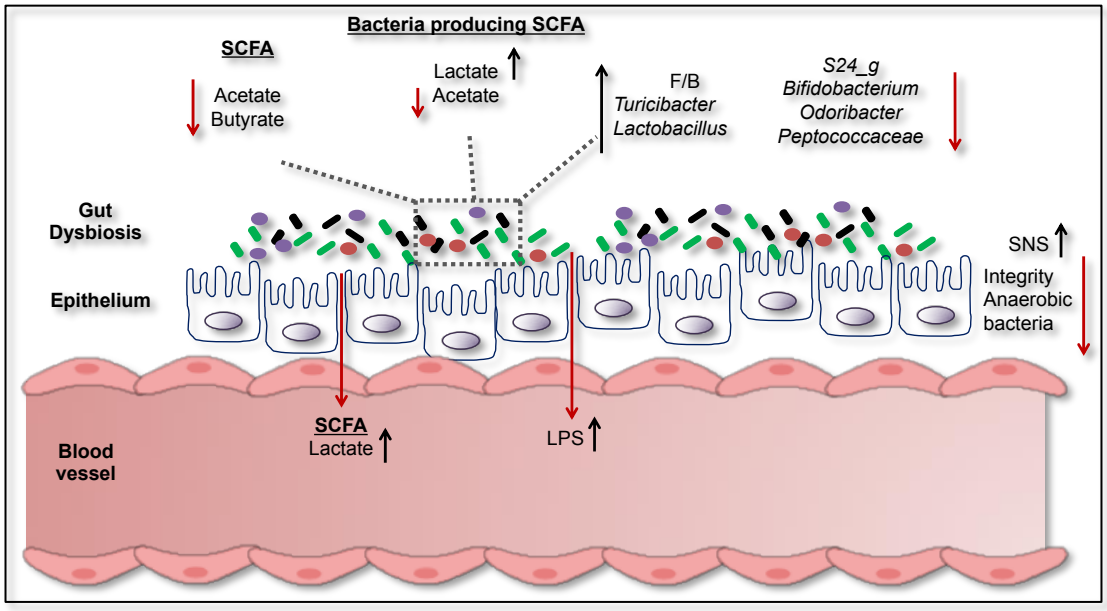


Figure 4. *Summary of the alteration in the gut microbiota in hypertensive conditions. SCFAs, short chain fatty acids, F/B, Firmicutes/Bacteroidetes ratio, SNS, sympathetic nervous system, LPS, lipopolysaccharide.*

6.2. Microbiota-Immune System-Hypertension

Overall, it seems clear that BP regulation by gut microbiota is, at least in part, mediated by its effect on the immune system.

Under hypertensive conditions, it was previously described that the intestinal integrity could be damaged, for that reason certain bacteria or bacteria components could be able to cross the gut epithelium and induce the activation of antigen presenting cell (APC) CX3CR1-expressing cells (Persson et al., 2017; Galkina et al., 2006). The APC could be able to capture bacteria from the gut lumen and transport them to the MLN, where they present antigens and activate $CD4_+$ T cells to produce IL-6 that suppresses Treg, and increases Th17 cells. In the present Thesis it has been described how in SHR as well as in L-NAME appears an elevation in the activation of APC joined to a higher expression of proinflammatory cytokines as IL-6 and IL17. The bacterial environment found in HTN would induce T-cell activation and polarization in a key immune inductive site, the MLNs, increasing Th17 and reducing Treg, increased circulating T cells and vascular T-cell accumulation. Moreover, as previously described, low levels of acetate and/or butyrate in the gut could also contribute to this T-cells polarization in MLNs. In the different experiments carried out in this thesis it was found that the modulation of microbiota by different ways such as FMT from SHR to WKY was able to regulate it. In SHR-SHR and WKY-SHR was found an increased T-cell activation in MLNs, as a result of CX3CR1-expressing cells accumulation, found an elevation of $CD4_+$ T cell activation, and produce IL-6 that translates into a suppression of Treg, and an increase in Th17 cells. Due to this, when the population of Th17 and Treg were studied in MLNs we found a change in the polarization of T cell to Th17 and a reduction of Treg. That polarization was reversed in SHR-WKY animals, which showed the role of the gut microbiota to modulate lymphocyte populations at the MLNs levels. Besides, the treatment with Losartan performed in the third experiment also was able to modulate the polarization to Th17 in the $CD4_+$ T cell population at MLNs. Classically, it has been described how T-cell activation requires T-cell receptor ligation and co-stimulation. In this context, it has been shown how the interaction between B7 ligands (CD80 and CD86) on antigen-presenting cells with the T-cell co-receptor CD28 is involved. In the FMT performed in the first experiment, we described the key role of that co-stimulation in HTN induced by gut microbiota. We showed how the blockade of B7-dependent co-

stimulation with CTLA4-Ig abolished T cells activation and polarization to Th17 in MLNs and the development of HTN found in WKY-SHR rats (**Figure 5).**

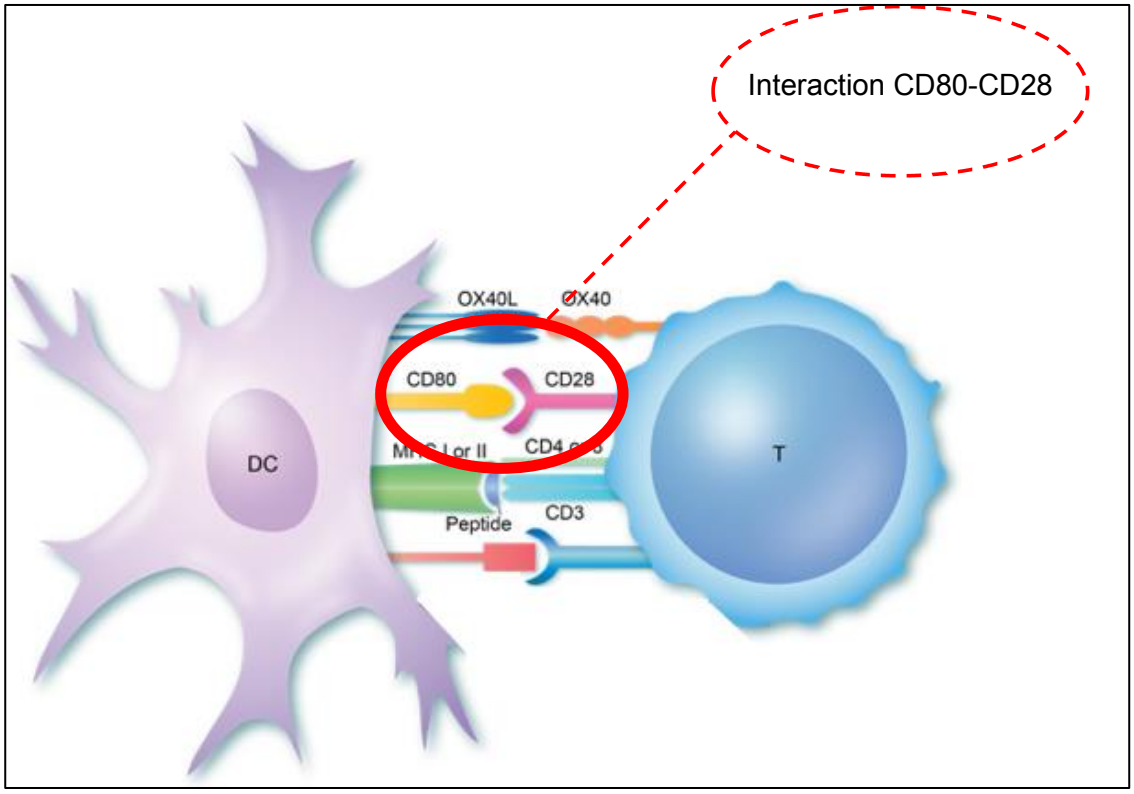


Figure 5. *T-cell activation requires T-cell receptor ligation and co-stimulation. In this context, it has been shown how the interaction between B7 ligands (CD80 and CD86) on antigen-presenting cells with the T-cell co-receptor CD28. DC, Dendritic cell, T, T cell, CD80, cluster of differentiation 80, cluster of differentiation 28.*

The modulation induced by microbiota at MLN level has shown to be able to induce effects in BP and, also, in the impaired endothelial function. The different results from this thesis showed that the observed endothelial dysfunction was mediated by increased IL-17 production in the vascular tissue. The axis Th17/IL17 was showed to be essential for the development of endothelial dysfunction and HTN induced by stool transplantation from SHR to WKY. The FMT study demonstrated the essential role of IL-17 in endothelial dysfunction and HTN induced by dysbiotic microbiota. In this experiment we found higher levels of RORγ, in aortas with impaired relaxation to ACh from SHR-SHR group as compared to WKY-WKY group and also in WKY-SHR. But, as was described previously by Katsuki et al., 2015 the Treg level in aorta was decreased in SHR-SHR groups as compared to WKY-WKY. Improvement of endothelial function by FMT from WKY to SHR reduced Th17 accumulation and increased Treg in the

vasculature, reducing the ratio of pro-inflammatory/anti-inflammatory cells. In agreement with this result was also found an improvement of T cells profile in aorta of SHR-Losartan characterized by an elevation of Treg.

The effects on the APC and on the T cell population was shown to be crucial in protective vascular effect and hypertension induced by normal microbiota, because it reduced accumulation of IL-17a producing cells in the aorta, and inhibited NADPH oxidase activity (Colliou et al., 2017). This fact was confirmed by the administration of an IL-17a-neutralizing agent, which reduced BP and restored endothelial function in WKY-SHR animals, showing the involvement of Th17-IL-17 axis as a pro-hypertensive pathway. These data are in agreement with Karbach et al., 2016, who described that microbiota could regulate the immune system and/or gut-brain communication reducing BP. In fact, they showed that Ang II infusion to germ-free mice reduced ROS formation in the vasculature, attenuated vascular expression of NADPH oxidase subunit Nox2, and reduced upregulation of RORγ, transcription factor for IL-17. These vascular changes exerted protection from endothelial dysfunction and attenuation of BP increase in response to Ang II, involving the immune system in the effects of microbiota in vascular function and BP regulation. We found increased ROS levels in aortas from L-NAME, SHR, WKY-SHR and SHR-SHR groups linked to impaired relaxation to ACh joined to a marked raise in both the NADPH oxidase activity and the expression of its subunits NOX-1, NOX-4, $p22_{phox}$ and $p47_{phox}$ in aortas compared to their respective control group. By contrast, when we found a reduction of NAPDH oxidase activity in aorta, this was associated with BP reduction and improvement of endothelial function. Interestingly, FMT from WKY to SHR, which reduced BP, was able to reduce aortic ROS content, NADPH oxidase activity and NADPH oxidase subunits expression.

The infiltration of Treg cells in the aorta produced higher level of IL-10, the main cytokine released by Treg, which is able to attenuate NADPH oxidase activity, which is a critical process in the improvement of vascular endothelial function in HTN (Kassan et al., 2011). In the different experiments were analyzed the effects of conditioned media of cultured lymphocytes from MLN from all experimental groups in aorta and RAEC from control rats. The incubation with the conditioned media of cultured MLN lymphocytes from L-NAME or SHR group impairs aortic endothelium-dependent relaxation to ACh, reduced NO production, and increased NADPH oxidase-driven ROS production in RAECs, showing that changes in Th17/Treg balance in favor of Th17 impair endothelial function. In agreement with this, the presence of IL-17a neutralizing

antibody improves this endothelial dysfunction as was described in FMT from SHR to WKY. Altogether, our data show the key role of IL-17/RhoA kinase/NADPH oxidase pathway in the deleterious effects of gut microbiota in hypertensive conditions (**Figure 6).**

However, the interaction between microbiota and immune system is not limited only to T cells, there has been described how IECs and Paneth cells could secrete antimicrobial peptides, defensins, which selectively kill Gram-positive bacteria (Pamer, 2007; Ayabe et al., 2000; Vera et al, 2004; Vaishnava et al., 2008). The defensins synthesis could be induced by different components of the microbiota, such as LPS, mediated by TLR-4 expression. In fact, in the present Thesis we found changes in mRNA levels of defensins in colonic samples from SHR compared to WKY. Losartan restored defensins expression to become similar to those found in WKY.

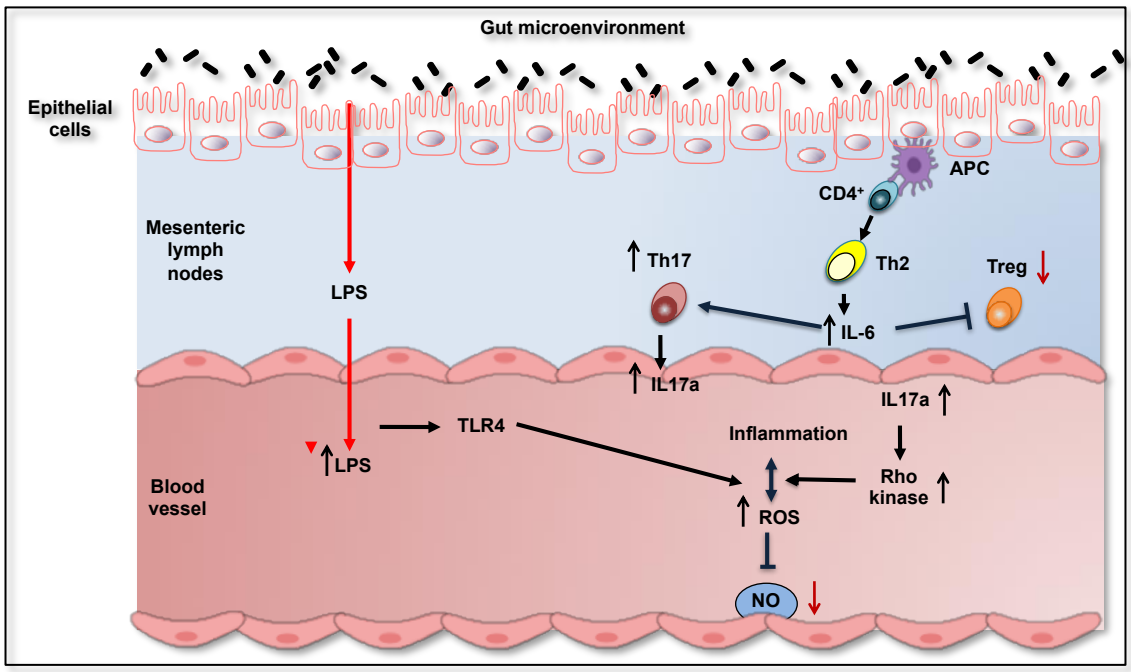


***Figure 6**. Summary of the alterations in the immune system-microbiota axis in hypertensive conditions at mesenteric lymph nodes level. LPS, lipopolysaccharide, Th17, T-helper 17, CD4, cluster differentiation 4, Th2, T-helper 2, Treg, T-regulatory, IL17a, Interleukin 17a, ROS, reactive oxygen species, NO, nitric oxide.*

6.3 Microbiota-Sympathetic nervous system-Hypertension.

Several authors have linked the gut microbiota with the development of hypertension due, at least in part, to alteration in the Ang II pathway in the brain. In particular, Gao et al., 2005 described that Ang II via AT1 receptor mechanism activates the sympathetic outflow by stimulation of the NADPH oxidase-dependent ROS production. In fact, the elevation of SNS activity is a hallmark of both animal and human HTN (Grassi et al., 2015; DiBona, 2013). Specially Santisteban et al., 2017 observed enhanced gut-neuronal communication in HTN originating from the PVN of the hypothalamus and presenting as increased sympathetic drive to the gut. Previous studies have also characterized HTN with alterations in the gut microbiota and sympathetic dysregulation (Zubcevic et al., 2014; Yang et al., 2015; Santisteban et al., 2017).

In this thesis, the link between gut microbiota and brain inflammatory and oxidative status under hypertensive conditions has been studied. The main findings are summarized in **Figure 7**. In brains from the SHR-SHR group it was found increased NADPH oxidase activity driven-ROS production, expression of NADPH oxidase subunits, pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and sympathetic activity, as compared to WKY-WKY rats. These data could be related to higher PRA found in the SHR-SHR group as compared to the WKY-WKY group. Conversely, FMT from SHR to WKY also resulted in higher PVN inflammation, NADPH-oxidase activity and sympathetic outflow than induced by FMT from WKY to WKY.

In addition, we have demonstrated the capacity of the gut microbiota to regulate the BP by an increase in the sympathetic drive. In the FMT carried out in the second article was observed a higher reduction in BP after pentolinium administration and higher plasma NA levels, both markers of increased sympathetic drive, associated with microbial dysbiosis. This higher SNS activity correlates with higher SBP and DBP in the WKY-SHR and SHR-SHR group compared to WKY-WKY. Moreover, increased catecholaminergic neurotransmission has been reported in SHR, characterized by increased TH activity as well as gene and protein expression (Yu et al., 1996; Reja et al., 2002; Lopez Verrilli et al., 2009), suggesting that TH plays a key role in the genesis, development and/or maintenance of HTN. In our experiment was found an increased colonic expression of TH and NA content from the SHR-SHR, SHR and WKY-SHR groups compared to WKY-WKY.

Santisteban *et al.,* 2016 described how under hypertensive conditions the increased sympathetic activity could affect the BM driving an increase in inflammatory cells, which migrate to the PVN and enhance neuroinflammation. In agreement with that result, an increased inflammation in the PVN from the SHR-SHR and WKY-SHR groups, associated with an increased expression of CCL2, CD11b and IL-17a, that could generate or contribute to neuroinflammation, was described in this thesis. Overall, all these data showed a gut-brain communication characterized by increased pro-oxidant, pro-inflammatory and immune cell infiltration profile in the brain PVN after FMT from SHR to WKY. Interestingly, chronic normal microbiota transplantation to hypertensive rats induced a stable BP reduction linked to reduced brain PVN inflammation, NADPH oxidase activity and sympathetic excitation.

Along this thesis, it has been discussed that the microbiota through bacterial metabolic products such as SCFAs or bacterial components, such as LPS, are able to modulate the gut epithelium integrity and have several effects on the extra-intestinal tissues. Concretely, LPS could be involved in the activation of SNS through toll-like receptor (TLR)-4 stimulation in the vasculature (Liang et al., 2013) or brain inducing systemic inflammation, accompanied by microglia activation, oxidative stress in PVN (Zhang et al., 2010), and others cardiovascular regions of the brain (Wu et al., 2012).

On the other hand, as was also previously commented, butyrate, acetate and propionate are metabolic products of the gut bacteria that have anti-hypertensive properties (Natarajan and Pluznick, 2014; Natarajan *et al*., 2016; Pluznick, 2014). Although several authors have hypothesized about the expression of SCFAs receptors in the brain, no studies have examined the expression of these receptors in cardioregulatory brain regions such as the PVN of the hypothalamus. The analysis of the expression of receptors for SCFAs in the PVN found changes in the main receptors to SCFAs, suggesting a probable connection with BP control. Specifically, it was found an elevation of Olfr59 and reduction of GPR41 and GPR43 genetic expression in SHR-SHR and WKY-SHR. An important limitation about the role of SCFAs and LPS as molecules involved in gut-brain communication is that we have not measure brain levels of LPS and SCFAs.

Overall, the transplantation of gut microbiota from WKY to SHR reduced gut inflammation, plasma LPS levels, brain PVN inflammation, NADPH oxidase activity and sympathetic excitation, leading to a stable drop of BP. Moreover, the sympathetic activity on the gut was normalized due to a lower level of TH transcript and NA

content, but without correction of gut parameters, in the SHR with a WKY microbiota. That result induced some conflict with this hypothesis and suggests that gut integrity in SHR is independent of sympathetic tone, BP, and microbiota composition. However, as was described previously by Santisteban et al., 2017, a dysfunctional sympathetic-gut communication is associated with gut pathology, dysbiosis, and inflammation, and plays a key role in HTN. In fact, Kim et al., 2018 found a reduction in the intestinal integrity in the SHR, and we also found that the losartan treatment was able to reduce that higher sympathetic activity in the gut and improve gut integrity. Other result in favor of the key role of SNS activity in gut integrity was that, the hydralazine treatment, that increases sympathetic drive in the gut, was unable to improve gut integrity.

We also investigated if local RAS inhibition induced by losartan would be involved in its protective effects in the gut. Losartan is an angiotensin receptor blocker, having gut anti-inflammatory properties via inhibition of the Ang II/ Ang II type 1 receptor (AT1) axis (Takeshita and Murohara, 2014). In addition, several authors have described the ability of AT1 blocking to induce inhibition of the classical RAS pathway, inducing the upregulation of ACE2, which activates the Ang-(1-7)/Mas pathway to counteract the inflammatory signalling (Yisireyili et al., 2018). However, no significant changes in AGT, ACE and ACE2 expression were found among colonic samples from WKY, SHR and SHR-losartan groups ruling out that changes in this pathway were involved in the protective effects of losartan in gut integrity. So, having in account that sympathetic tone in SHR is attenuated by RAS-inhibition (Tsuda et al., 1988; Demirci et al., 2005; Santisteban et al., 2017), and increased by hydralazine (Tsoporis and Leenen, 1988), we focused in the SNS activity as the key regulator of gut integrity and microbiota composition. Moreover, Sharma et al., 2019 describes how central administration of a modified tetracycline inhibited microglial activation, normalized sympathetic activity, attenuated pathological alterations in the gut wall, and restored certain altered gut microbial communities.

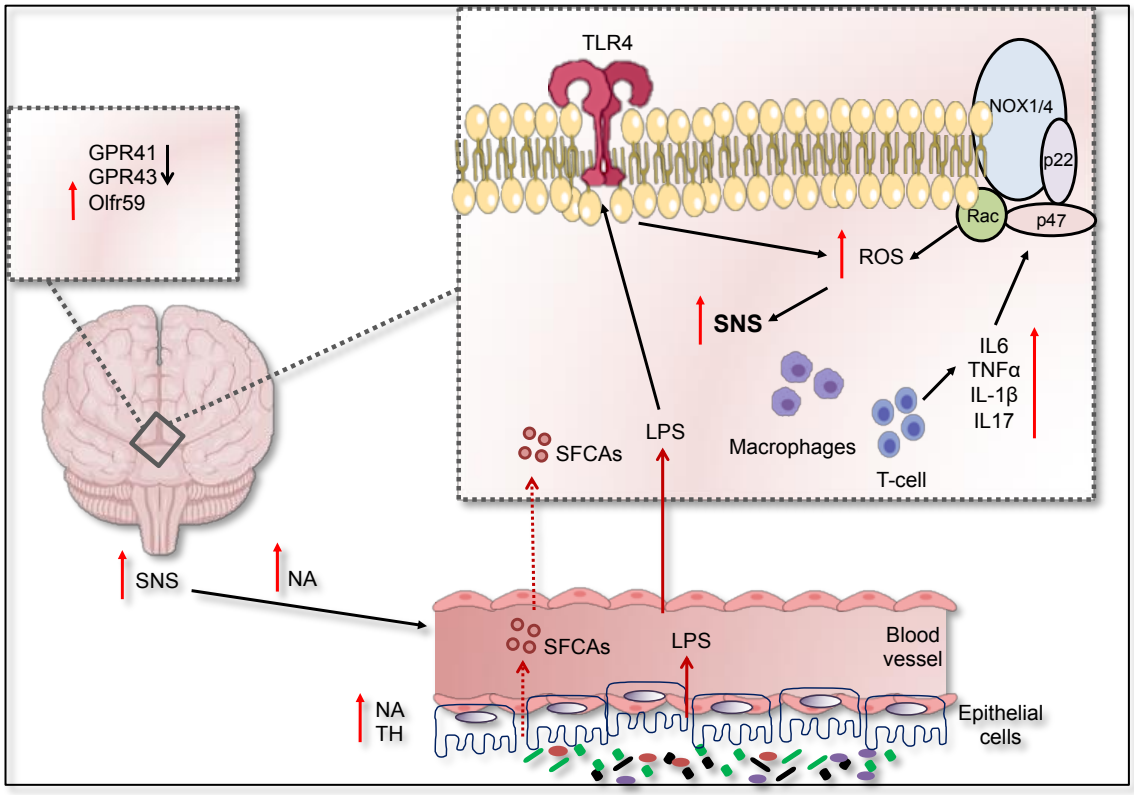


Figure 7. *Summary of the alteration in the brain-microbiota-immune system axis in hypertensive conditions at PVN level. GPR, G-protein-coupled receptor, Olfr59, olfactory receptor 59, SNS, sympathetic nervous system, NA, noradrenaline, TH, tiroxine hydroxylase, SCFAs, Short chain fatty acids, ROS, radical oxygen species, TLR4, Toll Like Recpetor-4, IL, Interleukin, TNF, Tumour Necrosis Factor alpha.*

6.4 Probiotics and Hypertension

In the introduction and justification sections it has been described the need to find new therapies to regulate BP, being the gut microbiota a possible target due to its role in the regulation of multiple systems involved in BP control, such as the SNS or the immune system among others. In fact, in multiple studies the association between gut dysbiosis and HTN has been demonstrated (Yang T el al., 2015; Mell B et al., 2015; Li J et al., 2017). Even in the diverse experiments performed in the present thesis it has been demonstrated the link among gut microbiota and HTN. Gomez-Guzman et al., 2015 described that when SHR with established HTN 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 BP were found. The administration of *Lactobacillus* strains increased *Lactobacillus spp* and reduced *Bacteroides* and Clostridium ssp.

In the two last articles of this thesis, we analyzed the role of different strains of probiotics: LC40, a strain of *Lactobacillus fermentum*, and BFM, a strain of *Bifidobacterium breve*, and also SCFAs (acetate and butyrate) in BP. Both probiotics did not change the relative abundance of their respective genera in the gut, *Lactobacillus* or *Bifidobacterium*, 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, as commented above. Neither the probiotics nor SCFAs was able to normalize the diversity, richness or evenness in the gut microbiota but all except LC40 were able to normalize the F/B ratio. However, the main change was at genera level, induced by chronic consumption of LC40 and BFM or SCFAs, the *Lactobacillus/S24-7g* ratio was reduced, which was described previously in this thesis as a possible biomarker of HTN in SHR.

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 as was discuss above. In fact, 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 were found elevated compared to SHR. One possible mechanism to explain the effect of the different treatments could be the alteration in the SCFAs-producing bacteria and also the SCFAs concentration at plasma and fecal level. When both were

analyzed together, we found differences between the concentrations in feces and plasma, the possible explanation might be related to the reduced expression of MCT1 in SHR, the major SCFAs transporter expressed in the gut for the uptake of lactate (Halestrap, 2013). Interestingly, a decreased membrane transporter specific for butyrate (MCT4) was observed in colonic samples from SHR, which was significantly upregulated by butyrate, similar to that previously described (Kim et al., 2018). Is important to remark that both probiotics were able to increase butyrate-producing bacteria, and butyrate consumption increased plasma levels of butyrate in SHR. Butyrate could inhibit the differentiation of Th17 cells (Chen et al., 2017; Zhang et al., 2019). In fact, only these treatments changed T cells polarization in spleen, being without effects acetate consumption. In addition, systemic butyrate showed the capacity to act directly at the vascular wall reducing oxidative stress and improving endothelial dysfunction, as was previously described (Aguilar et al., 2016).

The effect of the probiotic and both SCFAs on the microbiota was able to increase the intestinal integrity measured by mRNA levels of the tight junction proteins Occludin and ZO-1 in the colon. This effect could be mediated by their effect on IL-18, a cytokine important for tissue repair (Elinav et al., 2013) and also in the differentiation through Th17 or Treg, which was also reduced and elevated, respectively, in colon from SHR-treated groups as compared to untreated SHR.

Overall, the different results obtained in this thesis suggest that probiotics might induce an improvement in the gut integrity with the subsequent reduced translocation of bacterial endotoxin into the circulation. In addition, it was previously described that under dysbiosis conditions, some bacteria or their components 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 leading to T cell activation (Niess et al., 2005). As was discussed during this thesis, the gut microbiota is a highly relevant factor in BP regulation, due to its effects on T-cell activation in the gut immune system and in vascular T-cells accumulation. The treatment with the probiotics was able to reduce the high Th17 cells infiltrated in aortas of SHR. The proinflammatory cytokine IL-17, produced by Th17, causes Rho-kinase-mediated endothelial dysfunction by increasing phosphorylation of the inhibitory eNOS residue Thr^{495} (Nguyen et al., 2013; Toral et al., 2018) inducing an impaired relaxation to ACh from SHR as compared to WKY. Also, the probiotics and SCFAs were able to restore the level of Treg accumulation, which was decreased in

SHR group, and IL-10, the main cytokine released by Treg, that attenuates NADPH oxidase activity (Kassan et al., 2011). All those effects on the vascular wall entailed the improvement of endothelial function induced by LC40, BFM, and butyrate. In addition, reduced phenylalanine plasma levels induced by both probiotic treatments might contribute to improve endothelial dysfunction, since vessels from hyper-phenylalaninemic mice showed reduced levels of the essential cofactor of eNOS tetrahydrobiopterin, improving eNOS uncoupling (d'Uscio et al., 2011).

Finally, the role of NO in the protective effect of probiotics was explored in the last experiment by the administration in drinking water of L-NAME, inducing a gradual increase in BP via the inhibition of NO synthase activity. In this case LC40 administration prevented the gut dysbiosis induced by L-NAME, restored the Th17/Treg polarization in MLN and reduced Th17 infiltration in aorta. In addition, the LC40 treatment was able to restore the intestinal dysbiosis as well as the intestinal integrity. Even LC40 consumption was able to decrease CX3CR1 expression in the MLN, reducing the activation and migration of APC to the MLN, hence suppressing IL-6 production, which restore Th17/Treg polarization. Also, it was able to induce a reduction of *Propionibacterium,* whose surface protein dihydrolipoamide acetyltransferase has been described to stimulate intestinal APC, leading to T cell activation and Th17 differentiation (Peng et al., 2007).

However, in L-NAME rats the treatment with LC40 was unable to restore the endothelium-dependent relaxation to Ach, which was reduced in L-NAME. LC40 treatment only weakly prevented this effect, without affecting NO sensitivity, since it could not alter the relaxation to NO donor nitroprusside. This weak protective effect induced by LC40 was similar to the one induced by the non-selective NADPH oxidase inhibitor apocynin, suggesting that chronic LC40 administration improved endothelial function reducing NADPH oxidase-driver ROS production. In fact, aortic Th17 infiltration, ROS accumulation and NADPH oxidase activity were restored by the LC40 treatment. However, the reduction in ROS due to the LC40 treatment was unable to increase NO bioavailability enough to reduce BP, under chronic eNOS inhibition induced by L-NAME. So, this experiment showed the key role of NO in the antihypertensive effects of this probiotic in SHR (Gómez-Guzmán et al., 2015). Thus, from these experiments we can conclude that the action of probiotics is meditated by their ability to restore the gut dysbiosis, the intestinal integrity and normalized the

balance in the activation of APC cell and Th17-Treg in MLN as well as in the vascular wall without being able to work directly modulating eNOS activity (**Figure 8**).

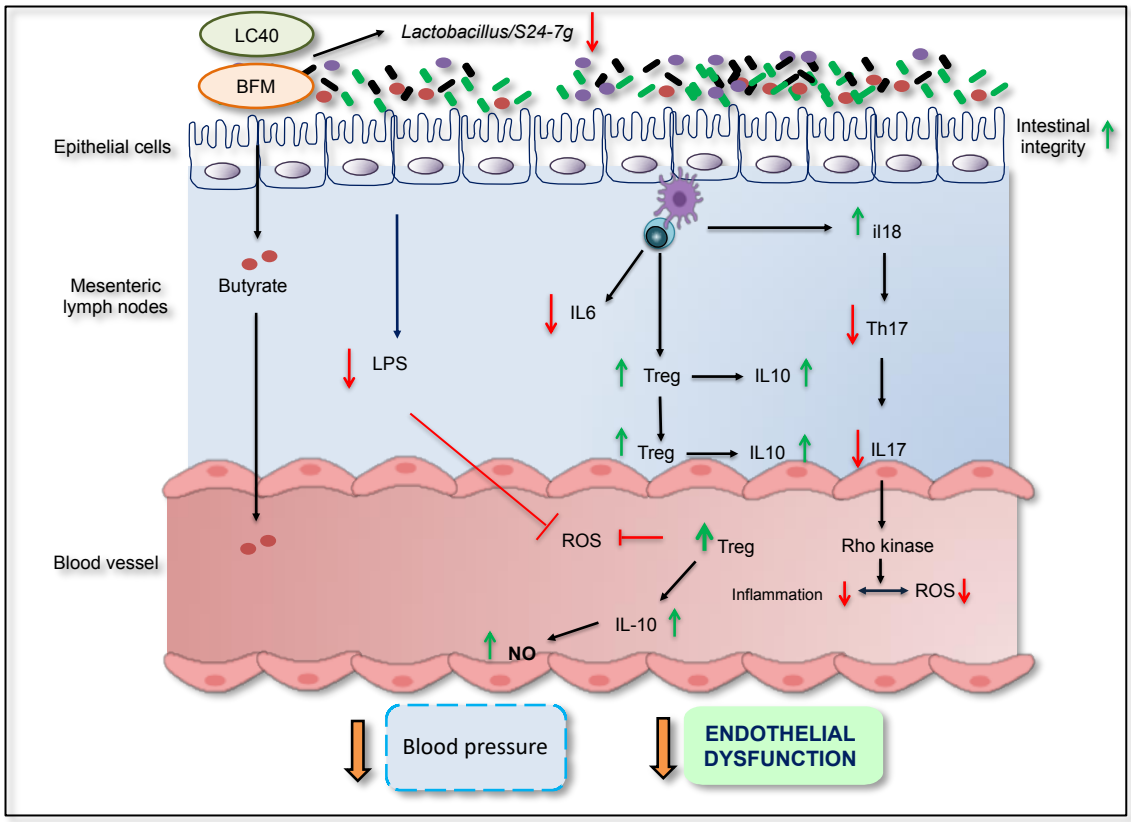


Figure 8. *Summary of effects of probiotics on the microbiota-immune system axis in hypertension. SCFAs, Short chain fatty acids, ROS, radical oxygen species, IL, Interleukin, Th, T-helper, Treg, T-regulatory, LPS, lipopolysaccharide, NO, nitric oxide, BFM, Bifidobacterium breve, LC40, Lactobacillus fermentum*

CONCLUSIONS

Conclusions

1. Microbiota is a crucial factor involved in the control of BP, as consequence of its effect in T-cell activation in gut immune system and vascular T-cells accumulation. Dysbiotic microbiota, characterized by increased *Turicibacter* and reduced *S24-7_g* , strongly correlated to SBP in genetic hypertension.

2. In the genetic model of hypertension (SHR) gut dysbiosis is correlated with sympathetic outflow via stimulation of NADPH-oxidase-derived ROS in the brain. These central effects seem to be associated with reduced expression of butyrate-sensing receptors in the hypothalamus, Th17 and macrophages infiltration in PVN and higher plasma levels of LPS.

3. In SHR, losartan treatment reduced gut dysbiosis and sympathetic drive in the gut, thus improving gut integrity. The changes induced by losartan in gut microbiota contributed, in part, to protecting the vasculature and reducing BP, possibly by modulating the gut immune system.

4. The probiotics *Lactobacillus fermentum* and *Bifidobacterium breve* 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.

5. In the model of chronic inhibition of NO synthesis, *Lactobacillus fermentum* treatment reduces early events involved in atherosclerosis development, such as vascular oxidative stress and pro-inflammatory status, as a result of prevention of gut dysbiosis and immune changes in MLN, but not hypertension. This data confirms the critical role of NO in the antihypertensive effects of *Lactobacillus fermentum* in genetic hypertension.

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