Received: 17 June 2020 | Revised: 23 July 2020 | Accepted: 27 July 2020

DOI: 10.1096/fj.202001532R

RESEARCH ARTICLE

THE FASEB JOURNAL

Probiotic *Bifidobacterium breve* prevents DOCA-salt hypertension

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Funding information
Ministerio de Economía, Industria y Competitividad, Gobierno de España (Ministeri d'Economia, Indústria i Competitivitat), Grant/Award Number: SAF2017-84894-R; HHS | NIH | National Heart, Lung, and Blood Institute (NHLBI), Grant/Award Number: HL102033; Consejería de Economía, Innovación, Ciencia y Empleo, Junta de Andalucía (Ministry of Economy, Innovation, Science and Employment, Government of Andalucia), Grant/Award Number: CTS164

Abstract

Many probiotics that affect gut microbial ecology have been shown to produce beneficial effects on renin-angiotensin-dependent rodent models and human hypertension. We hypothesized that *Bifidobacterium breve* CECT7263 (BFM) would attenuate hypertension in deoxycorticosterone acetate (DOCA)-salt rats, a renin-independent model of hypertension. Rats were randomly divided into five groups: control, DOCA-salt, treated DOCA-salt-BFM, treated DOCA-salt-butyrate, and treated DOCA-salt-acetate, for 5 weeks. BFM prevented the increase in systolic blood pressure, cardiac weight, and renal damage induced by DOCA-salt. BFM increased acetate-producing bacterial population and gut acetate levels, improved colonic integrity, normalized endotoxemia, plasma trimethylamine (TMA) levels, and restored the Th17 and Treg content in mesenteric lymph nodes and aorta. Furthermore, BFM improved nitric oxide-dependent vasorelaxation induced by acetylcholine in aortic rings and reduced NADPH oxidase activity in DOCA-salt animals. These protective

Abbreviations: CECT, collection of type cultures; CFU, colony-forming units; DOCA, deoxycorticosterone acetate; F/B, Firmicutes/Bacteroidetes; KEGG, kyoto encyclopedia of genes and genomes; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharides; MCT, monocarboxylate transporter; MLNs, mesenteric lymph nodes; MUC, mucin; NO, nitric oxide; OTU, operational taxonomic unit; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; RLU, relative luminescence units; ROS, reactive oxygen species; SBP, systolic blood pressure; SCFAs, short chain fatty acids; SHR, spontaneously hypertensive rat; TLR4, toll-like receptor 4; TMA, trimethylamine; ZO-1, zonula occludens-1.

effects were mimicked by acetate, but not by butyrate supplementation. These data demonstrate that BFM induces changes in gut microbiota linked with attenuation of endothelial dysfunction and increase in blood pressure in this low-renin form of hypertension. These beneficial effects seem to be mediated by increased acetate and reduced TMA production by gut microbiota, thus, improving gut integrity and restoring Th17/Tregs polarization and endotoxemia.

KEYWORDS

DOCA-salt hypertension, endothelial dysfunction, gut microbiota, immune system, probiotics

1 | INTRODUCTION

Hypertension is the most important risk factor for cardiovascular events, such as myocardial infarction and stroke. Emerging evidence indicates a link between gut microbiota to hypertension in both animal models and human hypertension.[1-6] In general, they demonstrate significant dysbiosis due to a reduction in microbial richness, diversity, evenness, and a rise in the Firmicutes/Bacteroidetes (F/B) ratio in renin-dependent hypertension in both animal model, such as spontaneously hypertensive rats (SHR), and essential hypertensive patients.[1,3,4,7,8] Patients with low renin, particularly African Americans, represent a significant number of essential hypertensives and exhibit low efficacy for inhibitors of the renin-angiotensin pathway. African Americans with high blood pressure had a distinct gut microbiota taxonomy and showed marked differences in the function of the microbiota when compared with hypertensive White Americans.[9] However, in renin-independent hypertensive mice, such as the deoxycorticosterone acetate (DOCA)-salt-induced hypertension model, characterized by a depressed renin-angiotensin system due to a high sodium retention rate,[10] there are no significant changes in F/B.[5] Studies using fecal microbiota transplantation from hypertensive patients or rats with genetic hypertension to germ-free mice[4] or normotensive rats[11-13] demonstrated that gut microbiota modulates blood pressure. In addition, our group has demonstrated the key role of T-cell activation in the gut immune system and vascular accumulation of T lymphocytes in the hypertensive response induced by the transplantation of fecal microbiota from SHR to WKY rats.[12] In the DOCA-salt hypertensive rats, T-cells play also a key role in the development of endothelial dysfunction, vascular remodeling, and high blood pressure,[14,15] but if the modulation of gut microbiota could alter T-cell activation and reduce blood pressure in this low-renin model is unknown.

Gut microbiota is able to communicate with the host by producing numerous metabolites that act locally on the gut wall or may pass into systemic circulation and cause biological effects.[16] The signaling molecules are metabolic products from bacteria, such as short chain fatty acids (SCFAs),[17] or bacterial wall elements like lipopolysaccharides (LPS).[18] As a matter of fact, hypertension-related gut dysbiosis is characterized by a decrease in acetate- and butyrate-producing bacteria and an increase in lactate-producing bacteria populations.[1,12] SCFAs can stimulate host G-protein-coupled receptor pathways that impact renin secretion and blood pressure regulation.[17] In fact, butyrate attenuates angiotensin II-induced hypertension in mice[19] and SHR,[8] acetate supplementation or a diet rich in fiber, which significantly raises SCFAs production by gut bacteria, such as acetate, prevented the development of hypertension in DOCA-salt mice,[5] and propionate supplementation exhibited antihypertensive effects in angiotensin II-infused mice.[20] Increased bacterial production of SCFAs has been linked to lower numbers of circulating CD4+ immune cells[21] and its antihypertensive effects were Treg-dependent.[8,20] Bacterial LPS, through toll-like receptor (TLR)-4 activation, contributes to high blood pressure and low-grade vascular inflammation in the SHR model.[8,22]

Thus, gut microbiota is potentially intertwined functionally to blood pressure regulation. In fact, it has been demonstrated through a meta-analysis that a significant decrease in blood pressure in patients treated with probiotics.[23,24] Moreover, a positive effect of probiotics for SHR with established hypertension in vascular protection and blood pressure control has already been described.[8,24-28] Nonetheless, whether probiotics consumption is able to prevent the rise in blood pressure in mineralocorticoid-induced hypertension, and the possible role of SCFAs in this is still unknown. *Bifidobacterium* is commonly considered a beneficial bacterial genus and plays a critical role in the maturation and regulation of the immune system.[29,30] We hypothesized that oral supplementation with *Bifidobacterium breve* CECT7263 (BFM) would be capable of preventing the rise in blood pressure in DOCA-salt by a shift in SCFAs-producing bacteria populations, thus, modulating gut communication with local secondary lymph organs

and/or distal organs. Hence, the aim of this study was to evaluate if BFM would prevent cardiovascular pathology and attenuate hypertension in DOCA-salt rats.

2 | MATERIALS AND METHODS

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

2.1 | Preparation and administration of the probiotic

Biosearch, S. A. (Granada, Spain) provided both BFM and *Lactobacillus fermentum* CECT5716 (LC40). For the probiotic treatment, the lyophilized bacteria were aliquoted and stored at −20°C until usage, at that moment, they were resuspended in tap water and administered by oral gavage.[8]

2.2 | Animals and experimental groups

Male Wistar rats (210-260 g) were obtained from Envigo Laboratories (Barcelona, Spain). A 2-week adaptation period for vehicle administration and SBP measurements was established prior to the initiation of experimental procedures. Animals were randomly divided into five groups: control, DOCA-salt, treated DOCA-salt-BFM (10^9 colony-forming units (CFU) day^{-1} in 1 mL by oral gavage), treated DOCA-salt-butyrate (0,5 mg kg^{-1} day^{-1}), treated DOCA-salt-acetate (100 mM) both in drinking water, for 5 weeks. We did not include groups of treatment in normotensive animals because none of the treatments were able to change blood pressure in normotensive WKY rats.[8] In an additional experiment, the effects of LC40 (10^9 CFU day^{-1} in 1 mL by oral gavage) were also studied. The selected doses of probiotics and SCFAs were similar to those previously utilized to decrease blood pressure in SHR.[8,25] DOCA-salt hypertension was induced by intramuscularly DOCA administration at a dose of 12.5 mg/0.5 mL per rat and week in uninephrectomized animals for 5 weeks, as previously described.[31] A sham operation was performed on the control counterparts and received a weekly saline injection. During the experimental period, DOCA-salt-treated rats had free access to water containing 1% NaCl. Rats were maintained in a specific pathogen-free environment at University of Granada Biological Services Unit, were kept in individual ventilated cages at a constant temperature (24 ± 1°C), with a 12-hour dark/light cycle, and were provided with water and standard laboratory diet (SAFE A04, Augy, France) ad libitum. In order to avoid horizontal transmission of the microbiota among animals, each rat was housed in a separate cage. Water was changed every day, and both water and food consumption were documented daily for all groups. Weekly, body weight was recorded.

2.3 | Blood pressure measurements

Systolic blood pressure (SBP) was measured weekly by tail-cuff plethysmography at room temperature, and, at the end of the experiment, by direct register in carotid artery, as described previously.[12]

2.4 | Cardiac and renal weight indices

After the experimental period, 18-hour fasting rats were anaesthetized with 2.5 mL/kg equitensin (ip) and blood was collected from the abdominal aorta. Finally, the rats were killed by exsanguination. The colon, kidneys, and ventricles were then removed and weighed. The heart was divided into right ventricle and left ventricle plus septum, and the cortex was excised of the rest of the kidney.

2.5 | Plasma and fecal determinations

After the treatment was finalized, rats were killed under isoflurane anesthesia. Blood samples were collected in heparinized tubes, chilled on ice and centrifuged for 10 minutes at 3500 rpm at 4°C, and the plasma frozen at −80°C. Plasma potassium was measured with an auto-analyzer (Beckman, model CX4, Fullerton, California, USA). Plasma LPS concentration was measured with the Limulus Amebocyte Lysate chromogenic endotoxin quantitation Kit (Lonza, Valais, Switzerland), according to the instructions of the manufacturer.

Plasma levels of SCFAs were measured by Nuclear Magnetic Resonance (NMR), as described previously.[8] Spectral processing was performed using the "Metabonomic" R package.[32] SCFAs concentrations in the feces were quantified by gas chromatography.[8]

2.6 | Vascular reactivity studies

Segments of thoracic aortic rings were mounted in organ chambers with Krebs solution (in mmol/L: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2, and glucose 11) at 37°C and gassed with 95% O_2 and 5% CO_2 and maintained at 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.[12] The concentration-relaxation response curves to acetylcholine (10^{-9} -10^{-5} mol/L) were performed in rings pre-contracted with 10^{-6} mol/L phenylephrine, in absence or presence of the NADPH oxidase inhibitor VAS2879 (5 μmol/L), or the endothelial NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) for 30 minutes. In addition, concentration-relaxation response curves to nitroprusside (10^{-9}-10^{-6} mol/L) were performed in rings without endothelium protected against light precontracted by 1 μmol/L phenylephrine. Vasorelaxing responses were expressed as a percentage of precontraction. The presence of functional endothelium was considered by the ability of acetylcholine (1 μM) to induce more than 50% relaxation in vessels maximally precontracted with phenylephrine. Vessels were considered to be denuded of functional endothelium when there was no relaxation response to acetylcholine.

2.7 | NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assays were used to determine NADPH oxidase activity in intact aortic segments, as previously described.[12] NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic tissue.

2.8 | Flow cytometry

MLNs were collected from rats. The tissues were mashed with wet slides to decrease friction, and then, the solutions were filtered through a 70 μm cell strainer. 1×10^6 cells were counted and incubated with a protein transport inhibitor (BD GolgiPlug) for an optimum detection of intracellular cytokines by flow cytometry, as previously described.[8] 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 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 (20 U/mL) for 60 minutes at 37°C.[33] 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 already described.

Antibodies for staining were purchased 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); CD44-PE-Vio770 (clone REA505); and viability dye (LIVE/DIED Fixable Aqua Dead cell Stain Kit, Molecular Probes, Oregon, USA).

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

For RT-PCR analysis, total RNA was extracted from the colon, aortae, and mesenteric lymph nodes by homogenization and converted to cDNA by 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, as described previously.[8] The sequences of the sense and antisense primers used for amplification are described in Table S1. The housekeeping gen glyceraldehyde-3-phosphate dehydrogenase was used for internal normalization.[12]

2.10 | 16S rDNA V4-V5 region sequencing

Fecal DNA was extracted from the samples collected from all experimental groups by using quick-DNA fecal/soil microbe kit (Zymoresearch, Irvine, CA). Primers compatible with illumina Miseq v2 2x250bp kit (Illumina, San Diego, CA) were used to amplify bacterial 16S V4-V5 variable regions.[1] The PCR amplicons were purified by QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and quantified by Qubit (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.

2.11 | Bioinformatics analysis

The raw paired-reads from Miseq were processed using QIIME 1.9.1. Briefly, reads were trimmed to remove bases with Phred score lower than 30 and quality-filtered with parameters set as previously optimized.[34] 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. Bacteria were classified based on the SCFAs end-product as previously described.[35,36] Bacteria were classified based on the capacity to produce TMA according to the classification of potential TMA-producing bacteria described previously.[37]

The metagenomic prediction was performed by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt). Predicted metagenomes were subsequently subjected to collapse predictions into Kyoto Encyclopedia of Genes and Genomes pathways (KEGG). The obtained data were presented as relative abundance of predicted functions within the samples. The abundance of KEGG modules was calculated by summing the abundance of genes annotated to the same feature. The interest pathways were presented as relative abundance of predicted functions within the samples. The rest of the obtained data was used to analyze the variation among the groups represented by z-score analyses.

2.12 | Chemicals

All chemicals, unless otherwise specified, were obtained from Sigma (Alcobendas, Madrid, Spain), and were dissolved in distilled deionized water.

2.13 | Statistical analysis

The Shannon, Chao, Pielou, and observed species whole indexes were calculated using QIIME (PAST 3x). Reads in each OTU 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 analysis was also 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.[38] 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 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. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | BFM induced changes in gut microbiota in DOCA-salt rats

The composition of bacterial communities was evaluated by calculating three major ecological parameters, Chao richness, Shannon diversity, and Pielou evenness. No significant differences among all experimental groups were found (Figure S1). Phyla composition analysis (Figure 1A, Table S2) showed that Firmicutes and Bacteroidetes were the most prevalent phyla in feces from control animals. Moreover, the proportion of bacteria from the Firmicutes and Bacteroidetes and F/B ratio (Figure 1B) were unchanged by DOCA-salt treatment. However, bacterial community belonging to Actinobacteria phylum was ≈4.8-fold higher in DOCA-salt than in control. As anticipated, treatment with BFM further increased its levels by ≈56% since BFM belongs to Actinobacteria phylum. The F/B ratio was not significantly modified by any treatment in DOCA-salt rats. However, significant increases in acetate-, butyrate-, and lactate-producing bacterial communities (≈2.5, 23, and 3.6, respectively) without changes in propionate-producing bacteria were observed in DOCA-salt as compared to control group (Figure 1C).

Next, we examined effects of BFM and acetate supplementation. Both treatments increased acetate-producing bacteria in DOCA-salt rats by ≈1.8- and 3.3-fold, whereas acetate normalized the proportion of lactate-producing bacteria. In contrast, butyrate treatment did not change SCFAs-producing bacterial species in the gut. Acetate levels in feces were increased 2.2-fold in DOCA-salt as compared to control rats, and it was increased ≈4.1, and 5.1 by BFM, and acetate treatment, respectively. However, butyrate treatment did not change the acetate levels in feces. Butyrate and propionate levels in feces were similar between control and DOCA-salt rats. BFM treatment reduced by ≈80% butyrate concentration (Figure 1D).

Linear discriminant analysis effect size (LEfSe) showed prominent changes in bacterial taxa, relative abundance of 12 taxa was increased while 6 taxa were reduced in DOCA-salt when compared to the control group (Figure S2A). BFM treatment of hypertensive rats also provoked changes in the microbiota taxa compared to DOCA, where the relative abundance of a single taxon (*Ruminococcus_Other*) was increased while and 4 taxa *(Ruminococcus, Clostridiaceae, Clostridium,* and *S24_7*) decreased (Figure S2B). However, acetate induced higher changes in bacterial taxa, increasing 9 taxa and 17 taxa with relative lower abundance than DOCA group (Figure S2C).

At the genus level, *Blautia* and *Peptostreptococcaceae;g_* were increased by ≈5.8 and 6.4 fold, respectively; *Rikinellaceae;g_* was reduced by ≈−74% in DOCA as compared

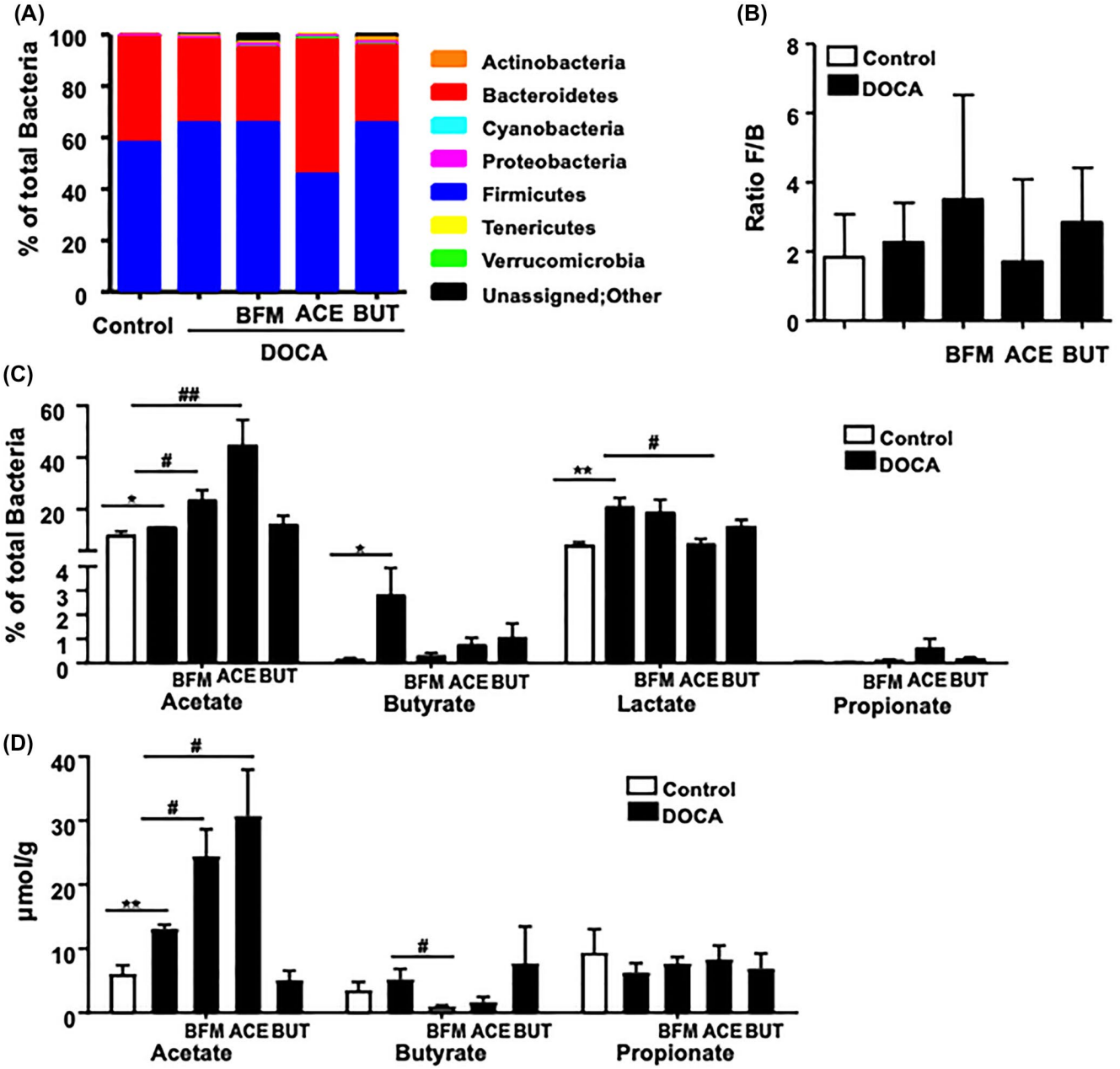

FIGURE 1 BFM induced changes in gut microbiota in DOCA-salt rats. Bacterial 16S ribosomal DNA were amplified and sequenced to evaluate the composition. Phylum breakdown of the seven most abundant bacterial communities in samples from all experimental groups (A). The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker for gut dysbiosis (B). Acetate-, butyrate-, lactate-, and propionate-producing bacteria expressed as relative proportions (C) and the concentration of acetate, butyrate, and propionate (D) in stool samples was determinate in the gut microbiota in Control and DOCA-salt groups. Results are shown as mean ± SEM (n = 6). $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the nontreated DOCA group. BFM, *Bifidobacterium breve* CECT7263

to control. BFM treatment reduced *Peptostreptococcaceae;g_*, at level similar to control group, whereas acetate treatment reduced *S24-7;g_*, *Blautia* and *Prevotella* and increased *Bacteroides* (Figure S3).

Finally, we evaluated gut microbial functions across experimental groups and expressed as z-score using KEGG database (Figure S4). However, no significant differences were observed among groups in KEGG modules. The capacity to synthetize and export LPS of the gut microbiome was increased in pre-hypertensive and hypertensive subject.[4] However, in our study, modules involves in LPS biosynthesis tended to decrease in DOCA-salt group, whereas that involves in LPS export tended to increase in DOCA-salt as compared to control animals.

3.2 | BFM improved intestinal integrity, reduced endotoxemia, and circulating lactate and trimethylamine levels in DOCA-salt rats

Hypertension is linked to alteration in gut tight junction proteins expression, increased permeability and gut pathology.[39] We observed reduced mRNA levels of barrier-forming junction proteins zonula occludens-1 (ZO-1) by ≈−70% in the colon of DOCA-salt rats compared to control, without any change in occludin expression (Figure 2A). Both BFM and acetate, but not butyrate, increased occludin and ZO-1 mRNA levels in the colon, suggesting preserved barrier integrity. Increased gut permeability has been associated with low numbers of goblet cells,[39] which produce mucins, protective agents against pathogen invasion in the gut, and thus, modulating the gut immune response.[40] Our data also showed downregulation of the principal structural component of the mucus layer, mucin (MUC)-2, by ≈−95%, and also of MUC-3 transcripts by ≈−96% in DOCA-salt rats (Figure 2B). BFM significantly increased MUC-3 mRNA levels, which were unaffected by both SCFAs. We measured endotoxin levels in plasma, and found them to be 2.7 ($P < .01$)-fold higher in DOCA-salt rats compared with the control group (Figure 2C). Interestingly, long-term treatment with BFM and acetate significantly prevented endotoxemia in DOCA-salt animals. These results point to an increase in intestinal permeability in DOCA-salt and allow bacterial components (eg, LPS) to be absorbed into the systemic circulation. Additionally, the colonic expression of IL-18 (Figure 2D), a cytokine important for tissue repair[41] and limiting colonic Th17 cell differentiation,[42] was ≈80% lower in DOCA-salt when compared to control rats. BFM and acetate treatments increased mRNA levels of IL18 in colon.

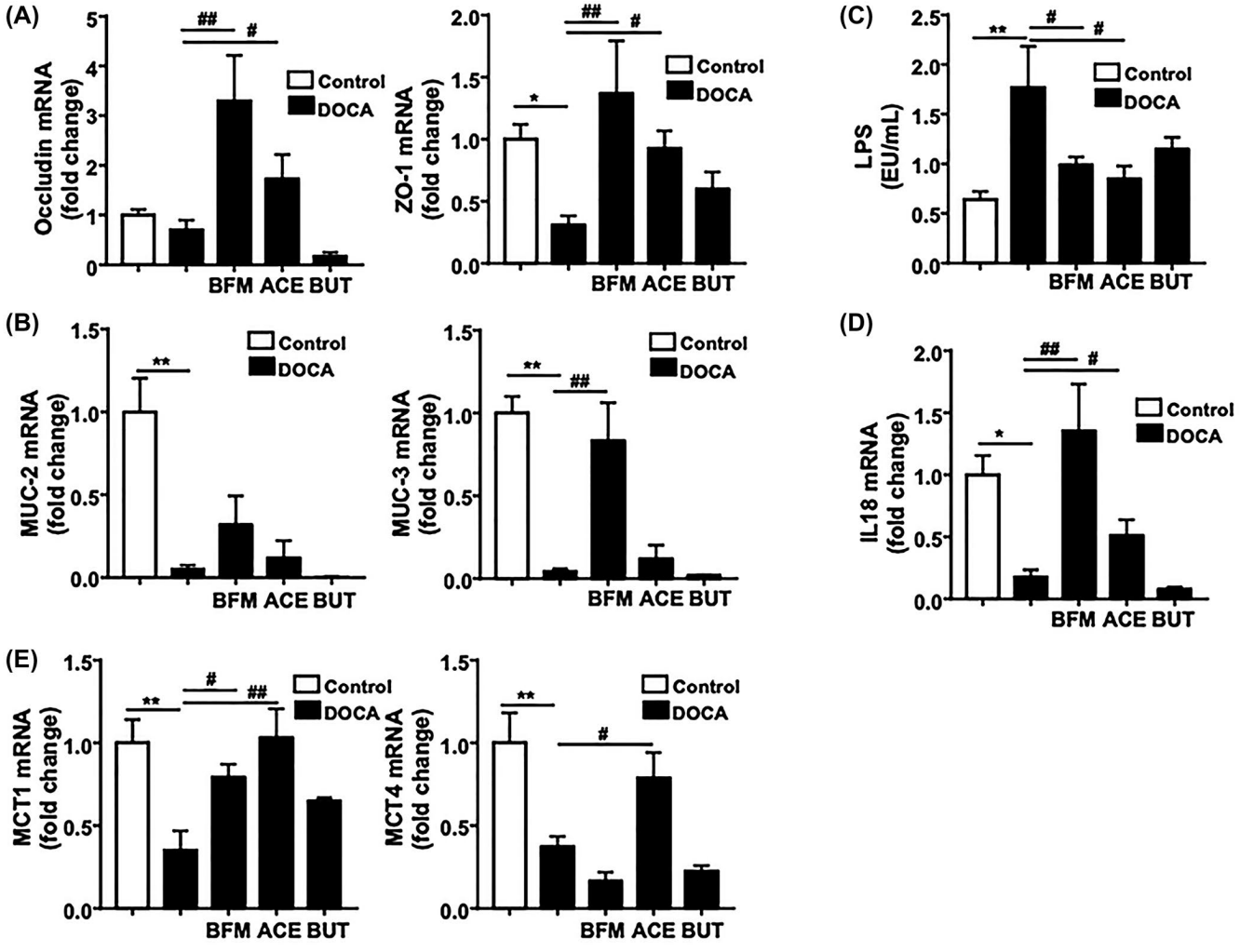

FIGURE 2 BFM improved intestinal integrity, reduced endotoxemia in DOCA-salt rats. Colonic mRNA levels of occludin, and zonula occludens-1 (ZO-1) (A). Mucin (MUC)-2, and MUC-3 (B). Plasma endotoxin concentrations (EU/mL, endotoxin units/mL (C). Expression of the short-chain fatty acids (SCFAs) transporters, MCT-1, and MCT-4 (D). Tissue repair cytokine IL-18 (D) was determinate in the gut microbiota in Control and DOCA-salt groups. Results are shown as mean ± SEM (n = 8). $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the non-treated DOCA group. BFM, *Bifidobacterium breve* CECT7263

Next, we analyzed expression levels of monocarboxylate transporter (MCT)1 and MCT4, key transporters of SCFAs in the colon. Both MCT1 and MCT4 transcription levels were decreased by ≈−60% in DOCA group as compared to control (Figure 2E). BFM increased MCT-1 and acetate increased both MCT-1 and MCT-4 colonic transcript levels.

Finally, a metabolomics study was performed in plasma from all experimental groups. We detected a total of 33 metabolites in plasma by NMR spectra (Figure 3A). Principal component analysis of the plasma metabolomics data indicates that the control and DOCA-salt groups generally cluster separately from one another and that DOCA-salt rats treated with BFM and acetate are closer to control than DOCA group (Figure S5). DOCA-salt showed significant higher concentration of 2-aminobutyrate, 2-hydroxyvalerate, 3-hydroxyisobutyrate, citrate, myo-inositol, pyruvate, and thymidine than control group (Figure 3A). Plasma levels of SCFAs acetate and lactate were measured from the NMR spectra, whereas butyrate levels were not detected. Lactate was increased by ≈2.9-fold ($P < .05$) in plasma from DOCA-salt when compared to control without changes in acetate levels (Figure 3B). Acetate treatment doubled plasma acetate levels, and both BFM and acetate restored lactate to values similar to control group. Interestingly, trimethylamine (TMA), a gut bacteria product that increased arterial blood pressure in rats, was ≈2.8-fold higher in DOCA-salt than control group, and normalized by both BFM and acetate treatment (Figure 3C). However the proportion of TMA-producing bacteria was unchanged among groups (Figure S6A), whereas pathways to generate TMA, such as betaine reductase and TMAO reductase, tended to increase in DOCA-salt rats, and pathways to generate methane from TMA was unchanged in hypertensive rats (Figure S6B). Acetate treatment increased significantly KEGG module of methanogenesis. Plasma creatinine concentration, a marker of kidney dysfunction, was increased ≈4.3-fold in DOCA-salt as compared to control group. This increase was prevented by BFM and acetate, but not by butyrate treatment (Figure 3D). Plasma K^+ levels were significantly reduced in DOCA-salt rats as compared to control (Figure S7). No treatment was able to modify hypokalemia induced by DOCA-salt.

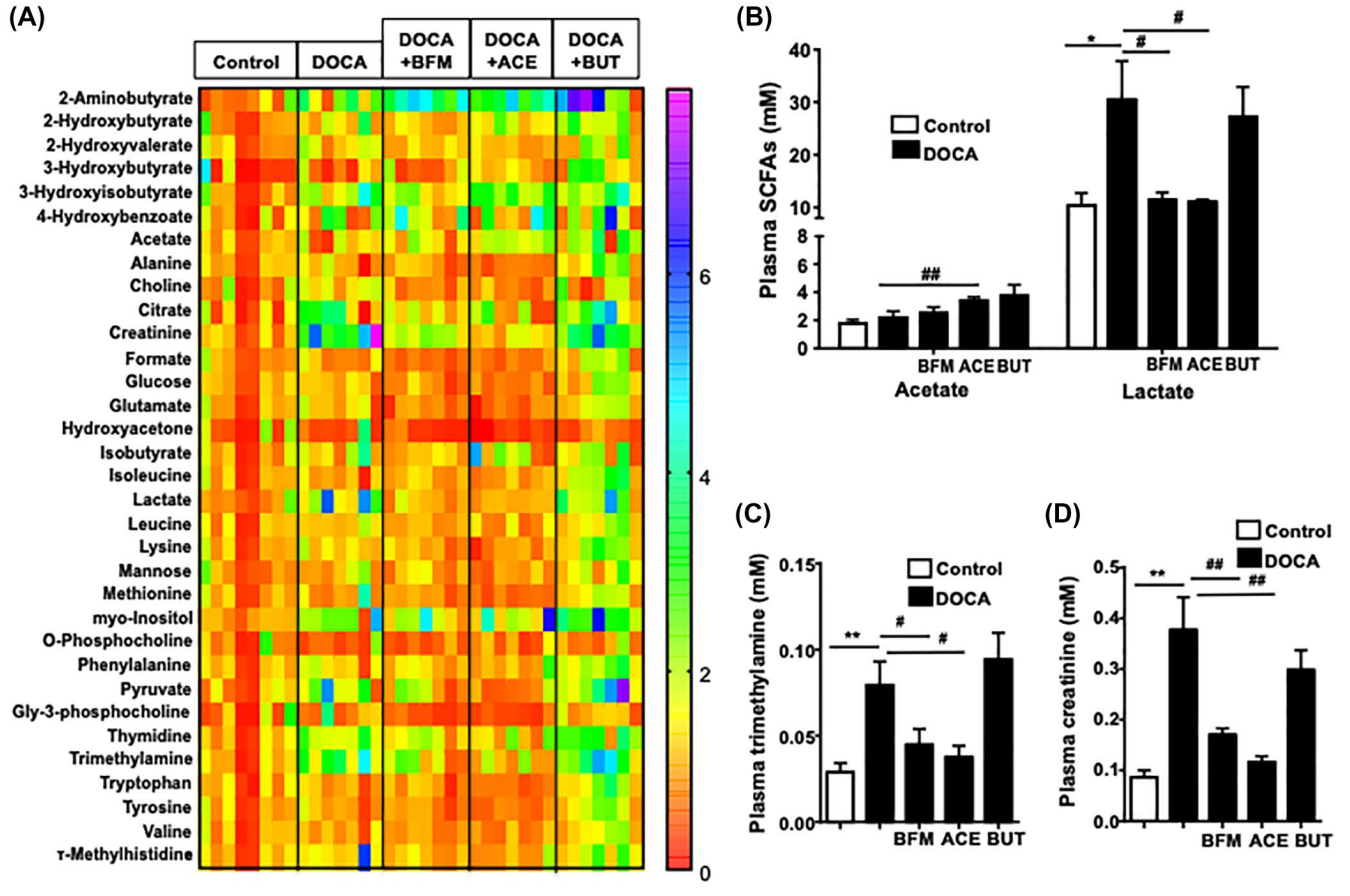

FIGURE 3 BFM reduced circulating lactate and trimethylamine levels in DOCA-salt rats. The plasma levels of 33 metabolites were measured by NMR spectra (A) SCFAs (B), Trimethylamine (TMA) (C), and creatinine (D) from the Nuclear Magnetic Resonance (NMR) spectra. Results are shown as mean ± SEM (n = 7-8). $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the nontreated DOCA group. BFM, *Bifidobacterium breve* CECT7263

3.3 | BFM restored changes in T-cell populations of mesenteric lymph nodes (MLNs) in DOCA-salt rats

Bacteria might translocate through the intestinal epithelium under altered conditions in gut mucosal integrity, which may lead to activation and migration of $CX3CR1^+$ cells, such as dendritic cells and macrophages, to draining lymph nodes of the lower intestinal tract.[43] Additionally, they are able present soluble antigens to naïve CD4+ T-cells, leading to T-cell activation. We found that the number of total T lymphocytes in MLNs was similar in DOCA-salts compared to control rats (Figure 4). No change was observed by treatments in total T-cells from this secondary lymph organ. However, the Th17 ($CD4^+IL\text{-}17^+$) was increased by ≈2.8-fold ($P < .05$), while Treg ($CD4^+FoxP3^+$) lymphocytes were reduced by ≈−44% ($P < .05$) in MLNs from DOCA-salt group compared to control. BFM and acetate reduced Th17 and increased Treg in MLNs (Figure 4). Butyrate was unable to change the polarization of T-cells in this lymph organ.

3.4 | BFM treatment improved vascular function, oxidative stress, and T-cells infiltration in DOCA-salt rats

The contractile response induced by phenylephrine was higher in endothelium-intact aorta from DOCA-salt rats than from control animals (Emax = 3.30 ± 0.26 g *vs* 2.49 ± 0.27 g, $P < .05$) (Figure 5A). BFM treatment reduced the phenylephrine-induced contraction (Emax = 2.18 ± 0.40 g, $P < .05$ *vs* DOCA group). However, both SCFAs were unable to change significantly this contractile response. Aortae from control DOCA-salt showed significant reduced endothelium-dependent vasorelaxant responses to acetylcholine in arteries stimulated by phenylephrine as compared with aortae from control rats (Emax = 51.7 ± 6.1% vs 84.4 ± 3.3%, respectively, $P < .01$). BFM and acetate produced a significantly higher relaxation capacity by acetylcholine in DOCA-salt rats (Emax = 74.1 ± 7.4%, and 68.8 ± 4.3%, respectively, $P < .05$ vs DOCA group) (Figure 5B). The relaxation response induced by acetylcholine was fully inhibited by L-NAME in all experimental groups (data not shown), showing that in aorta, acetylcholine-induced relaxation in both control and DOCA-salt was entirely dependent on endothelium-derived nitric oxide. The endothelium-independent vasorelaxant responses to nitroprusside, which directly activates soluble guanylyl cyclase in vascular smooth muscle, displayed no significant differences among groups (data not shown). Interestingly, LC40 treatment did neither change the contractile response induced by phenylephrine nor the acetylcholine-induced relaxation in DOCA-salt rats (Figure S8C,D).

NADPH oxidase-driven reactive oxygen species (ROS) production is a key event in endothelial dysfunction in DOCA-salt hypertension.[31] In fact, we found that the presence of the selective NADPH oxidase inhibitor VAS2870 in the organ bath increased the relaxant response to acetylcholine in untreated DOCA-salt, reaching similar relaxation levels seen in control. In the presence of this agent, no differences were observed among all groups of treatment, when compared to DOCA-salt rats (Figure 5B). Consistent with this is the observation that NADPH oxidase activity was higher by ≈41% in aortic segments from DOCA-salt than in control rats (Figure 5C). Chronic treatments with BFM and acetate prevented this increase in NADPH oxidase activity in DOCA-salt animals. However, neither butyrate (Figure 5C) nor LC40 (Figure S8E) treatment changed this activity. Significant increase in mRNA levels of NADPH oxidase subunits, NOX-4, and $p22^{phox}$ (≈3, and 2.7-fold, respectively) was observed in aortic tissue from DOCA-salt as compared with control rats (Figure 5D). Again,

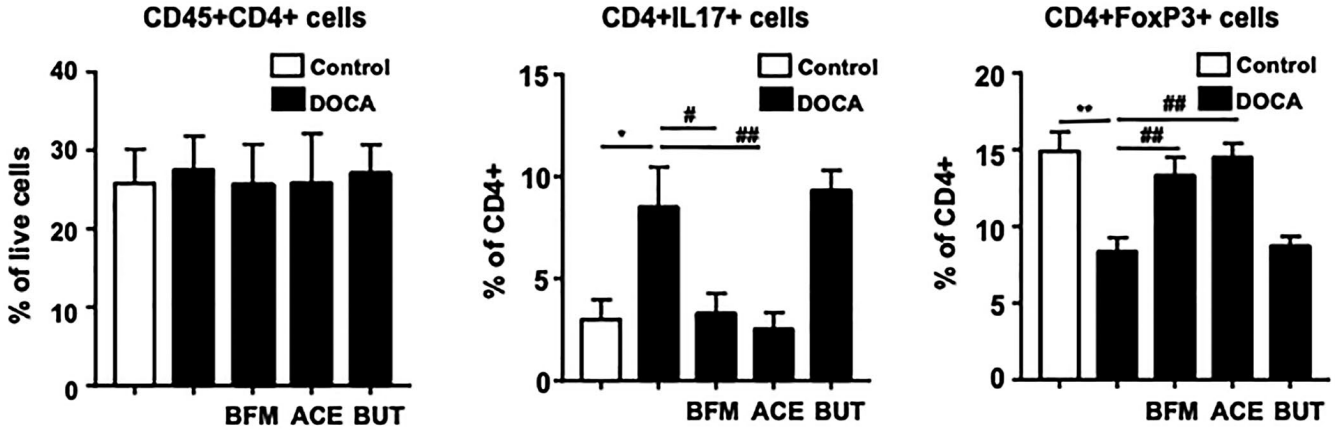

FIGURE 4 BFM restored changes in T-cell populations of MLNs in DOCA-salt rats. Total T-cells (CD45+CD4+), T-helper (Th)17 cells (CD4+IL17+), and regulatory T (Treg) cells (CD4+FoxP3+) measured in mesenteric lymphoid nodes in Control and DOCA-salt groups. Results are shown as mean ± SEM (n = 7-8). $^*P < .05$ and $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the nontreated DOCA group. BFM, *Bifidobacterium breve* CECT7263

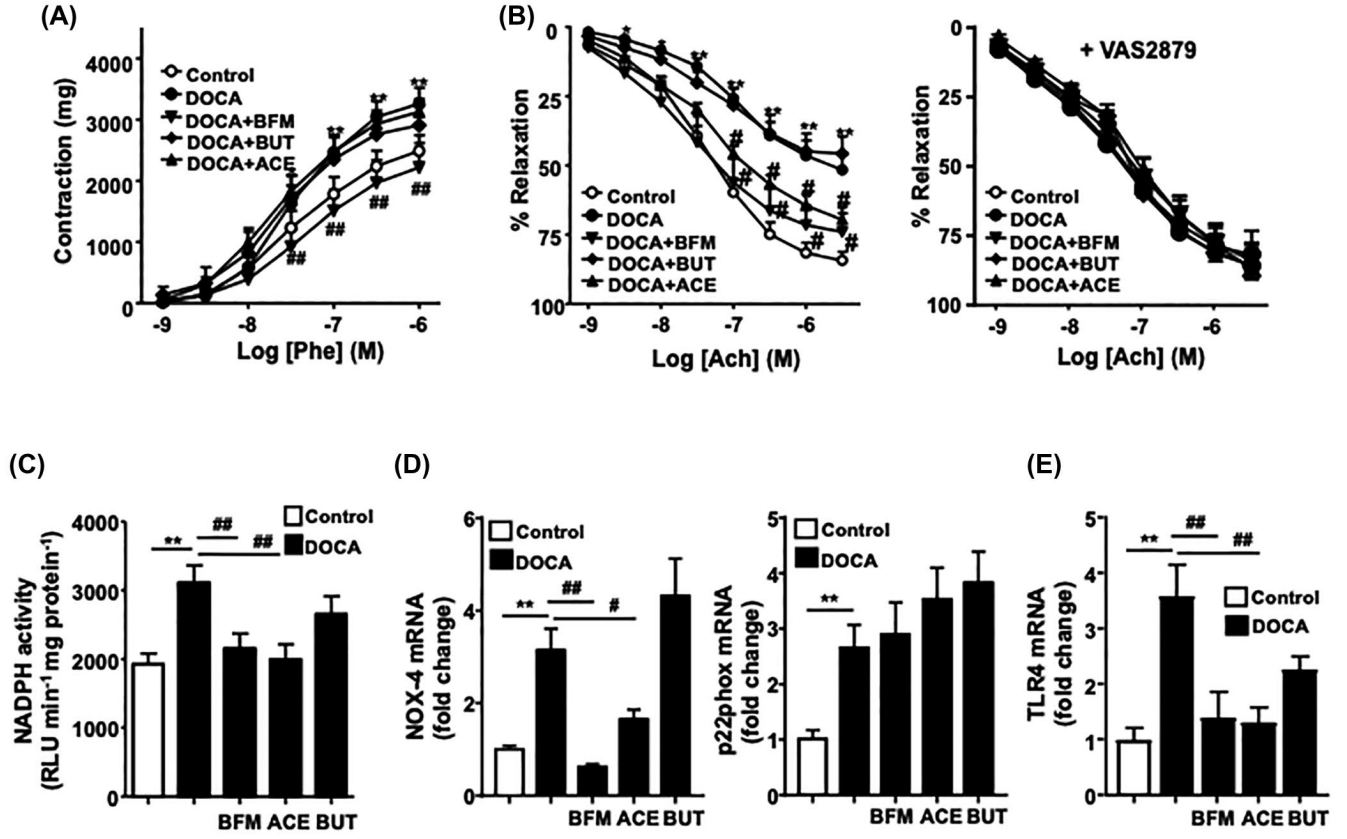

FIGURE 5 BFM treatment improved vascular function, oxidative stress in DOCA-salt rats. The contractile response induced by phenylephrine (A) Vascular relaxant responses induced by acetylcholine (Ach), in endothelium-intact aortae pre-contracted by phenylephrine (Phe) in the absence and in the presence of the NADPH oxidase inhibitor VAS2870 (5 μM) (B) in Control and DOCA-salt groups. NADPH oxidase activity measured by lucigenin enhanced chemiluminescence (C), and aortic mRNA levels expression of NADPH oxidase subunits NOX-4 and $p22^{phox}$ (D), and TLR-4 (E), in Control and DOCA-salt groups. Results are shown as mean ± SEM (n = 8). $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the nontreated DOCA group. BFM, *Bifidobacterium breve* CECT7263

both BFM and acetate reduced the gene expression of catalytic subunit NOX4 in DOCA-salt rats. TLR4 mRNA levels in aortic homogenates were ≈3.6-fold higher in DOCA-salt group as compared with control (Figure 5E), which were reduced by both BFM and acetate treatments.

Gut microbiota is an important factor involved in the control of blood pressure, as a consequence of its effects on T-cell activation in the gut immune system and in vascular T-cells accumulation.[12] We found reduced the Treg and increased Th17 cells in aortas from DOCA-salt as compared to control rats (Figure 6). BFM and acetate restored the aortic Treg and Th17 accumulation to levels similar to control.

3.5 | BFM prevented the rise in blood pressure, and both cardiac and renal hypertrophy in DOCA-salt rats

Five weeks of DOCA-salt treatment increased SBP by ≈39 mm Hg. Chronic BFM or acetate consumption were able to prevent significantly ($P < .01$) the development of hypertension (by 38% and 34%, respectively), whereas butyrate did not change the time course of SBP (Figure 7A). These observations on attenuation of SBP were confirmed by direct register in the carotid artery at the end of the experiment (Figure 7B). Left ventricle weight (Figure 7C) and kidney weight (Figure 7D) relative to tibia length were higher (≈33% and 2.4-fold, respectively) in DOCA-salt group as compared with control rats. Treatment with BFM and acetate, but not with butyrate, significantly reduced both indices. Kidney damage in DOCA-salt rats, measured by protein excretion, was also prevented by both BFM and acetate treatments (Figure 7E). Interestingly, LC40 treatment was unable to prevent the rise in SBP and in cardiac and renal weight indices in DOCA-salt (Figure S8A,B).

4 | DISCUSSION

The most significant aspect of this study is that it presents evidence of gut microbiota initiated mechanism of beneficial effects of probiotic consumption on a low renin form of

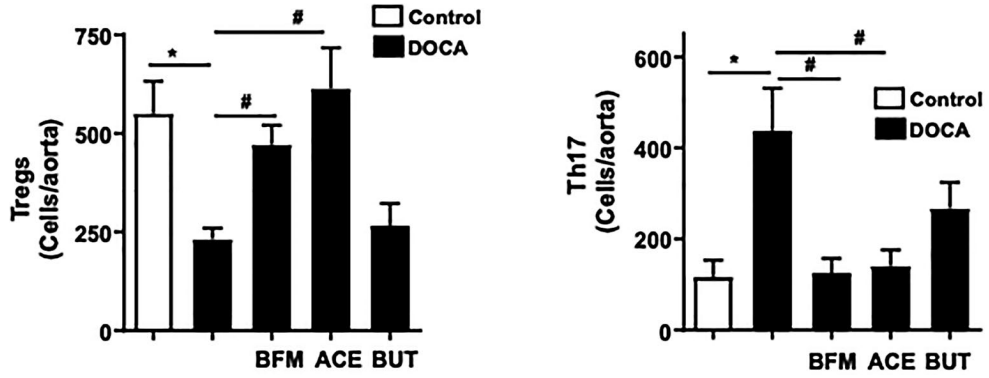

FIGURE 6 Effects BFM treatment in T-cells infiltration in DOCA-salt rats. Treg and Th17 cells in aortas from Control and DOCA-salt. Results are shown as mean ± SEM (n = 6-7). $^{*}P < .05$ compared with Control group. $^{\#}P < .05$ compared with the non-treated DOCA group. ACE, acetate; BFM, *Bifidobacterium breve* CECT7263; BUT, butyrate

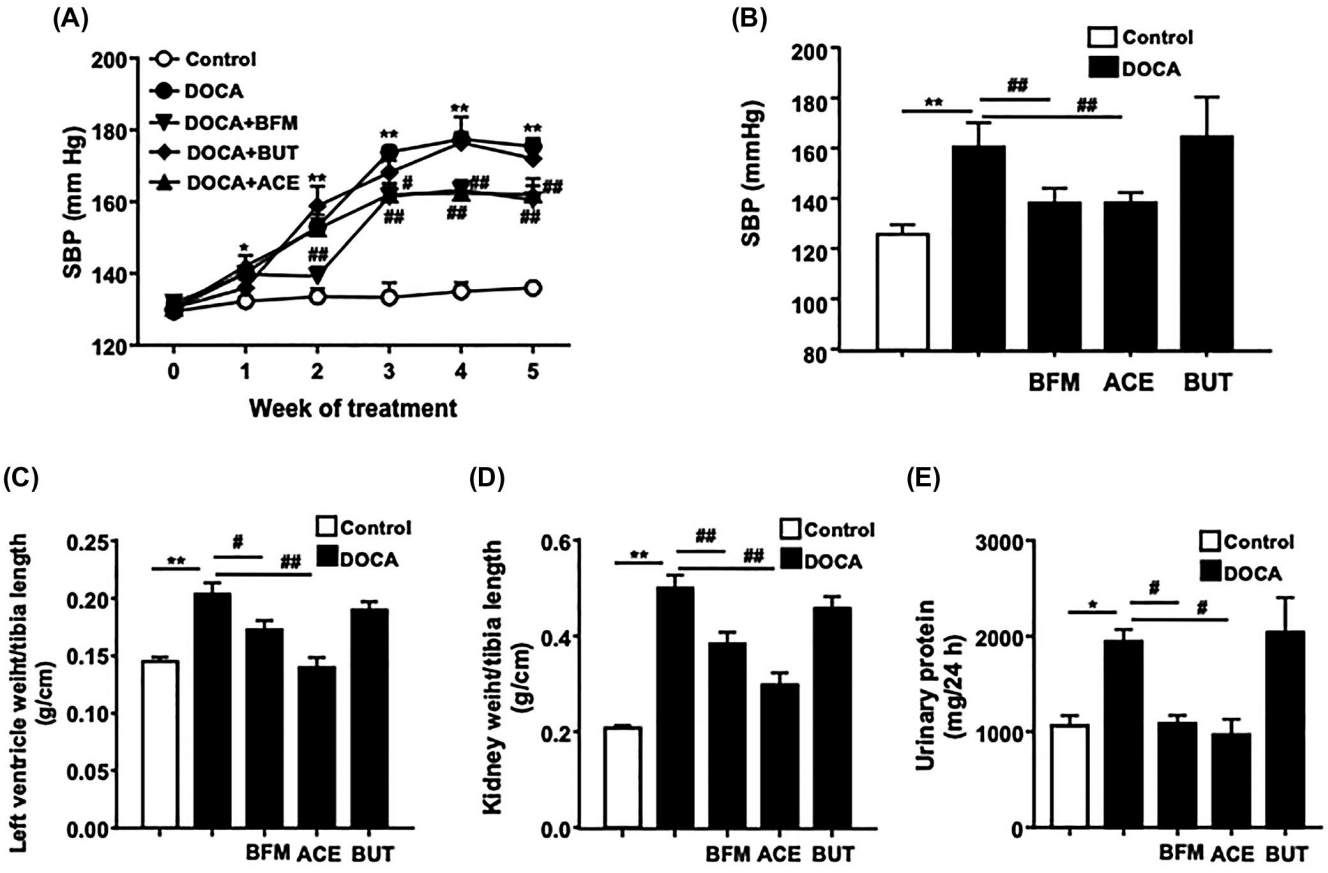

FIGURE 7 BFM prevented the rise in blood pressure, and both cardiac and renal hypertrophy in DOCA-salt rats. Time course of systolic blood pressure (SBP), measured by tail-cuff in all experimental groups (A) and measured by intra-arterial recording into left carotid artery (B), at the end of the experimental period. Morphological data in the left ventricle hypertrophy (C), and kidney (D) and 24 hours proteinuria (E). Results are shown as mean ± SEM (n = 8-10). $^{**}P < .01$ compared with Control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the nontreated DOCA group. BFM, *Bifidobacterium breve* CECT7263

hypertension. Thus, it provides first evidence for the involvement of gut microbiota in low renin model and potential of the usefulness of a probiotic-based strategy for the control of hypertension in this class of hypertensive patients. The main new findings of this study are as follows: (1) chronic treatment with the probiotic BFM, but not with LC40, prevented both the increase in blood pressure and the target organ (heart, kidney, and aorta) damage induced by DOCA-salt administration; (2) oral supplementation with the SCFA acetate also prevented these changes in DOCA-salt animals;

(3) both BFM and acetate increased acetate-producing bacteria and acetate content in the gut; (4) BFM and acetate treatments improved colonic integrity, normalized endotoxemia and plasma TMA levels, and restored the Th17 and Treg content in MLNs; (5) BFM and acetate prevented the impairment of endothelium-dependent relaxation to acetylcholine, due to a decreased NADPH oxidase-driven ROS production; and (6) both treatments reduced Th17 and increased Treg infiltration in the vasculature of rats with mineralocorticoid-induced hypertension.

Our observation of gut dysbiosis in hypertension is supportive of emerging concept for the involvement the gut in blood pressure regulation. Low-renin hypertension is expression of a physiological response to sodium-volume overload, such as DOCA-salt rats. A proper diagnosis, primarily based on an accurate family history and the concomitant evaluation of plasma renin, aldosterone, and potassium, is important to targeted therapy. Hypokalemia is characteristic of this low renin model of hypertension, derived from kidney mineralocorticoid receptor activation. BFM treatment was unable to change plasma K^+ levels, suggesting no interaction with the mineralocorticoid receptor pathway. However, what is novel here is the evidence of unique changes in gut microbial populations in low renin model of hypertension. The features of the gut microbiota from DOCA-salt rats are different that than found in SHR,[8,11-13] angiotensin II-infused mice,[1,19,39,44,45] or hypertensive patients.[1,4] The main characteristics of dysbiotic microbiota in DOCA-salt were: (a) microbial richness, evenness, and diversity were similar to control rats; (b) no significant change in F/B ratio were observed, and (c) an increased proportion in acetate-, butyrate-, and lactate-producing bacteria were observed. Interestingly, BFM increased acetate producing-bacteria and acetate content in feces, reduced blood pressure, renal and cardiac weight indices, improved glomerular function (lower plasma creatinine) and injury (lower urine protein excretion) and endothelial dysfunction. Similarly, increased acetate-producing bacteria induced by high-fiber diet or acetate supplementation were also associated with decreased blood pressure, improvement of cardiac function and attenuation of cardiac and renal fibrosis in DOCA-salt mice,[5] suggesting that interventions that increase acetate concentration in the gut are cardioprotective in mineralocorticoid-induced hypertension. BFM, a strain of *Bifidobacterium breve* with probiotic capabilities, did not change the relative abundance of its own genus in the gut, which points to its effect not being just caused by a replacement of other bacteria, but rather to a positive effect on the whole microbiota.

The gut acts as a functional and physical barrier to regulate the passage of pathogens and their structural components into the systemic circulation, by the integrity of epithelial cells, mucus and tight junction proteins. Loss of epithelial integrity and increased permeability has been reported in high blood pressure patients, angiotensin II-infused mice[19] and in SHR.[8,12] We described by first time that mineralocorticoid hypertension is also associated to reduced ZO1 expression and mucins, suggesting loss of gut integrity. In fact, increased plasma levels of LPS were found in DOCA-salt group, which seems to be independent of changes in bacterial LPS biosynthesis and export by gut microbiota. SCFAs have been shown to produce many beneficial effects on the intestinal epithelium, including the inhibition of inflammation,[46] and the modulation of oxidative stress.[47] Furthermore, an improvement in barrier function by SCFAs has already been reported in vitro,[48-50] ex vivo,[51] and in animal studies.[19,52] In agreement with these data, we also found that BFM and acetate supplementation produced an increase in mRNA levels of tight junction protein occludin and ZO-1 in the colon. In addition, IL-18, a cytokine that has been proposed to protect the gut barrier function, was also higher in colon from DOCA-salt treated with BFM or acetate as compared to untreated rats. Overall, our results support that BFM could improve gut integrity by increasing gut acetate level, with the succeeding decreased translocation of bacterial endotoxin into the blood stream. Moreover, intestinal epithelial cells constitutively secrete IL-18, which directly acts on IL-18R1-expressing CD4+ T-cells to determine colonic Th17 cell differentiation.[42] Our results, showing decreased levels in colonic IL-18 expression in DOCA-salt, are consistent with increased levels of Th17 in MLNs from DOCA-salt, and treatments increasing colonic IL-18 significantly reduced Th17 in secondary lymph organs in the gut.

Mineralocorticoid receptor activation alters the Th17/Tregs balance lending to the activation of Th17/IL17 pathway, which is involved in mineralocorticoid-dependent hypertension.[15] In agreement with this, we also found higher Th17 and lower Tregs in MLNs and aorta from DOCA-salt animals compared to control. BFM significantly reduced the proportion of Th17 and increased Treg in MLNs. Interestingly, BFM also reduced Th17 infiltration in aorta and increased Treg accumulation. SCFAs, like butyrate or acetate, can impact immune function in the intestine.[53-55] However, we found that acetate, but not butyrate supplementation, mimicked the effects of BFM in T-cells polarization in MLNs, suggesting that acetate is the mayor mediator of BFM effects in this secondary immune organ.

The pro-inflammatory cytokine IL-17, produced in part by Th17, causes ROS-mediated endothelial dysfunction,[56] whereas Tregs, through IL10 release, attenuates NADPH oxidase activity, which is a critical process in the improvement of vascular endothelial function in hypertension. We found that NADPH oxidase inhibition with VAS2879 suppressed the impaired relaxation to acetylcholine in aorta from DOCA-salt group. Moreover, both BFM and acetate treatments increased Tregs and reduced Th17 infiltration in the aorta, which correlated to reduced vascular NADPH oxidase

activity and improvement of NO-dependent relaxations. By contrast, butyrate consumption was unable to improve endothelial function and T-cells Th17/Tregs in aorta. In addition, bacterial LPS stimulates TLR4 in the vasculature, which resulted in an elevated NADPH oxidase-dependent $O_2^{\cdot-}$ production.[57,58] We also found higher LPS plasma levels and aortic mRNA levels of TLR4 in DOCA-salt than control rats. Thus, endothelial dysfunction in DOCA rats appears to be induced, at least partially, by the gut bacterial product endotoxin. Moreover, all treatments that reduced endotoxemia and aortic TLR4 expression (BFM and acetate) improved the endothelium- and NO-dependent relaxation. However, further studies are needed to examine the role of LPS in endothelial dysfunction and hypertension-induced by DOCA-salt administration.

SCFAs also exert direct protective effect in the endothelium by decreasing endothelial activation induced by LPS or TNFα.[59,60] We found that acetate plasma levels in DOCA-salt rats were similar to control, despite higher proportion of acetate-producing bacteria. This may be linked to the reduced levels of SCFA transporters (MCT1 and MCT4) expressed in gut from DOCA rats. However, acetate consumption increased acetate-producing bacteria, colonic MCTs expression, and circulating levels of acetate, which would be involved in the protective effects of chronic acetate consumption at the vascular wall. Plasma lactate levels have been associated with increased blood pressure.[61] Of particular interest is the finding that lactate-producing bacteria and plasma lactate concentration were increased in DOCA-salt rats, whereas BFM and acetate reduced plasma lactate and prevented the rise of blood pressure. Moreover, TMA have also been implicated in blood pressure regulation. In fact, intravenous infusion of TMA, at concentrations similar to that found in plasma from DOCA-salt rats, produced a significant increase in blood pressure.[62] We also found reduced plasma level of TMA in DOCA-salt rat treated with both BFM and acetate. The effects of acetate might be a consequence of increased conversion of TMA in methanol by gut microbiota, since bacterial genes involved in methanogenesis were increased by acetate consumption.

In conclusion, this study demonstrates, for the first time, that the probiotic BFM prevents the development of endothelial dysfunction and hypertension in DOCA-salt rats. These beneficial effects are mimicked by supplementation with acetate suggesting that the increase in gut acetate induced by BFM is a key event in its cardiovascular protective effect. Moreover, reduction of the endotoxemia, plasma lactate and TMA concentrations seem to be mediators of the vasculoprotective effects of oral consumption of BFM. Furthermore, the reduced Th17 and the increased Tregs accumulation in the vasculature induced by BFM, as a result is effects in MLNs, contribute to protect the vasculature and reduce blood pressure. Interestingly, other probiotic bacteria, such as LC40, which prevented,[8] or reduced blood pressure[25] in SHR, were unable to improve endothelial function and prevent hypertension. Therefore, consideration of unique bacterial communities seems to be an important consideration in designing therapeutic strategies for treatment and control of hypertension with different etiology. African Americans, which tend to have lower renin levels than Caucasians, might be specially protected from hypertension by probiotic treatments, such as BFM. Similarly, the elderly, on average, also have low plasma renin. Recent studies show that aging alters the gastrointestinal microbiome in both human and animals.[63,64] Probiotic treatments may have an even more pronounced effect on hypertension and deleterious consequences of hypertension in older individuals, such as the progressive decline in the volume of memory-associated brain areas with age.[65] However, caution is advised when extrapolating these findings to humans because of the possible differences in the behavior of the animal and human gut microbiota. Further research is needed to evaluate the efficacy and the safety profile before probiotics can be marketed for the clinical treatment of hypertension.

ACKNOWLEDGMENTS

This work was supported by Grants from Comisión Interministerial de Ciencia y Tecnología, Ministerio de Economía y competitividad (SAF2017-84894-R), Junta de Andalucía (CTS-164) with funds from the European Union, Ministerio de Economia y Competitividad, Instituto de Salud Carlos III (CIBER-CV; Ciberes), Spain and NHLBI grant HL102033. MT is a postdoctoral fellow of Instituto de Salud Carlos III (Sara Borrell Program). IR-V is a predoctoral fellow of MINECO. The cost of this publication was paid in part with funds from the European Union (Fondo Europeo de Desarrollo Regional, FEDER, "FEDER una manera de hacer Europa").

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

I. Robles-Vera, N. de la Visitación, and M. Toral contributed equally as first authors. R. Jiménez, and J. Duarte contributed equally to the supervision of the study. I. Robles-Vera, M. Toral, R. Jiménez, and J. Duarte designed research; I. Robles-Vera, N. de la Visitación, M. Toral, M. Sánchez, M. Romero, M. Gómez-Guzmán, T. Yang, JL Izquierdo-García, E. Guerra-Hernández, and R. Jiménez performed research; I. Robles-Vera, M. Toral, M. Romero, R. Jiménez, J. Ruiz-Cabello, M. K. Raizada, F. Pérez-Vizcaino, and J. Duarte analyzed data; F. Pérez-Vizcaino, and J. Duarte wrote the paper. All authors approved the final version to be published.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Robles-Vera I, de la Visitación N, Toral M, et al. Probiotic *Bifidobacterium breve* prevents DOCA-salt hypertension. *The FASEB Journal*. 2020;34:13626–13640. https://doi.org/10.1096/fj.202001532R