Gut bacteria products prevent acute kidney injury induced by ischemia-reperfusion

Full methods

Mice

Male C57BL/6 (H-2Ab) mice were purchased from Federal University of Sao Paulo, UNIFESP (n=5 per group). All animals were housed in individual and standard cages and had access to water and food ad libitum. All animal procedures were performed in accordance with the Brazilian Committee for Experimental Animals, and the study was approved by the institutional ethics committee on animal use of the University of São Paulo (number 121/2011).

AKI model and Short Chain Fatty Acids Treatment

Bilateral kidney IRI was performed as previously reported.[1] Briefly, the animals had their renal pedicles clamped for 45 minutes. After removal of the clamps, the animals were sutured. Sham-operated mice were used as controls. SCFA (pH 7.4, diluted in PBS) were administered individually in two intraperitoneal dosages (200 mg/kg), 30 minutes before ischemia and at the moment of reperfusion. At 24 h later, the animals were anesthetized and the material (kidney and blood) was collected. We also treated mice with the same protocol but these mice were not subjected to IRI to evaluate the SCFA toxicity. These doses were chosen based on previous studies that observed improvement kidney function after SCFA treatment in different kidney injuries models.[2,3]

Renal Function

Serum creatinine was measured by Jaffé's modified method and serum urea was measured using a Labtest Kit (Labtest, Minas Gerais, Brazil) according to the manufacturer's instructions.

Culture and administration of probiotics

The species of bifidobacteria (Bifidobacterium longum) was isolated and characterized at the Laboratory of Ecology and Physiology of Microorganisms, Institute of Biological Sciences, Federal University of Minas Gerais using morphology and staining characteristics, respiratory and biochemical tests, followed by multiplex PCR, according to Kwon et al.[4] Bifidobacterium adolescentis was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Bacteria were cultured in MRS broth medium (Difco, Detroit, MI, USA) and grown under anaerobic conditions using an anaerobic jar at 37 °C without stirring for 48 h. Administration of bacteria: Mice received an inoculum of 10^8 bacteria by gavage daily starting 10 days before surgery until the surgery day. In some experiments bacteria were labeled in PKH-26 (Sigma) following previous reports[5] and administered by gavage to track them down in the large intestine in treated mice following collection of intestine for further immunohistochemistry analyses.

Gene expression

Total RNA from kidney tissue was extracted with Trizol® (Invitrogen, Carlsbad, CA). cDNA (2 μg of total RNA) was synthesized with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA). Taqman Real-Time PCR was employed for the following genes: IL-6 - Mm00446190_m1; CxCL1 (KC) -Mm04207460_m1; CD68 - Mm03047340_m1; CCl2 (MCP-1) -

Mm00441242_m1; BCL-2 - Mm00477631_m1; VEGF - Mm01281449_m1; TLR4 - Mm00445273_m1; BGN (Biglycan) Mm01191753_m1 and HPRT - Mm01545399_m1 (housekeeping gene), all from Life Technologies™ (Carlsbad, CA, USA). GPR41 (Ffar3) and GPR43 (Ffar2) detection was performed with SybrGreen® (Life Technologies) using primers designed with the following sequences: GPR41 sense 5' TGCCTTGGACTCAGCAAGTCA 3' and GPR41 antisense 5' CCTGCGGTCCACTCTTTTCTCT 3'; GPR43 sense 5' TTTGTACATGTGCTCCGCTGAT 3' and GPR43 antisense 5' CCAGTGACTGGTGACACAGAGG 3' and HPRT (control gene) sense 5' CTGATGGACTGATTATGGACAGGAC 3'; HPRT antisense 5' GCAGGTCAGGAAAAGAACTTATAGCC 3'. The relative expression was calculated using the 2-ΔΔCt method, and the reference was a control group. A PCR array for chromatin modification enzymes (PAMM-085) was measured using a kit from SABiosciences (Qiagen, Venlo, Netherlands). All PCR reactions were detected in an ABI Prism 7300 (Life Technologies), and data analysis was performed using software available online (Life Technologies) for PCR Array. The gene expression values are presented relative to levels in the reference group (control group).

Glutathione assay

Measurement of reductive glutathione (GSS) and oxidized glutathione (GSSG) in kidney tissue extracts was performed with a Glutathione (GSH/GSSG/Total) Fluorometric Assay Kit (BioVision, Milpita, CA, USA) following the manufacturer's instructions.

Bioplex

RIPA-lysed kidney extracts or sera were assayed using the BioPlex mice Plex cytokine assay kit (BioRad Laboratories Inc., Hercules, CA, USA). Detection was performed on the BioPlex suspension array system, and the data were analyzed using BioPlex Manager software version 4.0. Standard curves ranged from 32.000 to 1.95 pg/mL.

Assessment of apoptosis

Apoptotic cells (TUNEL) were detected through the Cell Death Detection Kit TMR Red (Roche Diagnostics GmbH, Mannheim, Germany).

Immunohistochemistry

All steps to perform immunohistochemistry were performed as reported[6] using primary antibodies for the PCNA, MPO and or a negative control (all from DAKO, dilution 1:100). Analysis of positive staining was performed using a computer program for image analysis (KS300, Zeiss system). Ten images per kidney were evaluated. The data are presented as the median of all positively stained images per group. Blinded analyses of necrosis of tubular epithelial cells in H&E slides in kidney tissue were performed by an expert pathologist. For intestine analyses with the PKH-26 labeled bacteria, a piece of distal colon with feces was harvested and prepared based on a previous report.[7,8] Briefly, intestine was fixed for 24 h at 4 °C followed by 70% ethanol and paraffin embedding. Deparaffinized sections (5µm) were washed in staining buffer (Tris-HCl, NaCl and SDS) at 50 °C. A probe for 16S bacteria (Sigma, FITC-conjugated, final concentration 100 nM) were incubated in a humid chamber at 50 °C for 4 h. After that, lectin *Ulex europaeus* agglutinin-I (UEA-I) TRITC-conjugated

(Sigma) was added and incubation followed for 2 h at room temperature. The images were obtained using confocal microscopy.

Flow Cytometry

Kidney tissue was processed as recently published[9]. Cells were labeled with anti-CD11b APC, anti-F4/80 PERCP, anti-CD11c Pacific Blue and anti-CD40 PE anti-monoclonal antibodies (BD Biosciences, San Jose, CA, USA). For NF-κB activation staining (phosphorylation of p65 subunit, Cell Signaling Technology®, Danvers, MA, USA, #4887, dilution 1:50), cells were fixed and nuclear membrane permeabilization was performed (eBioscience). NO production was assayed using an indirect measure (DAF). All samples were collected using a FACSCanto II device (BD Biosciences) and analyzed using FlowJo (Tree Star, San Carlo, CA). We collected a minimum of 10.000 events.

Western Blot

Kidney tissue extracts in RIPA lysis buffer containing protease and phosphatase inhibitor (Roche, Indianapolis, IN, USA) were quantified using BCA Protein Assay Reagent (Thermo Scientific). SDS-PAGE was performed with 50 μg of total protein. Primary antibodies were employed to detect IκBα (dilution at 1:1000, Cell Signaling) and ATG-7 (dilution at 1:1000, Abcam®, Cambridge, MA, USA). Beta actin was used as a loading control.

Kidney epithelial cell line culture

The MM55.k mouse kidney epithelial cell line (acquired from and kept according to ATCC) was stimulated (60-80% confluent) as follows: LPS (10 μg/mL, Sigma-Aldrich, St.Louis, MO, USA); Zymosan (10 μg/mL, Sigma) and

recombinant cytokines (rIL-6: 50 ng/mL; rIL-1β: 50 ng/mL and rTNF-α: 100 ng/mL, all from R&D Systems®, Minneapolis, MN, USA) in the presence and/or absence of SCFA acetate (25 mM), propionate (12 mM) and butyrate (3.2 mM) for 24 h. These SCFA concentrations were based on studies that observed a modulation of the expression of adhesion molecules and also in the effector function in neutrophil modulation.[10,11] Thus, these concentrations were used in all subsequent in vitro experiments.

In vitro hypoxia cell model and confocal microscopy

Kidney immortalized human epithelial cells (HK-2, purchased from and cultured according to ATCC) were seeded on glass coverslips and subjected to hypoxia (1% O2; 5% CO2; balanced N2, 37°C) over a 24 h period in an incubator (Ruskinn Technology, Bridgend, UK) or were maintained under normoxia (humidified atmosphere, 5% CO2 at 37°C), and treated with or without SCFA for the entire hypoxic period. Cells were incubated with 50 μM HypoxyprobeTM-1 Omni kit and 5 μM MitoSOX™ following the manufacturer's instructions. Anti-human HIF-1α (Abcam, ab81634; dilution 1:100) or PAb2627 (hypoxyprobe reaction, dilution 1:100) were incubated overnight followed by incubation with FITC-conjugated antibodies (abcam). Confocal images were obtained using a Zeiss LSM 780 system (Carl Zeiss, Jena, Germany) at Core Facilities to Support Research (CEFAP). Images acquired with a 63x (1.4NA) oil immersion objective were rendered with Zen Software (Carl Zeiss, Jena, Germany). Lactate was measured in the culture supernatant using a commercially available kit (Labtest kit), following the manufacturer's instructions.

Detection of Myeloperoxidase (MPO) in renal tissue

MPO in renal tissue was estimated as previously described by Hillegass et al.[12] The reading was performed in a spectrophotometer at a wavelength of 460 nM.

Bone Marrow-derived Dendritic Cell (BM-DC) generation/maturation and co-culture

BM-DC was generated as recently described[13] and activated with LPS (20 ng/mL, Sigma) in the presence or absence of SCFA for 24 h. For the allogeneic culture, spleen cells from RAG knockout C57Bl/6 mice were pre-treated with LPS (20 ng/mL) in the presence of SCFA for 24 h. The cells were then washed and co-cultured with CFSE-labeled spleen cells from Balb/c mice for 4 days in a 1:3 ratio, with a total of 2x105 cells/well in a 96-well U-bottom plate. Evaluation of proliferation was performed in a FACS CantoII (BD Bioscience).

Histone Deacetylase (HDAC) activity assay

A total of 70 μg of protein from kidney tissue was used to measure HDAC activity in an HDAC activity colorimetric assay kit (K-331-100) following the fabricant's recommendations (Biovision).

Global methylation and evaluation of mitochondrial DNA

DNA from kidney tissue was extracted using DNeasy® (Qiagen). Global methylation was assessed according to Sharma et al.[14] Mitochondrial DNA (mtDNA) content was measured by real-time PCR with specific primers designed for the mtDNA gene: sense 5'-CCCAATCTCTACCAGCATC-3' and antisense 5'-GGCTCATAGTATAGCTGGAG-3'. The normalization was performed with genomic DNA (gDNA) primers: sense 5'-

GTACCCACCTGTCGTCC-3', and antisense 5'-GTCCACGAGACCAATGACTG-3'. The data are presented as a ratio between mtDNA and gDNA.

Acetate dosage in feces and plasma

Acetic acid (99.7%) and citric acid were purchased from Sigma-Aldrich (St Louis, MO, USA), butanol from Carlo Erba (Corneredo, Italy), acetonitrile from Merck (Darmstadt, Germany) and tetrahydrofuran from Acros Organics (Fair Lawn, USA). Chromatographic analysis were performed using an Agilent 6850 system with EzChrom software, equipped with an 7683B automatic liquid sampler, flame ionization detector (FID) (Agilent Technologies, USA) and a fused-silica capillary column DB-23 (Agilent Technologies, USA) of 60 m X 0.25 mm i.d. coated with 0.15 μm thick of 80.2% 1-methylnaphatalene. The initial oven's temperature was 100 °C (hold 7 min), then increased to 200 °C at 25 °C/min (hold 5 min). The FID temperature was kept at 260 °C, and the flow-rates of H2, air and N2, make-up gas were 30, 350 and 25 mL/min, respectively. Sample volumes of 5 μL were injected at 250 °C using a split ratio at ca. 25:1. Nitrogen was used as carrier gas at 1 mL/min (hold 4 min), then reduced to 0,8 mL/min (hold 1 min) and to 0.6 mL/min (hold 1 min), and finally rose to 1 mL/min at flow of 1 mL/min. The run time for each analysis was 16.5 min.

Acetic acid (HAc) was diluted at 1.0 mg/mL in butanol:tetrahyidrofuran:acetonitrile (5:3:2), and further dilutions were done with the same solvent to make a series of standard solutions.

Human blank plasma was spiked with 1.0 mg/mL of HAc,. This solution was diluted further with blank plasma to prepare plasma standards of 0.015-1 mg/mL. To each tube were added 40 mg of sodium chloride, 20 mg of citric acid, 40 μL of 0.1 M hydrochloric acid and 200 μL of

butanol:tetrahydrofuran:acetonitrile (5: 3: 2) was added. To quantify the acids, a calibration curve at the concentration range of 0.015-1 mg/mL was constructed. Mouse plasma samples (100 μL) were used to dosage the acetate concentration, whereas the other components were reduced to half. The concentration of acetate plasma was expressed in mM. Samples of feces from the interior of the intestines at the time of sacrifice were weighed into 1.5 mL tubes, crushed and homogenized in 100 mL of distilled water. Subsequently, 40 mg of sodium chloride, 20 mg of citric acid, 40 μL of 0.1 M hydrochloric acid and 200 μL of butanol: tetrahydrofuran: acetonitrile (5:3:2) were added. The tubes were vortexed for 1 minute and centrifuged at 10.000 rpm for 10 min. The supernatant was transferred to microtubes and 5 μL was injected in triplicate into the gas chromatograph. The retention time was 7.2. The precision and accuracy of the assay were evaluated by analyzing samples (0.62, 0.25 and 0.75 mg/mL) in 5 replicates. The precision, expressed as relative standard deviation (RSD), was less than 14.34%, and the accuracy was between 87.51% and 113.56 %.

Statistics

The data are presented as average and standard deviation. Student's t-test and ANOVA (with Bonferroni post-test) were employed for comparisons between 2 and 3 or more groups, respectively. A p value <0.05 was considered significant. All graphs and statistical analyses were performed using Graph-Pad PRISM (GraphPad Software Inc., LaJolla, CA, USA).

Disclosure

All the authors declare no competing interests.

References related to full methods

1. Correa-Costa M, Azevedo H, Amano MT, et al. Transcriptome analysis of renal ischemia/reperfusion injury and its modulation by ischemic pre-conditioning or hemin treatment. *PloS one* 2012; **7**: e49569.

2. Machado RA, Constantino Lde S, Tomasi CD, et al. Sodium butyrate decreases the activation of NF-kappaB reducing inflammation and oxidative damage in the kidney of rats subjected to contrast-induced nephropathy. *Nephrol Dial Transplant* 2013; **27**: 3136-3140.

3. Sun X, Zhang B, Hong X, et al. Histone deacetylase inhibitor, sodium butyrate, attenuates gentamicin-induced nephrotoxicity by increasing prohibitin protein expression in rats. *Eur J Pharmacol* 2013; **707**: 147-154.

4. Kwon HS, Yang EH, Lee SH, et al. Rapid identification of potentially probiotic Bifidobacterium species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol Lett* 2005; **250**: 55-62.

5. Kierbel A, Gassama-Diagne A, Rocha C, et al. Pseudomonas aeruginosa exploits a PIP3-dependent pathway to transform apical into basolateral membrane. *J Cell Biol* 2007; **177**: 21-27.

6. Braga TT, Correa-Costa M, Guise YF, et al. MyD88 signaling pathway is involved in renal fibrosis by favoring a TH2 immune response and activating alternative M2 macrophages. *Mol Med* 2012; **18**: 1231-1239.

7. Salzman NH, de Jong H, Paterson Y, et al. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. *Microbiology* 2002; **148**: 3651-3660.

8. Swidsinski A, Weber J, Loening-Baucke V, et al. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol*. 2005; **43**:3380-3389.

9. Castoldi A, Braga TT, Correa-Costa M, et al. TLR2, TLR4 and the MYD88 signaling pathway are crucial for neutrophil migration in acute kidney injury induced by sepsis. *PloS one* 2012; **7**: e37584.

10. Vinolo MA, Rodrigues HG, Hatanaka E, et al. Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. *Clin Sci* 2009; **117**: 331-338.

11. Vinolo MA, Hatanaka E, Lambertucci RH, et al. Effects of short chain fatty acids on effector mechanisms of neutrophils. *Cell Biochem Funct* 2009; **27**: 48-55.

12. Hillegass LM, Griswold DE, Brickson B, et al. Assessment of myeloperoxidase activity in whole rat kidney. *J Pharmacol Methods* 1990; **24**: 285-295.

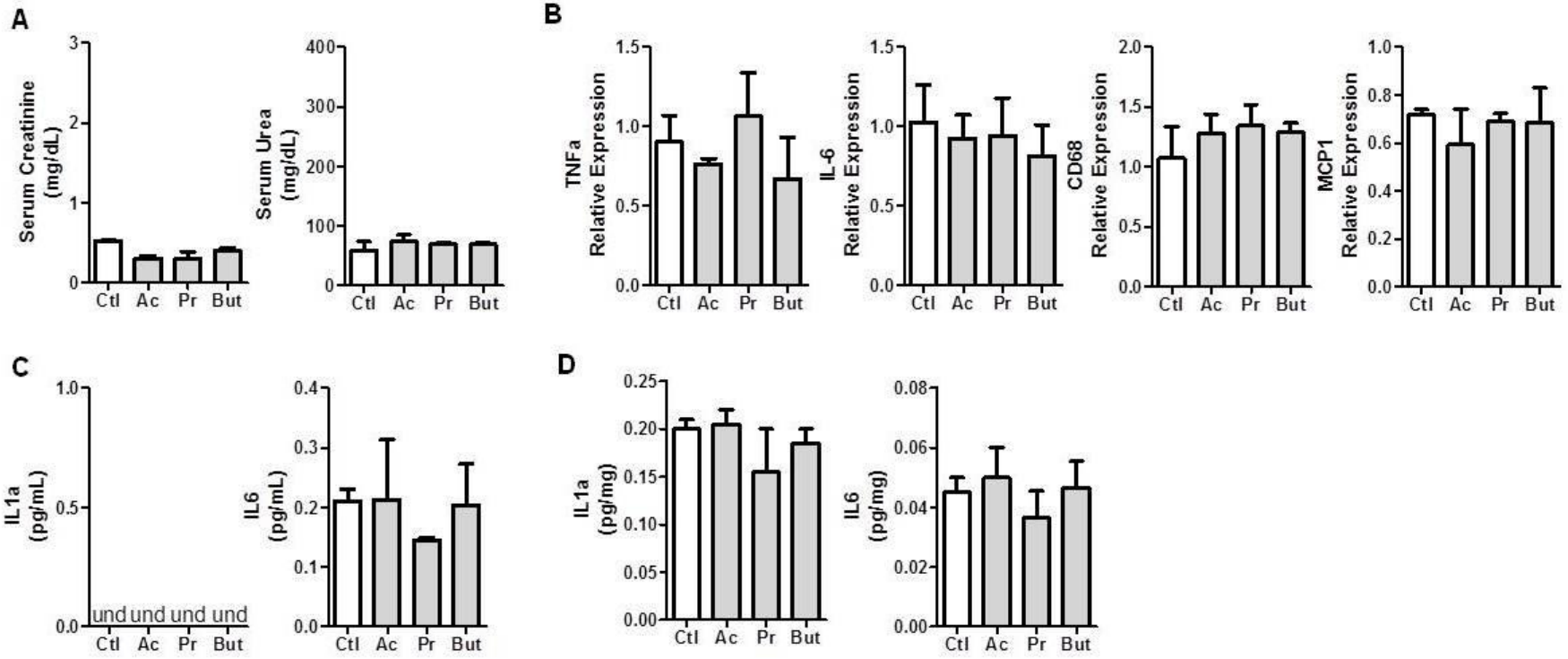
13. Moraes-Vieira PM, Larocca RA, Bassi EJ, et al. Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells. *Eur J Immunol*. 2014; **44**: 794-806.

14. Sharma S, De Carvalho DD, Jeong S, et al. Nucleosomes containing methylated DNA stabilize DNA methyltransferases 3A/3B and ensure faithful epigenetic inheritance. *PLoS Genet* 2011; **7**: e1001286.

Supplementary Figures and legends

Supplementary figure 1. Short Chain Fatty Acid treatment does not affect kidney function or inflammatory parameters. Mice were not subjected to kidney IRI and treated with acetate (Ac), propionate (Pr) and butyrate (But) at 0.5 h before ischemia and at the moment of reperfusion (200 mg/kg each) and parameters were evaluated after 24 h. **(A)** Serum creatinine and urea levels. **(B)** mRNA of TNFa, IL-6, CD68 and MCP1 in kidney tissue measured by real-time PCR. Protein levels of IL1-a and IL-6 measured in serum **(C)** and in kidney tissue **(D)**. Ctl: control; Ac: acetate; Pr: propionate; But: Butyrate; und: undetected.

Supplementary figure 1



Supplementary figure 2. Probiotic treatment reaches the intestinal niche and does not interfere in kidney function. Mice (n=5) were submitted to treatment with B. longun (BL) or B. adolescentis (BA) for 10 days. The last day, PKH26-labeled bacteria was administrated and after 24h. **(A)** Confocal microscopy was performed using universal 16S RNA probe to stain gut (colon) bacteria and tomate lectin (stain mucus). White arrows indicate PKH26-labeled bacteria co-localized with 16S RNA probe (yellow color). Scale bar = 10 μM **(B)** Serum creatinine and urea were measured before and after probiotic treatment. Ctl: control