Gut Bacteria Products Prevent AKI Induced by Ischemia-Reperfusion


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ABSTRACT
Short-chain fatty acids (SCFAs) are fermentation end products produced by the intestinal microbiota and have anti-inflammatory and histone deacetylase–inhibiting properties. Recently, a dual relationship between the intestine and kidneys has been unraveled. Therefore, we evaluated the role of SCFA in an AKI model in which the inflammatory process has a detrimental role. We observed that therapy with the three main SCFAs (acetate, propionate, and butyrate) improved renal dysfunction caused by injury. This protection was associated with low levels of local and systemic inflammation, oxidative cellular stress, cell infiltration/activation, and apoptosis. However, it was also associated with an increase in autophagy. Moreover, SCFAs inhibited histone deacetylase activity and modulated the expression levels of enzymes involved in chromatin modification. In vitro analyses showed that SCFAs modulated the inflammatory process, decreasing the maturation of dendritic cells and inhibiting the capacity of these cells to induce CD4+ and CD8+ T cell proliferation. Furthermore, SCFAs ameliorated the effects of hypoxia in kidney epithelial cells by improving mitochondrial biogenesis. Notably, mice treated with acetate-producing bacteria also had better outcomes after AKI. Thus, we demonstrate that SCFAs improve organ function and viability after an injury through modulation of the inflammatory process, most likely via epigenetic modification.


AKI is an inflammatory process frequently observed in hospitalized patients. AKI is associated with the development of CKD and causes distress to the patient.1,2 AKI induced by ischemia and reperfusion injury (IRI) is closely linked to the activation of tubular epithelial and endothelial cells by endogenous danger signals released after cell stress and death due to enhanced production of reactive oxygen species (ROS), among other inducers.3 Additionally, IRI involves the migration and activation of innate and adaptive immune cells into the kidneys.

Increased ROS production disrupts the ratio of oxidant/antioxidant enzymes, leading to mitochondrial-mediated apoptotic cell death. Activation of endothelial
cells enhances adhesion molecule expression, which recruits immune cells, contributing to ROS production. Similarly, kidney tubular epithelial cells (TECs) and resident antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), can produce cytokines and chemokines upon activation at the site of inflammation. Furthermore, APCs increase the expression of costimulatory molecules and migrate to the draining lymph node to activate CD4+ and CD8+ T lymphocytes, thereby contributing to tissue damage. Our group and others have demonstrated the role of different immune cell populations in kidney IRI.

Figure 1. SCFA ameliorates renal function. Mice (n=5) were subjected to kidney IRI and treated with acetate (Ac), propionate (Prop), and butyrate (But) at 0.5 hour before ischemia and at the moment of reperfusion (200 mg/kg each). Serum creatinine (A) and urea (B) were measured after 24 hours. (C) Quantification of necrosis in tubular epithelial cells. Ctl, control group.

Figure 2. Acetate treatment decreases cellular stress, cytokine and chemokine production, and cellular infiltrates. Mice (n=5) were subjected to kidney IRI and treated with acetate. (A) Glutathione reduced (GSS) and glutathione oxidized (GSSH) ratio. mRNA levels measured by real-time PCR (B) and protein levels measured by BioPlex (C) of proinflammatory cytokines and chemokines in kidney tissue and serum protein levels of the proinflammatory cytokines and chemokines (D). (E) mRNA levels of TLR4 and biglycan measured by real-time PCR in kidney tissue. (F) Right, Western blot analysis in kidney tissue for IκBα and β-actin; each band quantified (optical densitometry) for each protein in the group and normalized by quantification of β-actin for the respective group. (G) Myeloperoxidase (MPO) levels in kidney tissue. (H) Percentage of activated DCs (CD11c+/CD40+) and macrophages (CD11b+/F4/80+) in kidney tissue analyzed by flow cytometry. Ac, acetate; Ctl, control; KC, Cxcl1; MCP-1, monocyte chemotactic protein-1.
Recently, an intimate connection between the intestine and the kidneys has been proposed.10,11 Established data have already shown that modification of microbiota composition could affect the outcome of glomerulopathies, and recent data indicate that renal inflammation is associated with the production of uremic toxins by the intestine and with augmentations intestinal permeability, leading to the onset of systemic inflammation.12,13 In this sense, molecules produced by local intestinal flora could have a direct effect on kidney injuries.

Short-chain fatty acids (SCFAs) are end products produced from the fermentation of complex carbohydrates by the intestinal microbiota, especially by anaerobic bacteria.14 The most abundant SCFAs are acetate, propionate, and butyrate. Locally, SCFAs are energy sources for colonocytes. However, they can reach the bloodstream.15 SCFA treatment has ameliorated colitis, airway disease, and metabolic syndrome in diet-induced obese mice.16–18

So far, researchers suggested that SCFAs may operate in two manners: by binding G-protein membrane receptors (GPR41 and GPR43),19,20 or by entering cells directly through transporter channels in the cellular membrane and working as histone deacetylase (HDAC) inhibitors. Thus, they could act by modulating epigenetic processes.21

Because AKI has an important inflammatory component yet SCFAs have anti-inflammatory properties, we investigate whether SCFAs treatment could protect mice from IRI. Furthermore, we investigated whether this protection could involve direct modulation of the inflammatory process and/or amelioration of the oxidative stress presented in this model.

RESULTS

SCFA Treatment Ameliorates Kidney Function after IRI

To investigate the role of SCFAs in IRI, mice were subjected to IRI and treated with SCFAs. SCFA treatment diminished levels of serum creatinine and urea after IRI, with acetate treatment

Figure 3. Acetate treatment decreases apoptosis levels and increases tubular proliferating cells and activation of the autophagy pathway. (A) Immunofluorescence of apoptosis levels measured by TUNEL in kidney tissue. The percentage of apoptosis was measured relative to the photographic area (original magnification, ×20). (B) Immunohistochemistry for proliferating cell nuclear antigen in kidney tissue. The percentage of positive staining for the molecule was measured relative to the photographic area (original magnification, ×20). (C) Real-time PCR in kidney tissue for BCL-2. (D) Western blot analysis in kidney tissue for ATG-7 and β-actin. The ratio of ATG-7/β-actin was calculated through quantification of each band (optical densitometry) in the group and normalized to the quantification of β-actin. (E) Ratio of mitochondrial DNA (mtDNA) and genomic DNA (gDNA) in kidney tissue. n=5 per group. Ac, acetate; Ctl, control; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling.
resulting in the best protection (Figure 1, A and B). SCFA treatment did not interfere in kidney function or inflammatory parameters (Supplemental Figure 1, A–C). Hereafter, we consider in vivo analyses only for acetate treatment. Histopathologic analysis showed that acetate treatment preserved kidney structure as reflected in the reduced necrosis score in kidney tubular epithelial cells (Figure 1C).

**Acetate Treatment Reduces Cellular Stress and Local and Systemic Inflammation**

Acetate protection was associated with decreased ROS production (Figure 2A). Cytokines and chemokines were also diminished locally (Figure 2, B and C) and systemically (Figure 2D). Additionally, low levels of mRNA of toll-like receptor 4 and its endogenous ligand, biglycan (Figure 2E), and lesser activation of the NF-κB pathway were observed (Figure 2F). As a consequence, low levels of activated neutrophils and macrophages (myeloperoxidase in kidney tissue), a low frequency of infiltrating macrophages (CD11b+ F4/80+), and a low frequency of activated DCs (CD11c+ CD40+) (Figure 2, G and H) were observed in acetate-treated mice. Thus, acetate treatment diminished cellular stress and inflammation in kidney IRI.

**Acetate Treatment Reduces Apoptosis and Increases Autophagy and Tubular Proliferating Cells**

Apoptosis is a frequent event in kidney IRI. Acetate treatment diminished the number of apoptotic cells in kidney tissue (Figure 3A) but increased proliferation in kidney epithelial cells (Figure 3B) and BCL-2 gene expression (Figure 3C). Moreover, acetate treatment increased the levels of ATG-7 protein, which is involved in the autophagy pathway (Figure 3D).

Supporting these findings was an increase in mitochondrial

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**Figure 4.** Acetate treatment modulates epigenetic modification and upregulates GPR43 expression. (A) Real-time PCR of GPR41 and GPR43 in kidney tissue. (B) PCR-based array for the expression of genes encoding chromatin modification enzymes in kidney tissue. Comparison of gene expression levels for these genes between IR versus control (left), IR plus acetate versus IR (middle) and IR plus acetate versus IR (right). Up- and downregulated gene expression is shown in relation to the y axis group. (C) Measurement of histone deacetylase activity in kidney tissue. (D) Global DNA methylation in kidney tissue. n=5 per group Ac, acetate; Ctl, control.
DNA in kidney tissue after acetate treatment compared with the IRI group (Figure 3E). Taken together, these results show that acetate treatment inhibits apoptosis and increases cell proliferation, potentially through autophagy activation.

**SCFA Receptors and Epigenetic Modifications in Kidney Tissue**

Gene expression analysis for SCFA receptors revealed no difference in GPR41 expression, whereas the levels of GPR43 increased after acetate treatment (Figure 4A). Additionally, the PCR-based array showed that acetate treatment modulated the expression of genes encoding enzymes involved in epigenetic modifications (Figure 4B). Another important function for these SCFAs is their ability to act as HDAC inhibitors. Acetate treatment inhibited the activity of HDACs (Figure 4C) in kidney IRI. Another important epigenetic process is DNA methylation. Global methylation status decreased in kidney tissue undergoing IRI, and acetate treatment reversed this process (Figure 4D).

**Evaluation of SCFAs in DCs In Vitro**

Having observed in vivo a lower frequency of activated DCs in kidney tissue after IRI, we sought to determine whether SCFAs may modulate these cells in vitro. SCFA treatment reduced the expression of the costimulatory molecules CD80 and CD40 in bone marrow DCs (BM-DCs) (Figure 5A and B). These data corroborated the in vivo finding (Figure 2H). Furthermore, we evaluated whether this reduction is functional. We pretreated...
APCs from RAGKO mice with LPS, with or without SCFAs, for 24 hours. Treatment of APCs with LPS plus SCFAs was sufficient to reduce the proliferation of CD8+ and CD4+ cells (Figure 5, C and D). Therefore, SCFA treatment modulates the activation and function of APCs.

Evaluation of SCFA Treatment in a Kidney Epithelial Cell Line

It is well known that TEC activation enhances inflammation in kidney tissue after IRI. To date, SCFA action in TECs has not been reported. Thus, we investigated whether SCFA treatment is effective in these cells. TECs stimulated with an inflammatory cocktail (LPS, 10 μg/ml; zymosan, 10 μg/ml; IL-6, 50 ng/ml; IL-1β, 50 ng/ml, and TNF-α, 100 ng/ml) in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But; 3.2 mM) for 24 hours were evaluated by flow cytometry for (A) left: overlay histogram of NFκB activation measured through phosphorylation of the p65 subunit (percentage of p65+ cells) and right: percentage of the p65+ cells and (B) left: overlay histogram nitric oxide production (DAF-FM Diacetate) [4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate] and right: mean fluorescence intensity of the DAF-positive cells. *P<0.05.

DISCUSSION

Kidney IRI physiopathology has a complex network of events that involves a well known and important inflammatory process that includes tubular epithelial, endothelial, and resident immune cells. In addition to the high mortality rate in hospitalized patients, the extent of this event has been believed to be a risk factor associated with worse outcomes.1 Thus, its prevention is extremely important. In this study, we investigated whether SCFAs, which have anti-inflammatory properties, could ameliorate kidney IRI.

Treatment with SCFAs, especially acetate, reduced kidney damage after kidney IRI. To our knowledge, this is the first study demonstrating the protective role of SCFAs in kidney IRI. Other studies have observed a reduction in kidney injury in other models after SCFA treatment.22,23 The higher protection observed after acetate treatment might be due to the composition of acetate, favoring the rapid entry of the molecule into the metabolic pathway, whereas other types of SCFAs need to

Figure 6. SCFA treatment inhibits NFκB activation and nitric oxide production in epithelial kidney cell line. MM55.k kidney epithelial cells were stimulated with inflammatory cocktail (LPS, 10 μg/ml; zymosan, 10 μg/ml; IL-6, 50 ng/ml; IL-1β, 50 ng/ml, and TNF-α, 100 ng/ml) in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But; 3.2 mM) for 24 hours and were evaluated by flow cytometry for (A) left: overlay histogram of NFκB activation measured through phosphorylation of the p65 subunit (percentage of p65+ cells) and right: percentage of the p65+ cells and (B) left: overlay histogram nitric oxide production (DAF-FM Diacetate) [4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate] and right: mean fluorescence intensity of the DAF-positive cells. *P<0.05.
be converted into precursor molecules. The concentrations used in this study agree with the physiologic concentrations observed for these molecules in the intestine. SCFAs can reach the bloodstream even when produced and taken up by local cells in the intestine, but their presence in kidney cells has not been reported. Inflammation and apoptosis are hallmarks of this model, and acetate treatment inhibited both processes. This is an expected result because SCFAs have previously been found to possess anti-inflammatory properties. The reduction of apoptosis levels could be due to inflammation reduction or enhancement of the autophagy activation pathway. Restricted deletion of ATG7 in TECs showed increased susceptibility to kidney injury in cisplatin-induced AKI. Additionally, in cancer cells, butyrate treatment induced autophagy as a defense strategy to retard mitochondrial apoptosis-induced cell death. We observed lower apoptosis levels and higher levels of the ATG-7 protein after acetate treatment, suggesting that acetate could induce autophagy and thereby retard apoptosis.

Two mechanisms have been proposed for the operation of SCFAs: as a ligand for GPR41 and GPR43 or as an HDAC inhibitor. The latter is believed to occur in a GPR-independent manner. In our study, GPR41 had similar mRNA levels with kidney IRI and acetate treatment, whereas GPR43 levels were increased. Considering the other proposed function of SCFAs, HDAC activity was decreased in kidney tissues and the expression of chromatin modification enzymes was modulated.

Among the differentially expressed genes, none of them encoded for an HDAC, which suggests that HDAC inhibition exerted by SCFAs may be only at the protein level. Other studies have observed modulation of HDAC activity by acetate. It is not fully understood whether HDAC inhibition by SCFAs is GPR41/43 dependent. Recently, HDAC inhibition was partially GPR43 dependent in colon tissue, but other researchers have observed HDAC inhibition in a GPR43-independent manner. Although we observed high expression of GPR43 in our model, its expression has been reported as minimal or null in kidney tissue. Thus, we believe that acetate might be regulating the inflammatory process by regulating epigenetic modification in a GPR-independent manner. Another interesting finding in our work is the increase of global methylation of DNA after acetate treatment. One limitation of this finding is that this assay is unspecific, barring speculation as to which regions and consequently target genes could be altered by modifications in methylation status. Because we do not know whether this effect is direct or indirect, future investigations are necessary to unravel the possible relationship between GPR activation, HDAC inhibition, and DNA methylation by SCFAs in the kidney.

To further investigate possible SCFA protection, we examined the role of SCFAs in immune cells and a kidney epithelial cell line. As observed in vivo, SCFA treatment reduced the expression of the costimulatory molecule CD40 and (to a lesser extent) CD80 in BM-DC. Other studies have also demonstrated a reduction in DC development and activation. Furthermore, we observed that SCFA-treated APC induced less proliferation of CD4+ and CD8+ T lymphocytes, demonstrating that the modulation of DC treated with SCFAs was functional. DC links innate immunity

Figure 7. SCFA treatment inhibits ROS production in an epithelial kidney cell line after hypoxia. HK-2 human kidney epithelial cells were seeded on a cover slip and subjected to hypoxia for 24 hours in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But, 3.2 mM). Thirty minutes before completion of the 24 hours, cells were incubated with Hypoxyprobe and MitoSOX markers and analyzed with confocal microscopy. Scale bar, 10 μm.
with adaptive immunity, so lower activation of DC is important to reduce lymphocyte activation. Our group reported that Th1-lymphocyte factors, such as IFN-γ and IL-12, are important for AKI development in kidney IRI. Thus, it is plausible to speculate that low frequencies of activated DC and reduced lymphocyte activation occur in vivo.

Kidney ischemia is an important event that contributes to kidney damage because of ROS production and inflammation. SCFA treatment reduced NFκb activation and nitric oxide production in TECs. Moreover, lower production of ROS, lower HIF-1α translocation, and lower lactate production after hypoxia in the presence of SCFAs were observed. Lactate production and HIF-1α activation are directly associated. The expression of GPRs in kidney tissue is minimal, which suggests that SCFAs could operate via an epigenetic mechanism in these cells, as observed in vivo. Another possibility is that SCFAs ameliorate mitochondrial function after kidney ischemia, through SCFAs metabolism in these cells, providing energy. This idea is supported by the finding of increased mitochondrial DNA in kidney tissue undergoing IRI after acetate treatment (Figure 3E). Butyrate seems to have exerted a greater effect in vitro than in vivo. Not all SCFAs could bind to the same receptor, as shown recently for the receptor Ohr78, which can be activated by acetate and propionate and is involved in kidney BP regulation after binding of SCFAs. This new finding raises the question of whether acetate could protect from kidney IRI through Ohr78 activation. If so, this could explain, at least for butyrate treatment, the better results observed in vitro because Ohr78 is not expressed in epithelial cells. Either way, SCFA treatment can modulate the pathways involved in ischemia injury and inflammation in TEC in vitro.

Finally, we treated animals with acetate-producing bacteria and observed an improvement of kidney function. B. longum, but not B. adolescentis, was recently reported to reduce death in a model of enterohemorrhagic

Figure 8. SCFA treatment inhibits HIF-1α translocation to the nucleus, lactate production, and VEGF expression under hypoxia. HK-2 human kidney epithelial cells were seeded on a cover slip and subjected to hypoxia for 24 hours in the presence of acetate (Ac; 25 mM), propionate (Pr; 12 mM), and butyrate (But, 3.2 mM). (A) Cells were labeled with anti-human HIF-1α followed by FITC-labeled secondary antibodies and analyzed with confocal microscopy. Scale bar, 10 μm. (B) Left lactate levels after hypoxia were measured in culture supernatant. Right relative expression of VEGF in normoxia and hypoxia. *P<0.05; ***P<0.001.
Escherichia coli infection, which was associated with acetate production. Here, both types of bacteria treatment protected from kidney IRI. One explanation is that although B. adolescentis produces less acetate, the amount was enough to protect from kidney IRI. However, further investigation will be necessary to clarify this issue. It is important to mention that because these treatments were performed without previous antibiotic administration, and because we have found very low frequencies of these probiotics in intestine (none of them close to the mucus region of treated mice), these bacteria probably did not colonize the intestine. However, future studies are necessary to elucidate this issue. Germ-free mice are more susceptible to kidney IRI. In this study, we demonstrated that metabolites from microbiota are also important to control inflammatory processes in distant organs where SCFAs are not produced. Acetate administration can also protect from colitis in the absence of microbiota, whereas the administration of SCFAs in wild-type mice also protects from other diseases. Despite the importance of the presence of microbiota in maintaining intestinal homeostasis and preventing inflammation, it appears that an increase in the concentration of SCFAs could enhance this protection. It is already known that

Figure 9. Acetate-producing bacteria ameliorate AKI induced by IR. Mice (n=5 per group) were administered B. longum or B. adolescentis (10^8 bacteria) by gavage for 10 days before euthanasia and were subjected to kidney IRI. (A) Weight change and (B) food intake during 10 days before surgery induction. (C) Acetate levels measured in the intestinal (colon) content. (D) Acetate levels measured from plasma samples harvested pre and post probiotic treatment. (E) Serum levels of creatinine and urea in mice subjected to kidney IRI. (F) Serum levels of proinflammatory cytokines and chemokines in mice subjected to kidney IRI, treated or untreated with B. longum (BL) or B. adolescentis (BA). Ctl, control. **P<0.01.

gut microbiota can change composition rapidly through diet alteration. Augmented SCFA levels could be reached through changes of diet, and probiotics have been investigated as alternative therapeutic strategies in some contexts, strengthening the idea that acetate-producing bacteria could become a tool for management or prevention of inflammatory processes.

In conclusion, acetate treatment diminishes inflammation in kidney epithelial and immune cells and ameliorates kidney IRI, most likely through modulation of epigenetic processes.

**CONCISE METHODS**

**Mice**

Male C57BL/6 (H-2Ab) mice were purchased from Federal University of São Paulo, UNIFESP (n=5 per group). All animal procedures were performed in accordance with the Brazilian Committee for Experimental Animals, and the institutional ethics committee on animal use of the University of São Paulo approved the study (number 121/2011).

**AKI Model SCFA/Probiotics Treatment**

Bilateral kidney IRI was performed as previously reported. SCFAs (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.

**Renal Function**

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

**Assessment of Apoptosis**

Apoptotic cells (terminal deoxynucleotidyl transferase-mediated dioxigenin-deoxyuridine nick-end labeling) 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. Details are provided in the full methods section (see Supplemental Material).

**Flow Cytometry**

Kidney tissue was processed as recently published. See the full methods section (Supplemental Material).

**Detection of Myeloperoxidase in Renal Tissue**

Myeloperoxidase in renal tissue was estimated as previously described by Hillegass et al.

**BM-DC Generation/Maturation and Coculture**

BM-DCs were generated as recently described. See the full methods section (Supplemental Material).

**Global Methylation and Evaluation of Mitochondrial DNA**

Global methylation was performed according to the method of Sharma et al. See the full methods section (Supplemental Material).

For detailed information about the other assays performed in this manuscript, see full methods section (Supplemental Material).

**Statistical Analyses**

The data are presented as mean and SD. A t test and ANOVA (with Bonferroni post-test) were used for comparisons between two and three or more groups, respectively. P<0.05 was considered to indicate a statistically significant difference. All graphs and statistical analyses were performed using Graph-Pad PRISM (GraphPad Software Inc., La Jolla, CA).

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**DISCLOSURES**

None.

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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. 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.  

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 reports5 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’ TGCGTGACACTCTTTTCTCT 3’ and GPR41 antisense 5’ CCTGCGGTCCACTCTTTTCTCT 3’; GPR43 sense 5’ TTTGTACATGTGCTCCGCTGAT 3’ and GPR43 antisense 5’ CCAGTGACTGGTGACACAGG 3’ and HPRT (control gene) sense 5’ CTGATGGACTGATTATGGACAGGAC 3’; HPRT antisense 5’ GCAGGTAGAAGAAGCTTATTAGCC 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 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. 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 published⁹. Cells were labeled with anti-CD11b APC, anti-F4/80 PERCP, anti-CD11c Pacific Blue and anti-CD40 PE anti-monomoclonal 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.\textsuperscript{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 Hypoxyprobe\textsuperscript{TM}-1 Omni kit and 5 µM MitoSOX\textsuperscript{TM} 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.\textsuperscript{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\textsuperscript{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 2x10^5 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\textsuperscript{®} (Qiagen). Global methylation was assessed according to Sharma et al.\textsuperscript{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:tetrahydofuran: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


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 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