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Inorganic nitrate prevents the loss of tight junction proteins and modulates inflammatory events induced by broad-spectrum antibiotics: A role for intestinal microbiota?

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ABSTRACT

Upon consumption, dietary nitrate is reduced to nitrite in the oral cavity and to nitric oxide (˙NO) in the stomach. Here, ˙NO increases mucosal blood flow, mucus thickness and prevents microbial infections. However, the impact of nitrate on gut microbiota, a pleiotropic organism essential to maintain gastrointestinal and systemic welfare, remains elusive. This study investigates the impact of nitrate on gut microbiota profile and ensued mucosal effects during dysbiosis. Male Wistar rats were randomly distributed in 4 groups and the drinking water was supplemented for 7 days as follows: 1) antibiotic cocktail (neomycin, bacitracin and imipenem), 2) antibiotic cocktail + sodium nitrate, 3) sodium nitrate and 4) regular drinking water. Animals were weighted daily and feces were collected before and after the treatment. The stomach was isolated and the expression of occludin, claudin-5 as well as myeloperoxidase and iNOS was studied. Bacterial DNA was analyzed in fecal samples by PCR-DGGE genetic fingerprinting. Nitrate prevented antibiotic-induced body weight loss (1.9 ± 1.8% *vs* 8.9 ± 1.8%, $p < 0.05$) and cecamegalia (7.1 ± 0.5% vs 5.6 ± 0.4%, $p < 0.05$). Gastric expression of occludin and claudin-5 tended to decrease during dysbiosis but both protein levels were recovered following nitrate consumption ($p < 0.05$). Similarly, nitrate inhibited the overexpression of myeloperoxidase and iNOS observed under dysbiosis ($p < 0.05$). Broad spectrum antibiotics significantly decreased microbiota richness and diversity in comparison to controls ($p = 0.0016$). After 7 days of treatment, whereas antibiotics reduced microbiota richness by 56%, it was observed that nitrate was able to prevent such microbial loss to only 48%, although without statistical differences ($p = 0.068$). This data suggests that dietary nitrate may be envisaged as a key component of functional foods with beneficial impact on gastric mucosal integrity during antibiotherapy but further studies are mandatory to better ascertain as to whether it modulates intestinal microbiota in terms of taxonomic and functional levels.

1. Introduction

Green-leaf vegetables are recognised for their indisputable health benefits, mitigating cardiovascular, metabolic and neurological disorders [1–4]. Indeed, diet is among the most significant and adjustable determinants of human health. It is now acknowledged that not only nitrate anion is one of the major components of such foods [5] but also that the stepwise reduction of dietary nitrate to nitric oxide (˙NO) in the gut modulates several physiological mechanisms [3,6–9]. Foods such as lettuce, spinach, celery and beets are major sources of nitrate which, through an enterosalivary circulation *in vivo*, is reduced to nitrite and ˙NO [10]. In the gut, nitrate-derived ˙NO induces mucosal vasodilation, increases mucus production [11], modulates mucosal imune responses [12] and prevents acute peptic ulceration [13–15].

When addressing the potential effects of diet on the gastrointestinal tract, it is imperative to ascertain the role of microbiota on these

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processes. In fact, it is now known that all surfaces of the human body are inhabited by microorganisms that constitute a dynamic ecosystem living in symbiosis with humans but about 70% of these microorganisms inhabit the gastrointestinal tract and are referred to as *gut microbiome* [16]. The human gut harbours up to 1000 different bacterial species that harvest otherwise indigestible nutrients, control epithelial cell renewal and modulate innate and adaptive immune responses [17]. In this regard, bowel disorders such as ulcerative colitis, Crohn's disease and irritable bowel syndrome have been associated with changes on gut microbial diversity, a phenomenon known as *dysbiosis* [18,19]. Several factors may induce dysbiosis including hygienic habits, stress, dietary behaviour and antibiotic consumption [16]. Regardless of the etiology, the trigger of dysbiosis appears to be located in the gut, where endogenous events such as accelerated intestinal transit, neurotransmitter secretion and mucus depletion, as well as exogenous stressors (*e.g.*, spices, pathogenic bacteria) increase epithelial permeability and activate inflammatory pathways, thus allowing the translocation of bacteria and bacterial-induced inflammatory products into the systemic circulation [20,21]. Yet, the impact of nitrate on the profile of gut microbial flora remains elusive despite the fact that many of these gastrointestinal events associated with dysbiosis are prevented by dietary nitrate under physiological conditions [3,11,22]. Hyde et al. have recently shown that dietary supplementation of nitrate increases the prevalence of nitrate-reducing bacteria in the oral cavity, thereby promoting endogenous nitrite and $^{\bullet}$NO production [23]. Interestingly, recent data also suggests that upon absorption into the blood stream, nitrate is not only actively taken up by the salivary glands and secreted into the oral cavity, but may also be secreted back into the intestinal lumen [24]. Here, gut bacteria may respire on nitrate yielding nitrite, $^{\bullet}$NO and ammonia [25] and thus fuel alternative metabolic bacterial pathways with a putative influence on local redox reactions. Thus, considering the wide-range impact of these microorganisms in human health (reviewed in Ref. [16]), it may be hypothesised that a nitrate-rich diet may help prevent dysbiosis and promote gastrointestinal health.

Recent data from the European Center for Disease Prevention and Control show that, in 2016, the consumption of antibiotics by outpatients was 21.9 Defined Daily Doses (DDD) *per* 1000 inhabitants. Similarly, according to the Center for Disease Control and Prevention, 5 out of 6 individuals in the US receive one antibiotic prescription each year, 30% of which are considered misused. Such clinical options represent a serious burden not only at a community level, by increasing antimicrobial resistance, but also at an individual level by eliciting symptoms such as diarrhea, dyspepsia and heartburn along with long-standing changes in gut microbiome with consequences yet to be unveiled. Hence, the hypothesis herein investigated is of foremost translational relevance by proposing that nitrate consumption should be recommended during antibiotherapy to prevent overt intestinal inflammation and increased epithelial permeability which may elicit gastrointestinal side effects and dysbiosis.

2. Materials and methods

Animals. All experiments were performed according to European Community Council Directive for the Care and Use of Laboratory Animals (86/609/ECC) and approved by the local institutional animal care committee (ORBEA) and the Portuguese Veterinarian agency (Direcção Geral de Agricultura e Veterinária). Wistar rats (n = 32) were purchased from Charles River, Barcelona and kept under 12 h cycles of light/dark for 7 days. During this period of acclimatization, the animals were fed a standard chow and had access to water *ad libitum*. Animals were then randomly distributed in 4 groups, with 8 animals each: 1) group 1 – control (with access to both food and water *ad libitum*), 2) group 2 – antibiotics (with access to food and an antibiotic cocktail dissolved in the drinking water – neomycin 5 mg/mL, bacitracin 5 mg/mL, imipenem 1.25 μg/mL), 3) group 3 – antibiotics + sodium nitrate (10 mM, dissolved in the antibiotic cocktail), 4) group 4 – sodium nitrate (10 mM in the drinking water). Based on a previous pilot study, the following humane endpoints were established: weight loss of more than 20% of the initial body weight, signs of dehydration (dorsal skin fold) combined with diarrhea for two consecutive days and severe behavioral changes. After 7 days of the dietary intervention, animals were anaesthetised with isofluorane and euthanised by cervical dislocation.

Sample collection. Feces from all animals were collected before and after the 7-day experimental period. Samples were collected directly from the anus by gentle stimulation of the anal sphincter and immediately snap frozen. Following euthanasia, stomach and cecum were excised and stored at −80 °C until further processing. Part of the glandular region of the stomach was immersed in OCT and immediately frozen (−80 °C) for immunohistochemical analysis. Ceca from all animals were weighted and photographed before storage.

DNA extraction. Typically, 100 mg of fecal material was homogenized with 900 μL of phosphate buffer solution and 100 μL of GES solution (guanidine thiocyanate 5M, EDTA 0.1M pH 8, N-lauroyl sarcosinate 34 mM) in the presence of 0.2 g of 1.0 mm glass beads using a bead beater for 5 min at 30 rev. A first step of the enzymatic lysis was performed by incubation for 1.5 h at 37 °C with lysozyme (5 mg/mL), RNase A (0.1 mg/mL) and mutanolysin (0.25 U/μL). Samples were then incubated with SDS 0.6%, NaCL 0.5M and Proteinase K 25 μL for 1.5 h at 65 °C. Following centrifugation at 15000*g* for 5 min, DNA was extracted from the supernatant by adding equal volume of phenol-chloroform-isoamyl. Finally, the samples were again centrifuged (15000 *g*, 10 min) and the top layer was recovered. DNA was then cleaned and concentrated by using Zymo DNA clean and concentrator kit (Zymo Research, Biognostica). All concentrations indicated are final.

Polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE). The V3 region of bacterial 16S rRNA gene was amplified using the primers HDA1-GC (5′CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3′) and HDA2 (5′-GTATTACCGCGGCTGCTGGCAC-3′) with a GC clamp attached to the forward primer. PCR was performed in 50-μL reaction mixtures containing 1X PCR buffer, 3 mM MgCl2, 5% dimethylsulfoxide, 200 mM each nucleotide, 15 pmol of each primer, 1 U of Taq polymerase, and 50–100 ng of purified DNA. The program was as follows: initial denaturation (94 °C for 5 min); 30 cycles of denaturation (92 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s); and a final extension (72 °C for 7min). The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using reagents purchased from MBI Fermentas (Vilnius, Lithuania). DGGE was performed on a DCode™ Universal Mutation Detection System (Bio-Rad). Samples containing approximately equal amounts of PCR amplicons were loaded onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) in 0.5xTAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na2EDTA) using a denaturing gradient ranging from 35% to 50% (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 60 °C, initially at 20 V (15 min) and then at 200 V (330 min). Gels were stained in an ethidium bromide solution (5 min) and then rinsed in distilled water. The image was acquired using a Molecular Image FX apparatus (Bio-Rad).

Preparation of tissue homogenates. The glandular region of the stomach (typically 100 mg of tissue for 1 mL lysis buffer) was excised and immersed in ice-cold RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS, supplemented with 1 mM $NaVO_4$, 5 mM NaF, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail 1:1000 (Sigma–Aldrich)). The tissue was roughly minced and then homogenized using a *Polytron*®. All crude homogenates showed minimal viscosity and protein extraction was performed under permanent agitation for 2 h at 4 °C. Cellular debris were then precipitated by a 10-min centrifugation at 15000*g* (4 °C). The supernatant was collected and frozen at −80 °C until further analysis. Protein concentration was determined using the Bradford

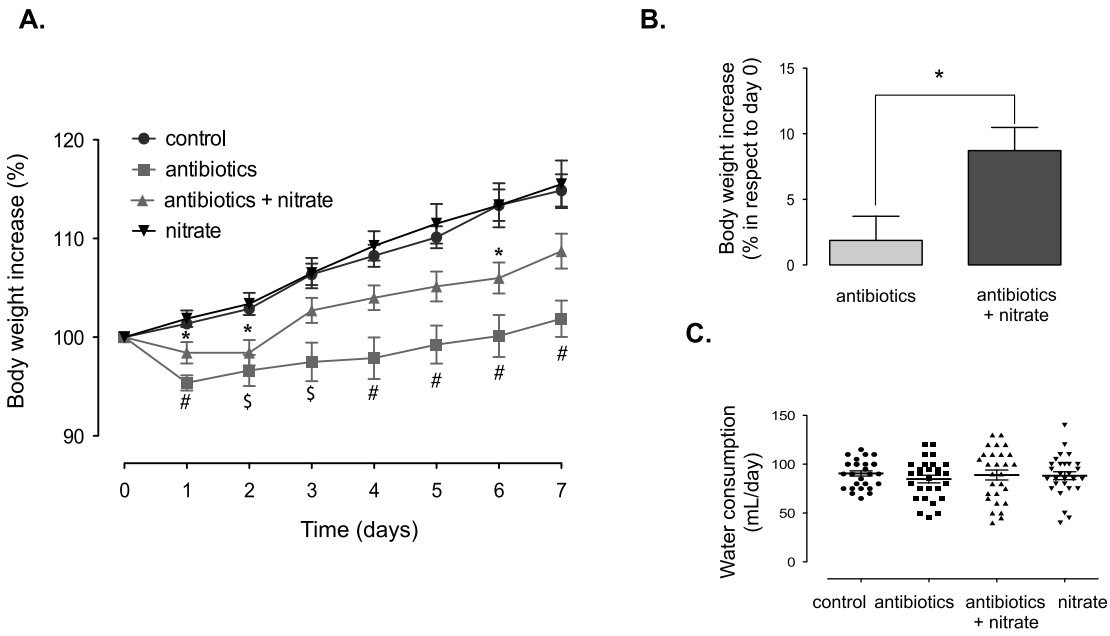

Fig. 1. Body weight variation during 7-day treatment with broad-spectrum antibiotics with or without nitrate supplementation. A) For each animal, initial body weight (D0), was considered 100% and the increase or decrease from then onwards was calculated in respect to this initial value. B) Comparison of body weight variation at the end of the treatment (D7) between groups exposed to antibiotics with or without nitrate. C) Water consumption *per* cage during the treatment. Each cage contained two animals and the volume consumed was measured daily. All symbols represented in panel A correspond to the comparison between the experimental condition and the control for the same day; $^{*}p < 0.05$; $^{\$}p < 0.001$; $^{\#}p < 0.0001$. Also, significant differences were observed between the groups exposed to antibiotics and antibiotics + nitrate for day 4 ($p = 0.035$), 5 ($p = 0.04$) and 7 ($p = 0.03$), $n = 8$.

method (Bio-Rad).

Immunoblotting. Tissue homogenates were denatured by adding loading buffer (Tris base 250 mM, SDS 6.25%, glycerol 25%, β-mercaptoethanol 12% and a pinch of bromophenol blue) followed by heating at 95 °C for 5 min. Samples were then applied on SDS–10% polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to PVDF membranes. Nonspecific binding sites were blocked with 3% defatted dry milk (30 min). Membranes were then incubated with iNOS, myeloperoxidase (1:250, Santa Cruz Biotechnology, Frilabo, Portugal), occludin and claudin-5 primary antibodies (1:100, Invitrogen). Membranes were washed with TBST (Tris-buffered saline with 0.1% Tween) and then probed with a goat anti-rabbit antibody (Abcam, United Kingdom) for 2 h. After another set of washes, labeling was detected by incubation with ECF (Enhanced Chemifluorescence substrate, GE Healthcare, United Kingdom) for 5 min and analysed using a fluorescence image analysis system (Typhoon; GE Healthcare). Primary and secondary antibodies were then stripped off and the membranes reprobed with an actin primary antibody (Santa Cruz Biotechnology, Frilabo, Portugal) and the same procedure was performed. Densitometry was performed using ImageJ software (National Institutes of Health, USA) and all band intensities were corrected to actin.

Immunofluorescence. Cryosections (8 μM) of the glandular stomach were obtained and fixed in paraformaldehyde 1% (10 min) followed by two washes of 15 min (PBS and 0.5% Triton). Non-specific binding was prevented by incubation with 10% donkey serum (Sigma-Aldrich, Portugal) diluted in TBST for 1 h. Then, tissue slices were incubated overnight at 4 °C with anti-occludin primary antibody (1:100, Invitrogen, Alfagene, Portugal) in a humidified chamber. Slices were then washed with PBS and incubated for 1 h with donkey anti-rabbit secondary antibody (Alexa Fluor, Santa Cruz Biotechnology, Frilabo, Portugal). Nuclei were stained with Hoescht (1:10000, Sigma-Aldrich, Portugal). Negative controls, with the secondary but not the primary antibody were performed in all experiments. Preparations were observed under a fluorescence microscope (Zeiss Axiovert 200; Carl Zeiss MicroImaging, Germany). Fluorescence intensity was evaluated with ImageJ software (National Institutes of Health, USA).

Statistical analysis. The results are expressed as the mean ± SEM of the values in each group. Unless otherwise stated, each experimental group comprised 8 animals ($n = 8$), except for the group exposed to antibiotics and nitrate in which $n = 7$. One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare three or more conditions. Two-sample comparison was performed using unpaired and two-tailed Student t tests. A probability value (p) of less than 0.05 was considered significant. GelComparII (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse and compare DGGE profiles. Cluster analysis was performed using the UPGMA method (group average method) applying Pearson correlation coefficient. Diversity (Shannon - H′) index of bacterial communities was calculated based on DGGE banding data using PAleontological STatistics (PAST) version 1.34 [26], considering the number of DGGE bands as an estimation of the number of species (S) and band intensity as an estimation of species abundance. Significance of differences among diversity index was performed using the *t*-test through PAST.

3. Results

3.1. Inorganic nitrate prevents body weight loss and cecamegalia induced by broad-spectrum antibiotics

One animal of group 3 (antibiotics + sodium nitrate) was excluded from the experimental procedures, as it did not comply with the humane endpoints established (presented progressive dehydration and lost 25% of body weight). Regarding the other animals, while rats in control and nitrate groups displayed constant weight gain during the course of the 7-day experimental period, animals exposed to broad-spectrum antibiotics (groups 2 and 3) showed weight-loss during the first 2 days of the experimental period. Overall, the animals treated with antibiotics exhibited lower body weight gain along the experimental period as compared to the other groups, while animals receiving antibiotics plus nitrate were able to gain weight in a way between antibiotics and control group (Fig. 1A). At the end of seven days of

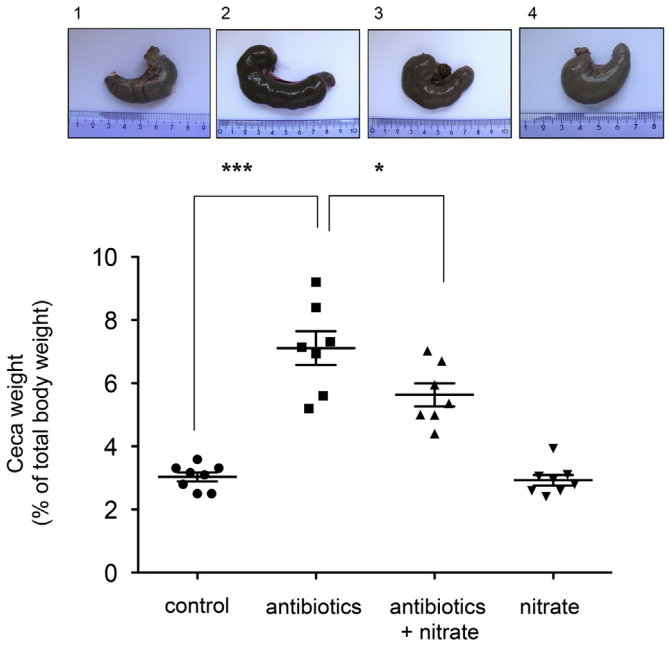

Fig. 2. Variation of relative ceca weight during antibiotic and nitrate consumption. Dysbiosis induces a significant increase of ceca weight while the simultaneous exposure to nitrate reduces antibiotic-induced cecamegalia. No differences were observed between control and nitrate groups. Representative photographs of ceca from control (1), antibiotic (2), antibiotics + nitrate (3) and nitrate (4) groups. Ceca weight was calculated for each animal in relation to its whole body weight. ***$p < 0.0001$; *$p < 0.05$.

treatment, animals exposed to antibiotics gained 1.9 ± 1.8% of body weight while the group simultaneously exposed to nitrate increased 8.9 ± 1.8% of body mass ($p = 0.02$) (Fig. 1B). No differences were observed between control and nitrate groups (14.9 ± 1.6% vs 15.5 ± 2.4, $p = 0.83$). No differences were observed on water consumption (Fig. 1C). Interestingly, animals exposed to broad-spectrum antibiotics exhibited larger and heavier ceca (Fig. 2) when compared to the control group (7.1 ± 0.5% vs 3.0 ± 0.1%, $p = 0.0038$). Cecamegalia was significantly reduced when antibiotics were administered in addition to nitrate (7.1 ± 0.5% vs 5.6 ± 0.4%, $p = 0.042$). No differences were observed between control and nitrate groups.

3.2. Gut microbiota

Stool samples were used to investigate gut microbiota profile. Bacterial DNA was amplified by PCR and the amplicons were resolved by DGGE. DGGE profiles reflect the structure of the communities, in which the number of bands is indicative of the richness in phylotypes. Hence, as shown in Fig. 3A, animals exhibited a rich and diverse microbiome before the dietary intervention. As expected, broad spectrum antibiotics altered the structure of the microbiome and eradicated most of bacteria from gut lumen, as can be appreciated by the reduction of the number of bands in samples collected after antibiotic treatment (Table 1, Fig. 3B). Shannon's diversity index decreased from 3.03 ± 0.06 in the control group (D7) to 2.22 ± 0.02 in the group exposed to antibiotics (D7). Accordingly, a significant decrease in microbial richness was also observed between these groups: 30 ± 4 vs 13 ± 2 ($p = 0.0016$) (Fig. 3B). At the end of 7 days of treatment, the microbiota richness was 43.7 ± 3.6% in the group of animals exposed to antibiotics (with a reduction of c.a. 56%) and 52.8 ± 1.8% (with a reduction of c.a. 48%) in animals exposed to antibiotics and nitrate (both values are expressed in comparison to D0). Despite this tendency suggesting that nitrate may preserve, at some extent, gut microbial richness during antibiotherapy, no significant differences were observed ($p = 0.068$) (Fig. 3C). No differences were observed between control and nitrate groups.

3.3. Nitrate increases the expression of occludin and claudin-5 in the stomach under dysbiosis

As expected, exposure to broad-spectrum antibiotics tended to decrease the expression level of both occludin and claudin-5 in the gastric epithelial lining. Interestingly, when the antibiotic cocktail is supplemented with a pharmacological dose of nitrate, the expression of both proteins increases significantly (75.5 ± 87.0% vs 129.7 ± 18.1% for occludin, $p = 0.0343$ and 38.8 ± 4.2% vs 92.6 ± 12.9% for claudin-5, $p = 0.0201$) (Fig. 4). No differences were observed between the control group and the animals exposed to nitrate alone. The immunofluorescence data adds, notwithstanding, that occludin remains located in the epithelial cell membrane in all conditions tested (Fig. 5).

3.4. Gastric mucosal inflammatory pathways are hindered by dietary nitrate during antibiotherapy

Myeloperoxidase is an enzyme expressed by polymorphonuclear cells that is involved in antimicrobial responses by generating oxidizing agents [27]. Similarly, inducible nitric oxide synthase (iNOS) is an enzyme that increases the production of $^{\bullet}NO$ during inflammatory events. Thus, both enzymatic activities are associated with inflammatory responses and, as shown in Fig. 6, it is noteworthy that the expression level of both myeloperoxidase and iNOS is reduced in the gastric mucosa when animals are exposed to antibiotics and nitrate in comparison to the exposure of antibiotics alone (97.5 ± 10.9% vs 64.2 ± 5.4%, $p = 0.0218$ for myeloperoxidase and 279.8 ± 88.1% vs 57.3 ± 18.4%, $p = 0.0280$ for iNOS).

4. Discussion

The description of the nitrate-nitrite-NO pathway in the mid 1990's has sparked intensive research in an attempt to understand the health benefits of a nitrate-rich diet and how it could be availed to boost gastrointestinal and systemic welfare [2,28–31]. In this regard, oral microbiota is essential for the physiological functions of nitrate as in the absence of nitrate-reducing bacteria, gastric $^{\bullet}NO$ production is markedly reduced and most of the systemic vascular effects of nitrate are attenuated [32,33]. Recent studies highlight the importance of dietary nitrate in ensuring a pool of nitrate-reducing bacteria in the oral cavity, thereby promoting endogenous nitrite and $^{\bullet}NO$ synthesis along the gut and systemically [23,31]. Moreover, nitrate may also have an impact in intestinal microbiome as it has been demonstrated that a fruit-juice based diet increases the abundance of the phylum *Bacteroidetes* and decreases *Firmicutes* in the intestinal microbiota [34]. However, neither the local effects of nitrate in the gastrointestinal mucosa nor the impact in luminal microbiome have been studied in the context of antibiotic-induced dysbiosis. Antibiotics are major contributors to the disturbance of the dynamic equilibrium between commensal and opportunistic species, sometimes allowing pathogenic bacteria to thrive, while diminishing the population of beneficial bugs [35]. It is herein shown that a cocktail of broad spectrum antibiotics, administered in a rat-therapeutic dose, induces body weight loss and cecamegalia while eradicating most of the bacterial flora. It is tempting to speculate that antibiotics may eliminate bacteria involved in nutrient harvesting thereby reducing energy extraction from the chow (reviewed in Ref. [36]) and thus preventing body mass increase. Also, in accordance to what has been described for germ-free animals [37], rats exposed to neomycin, bacitracin and imipenem showed larger and heavier ceca than controls but this effect was inhibited by nitrate. Cecamegalia observed in germ-free animals has been associated with the fact that, in the absence of gut commensals, endogenous and exogenous proteins as well as glycoproteins and mucopolysaccharides are not digested into monomers and

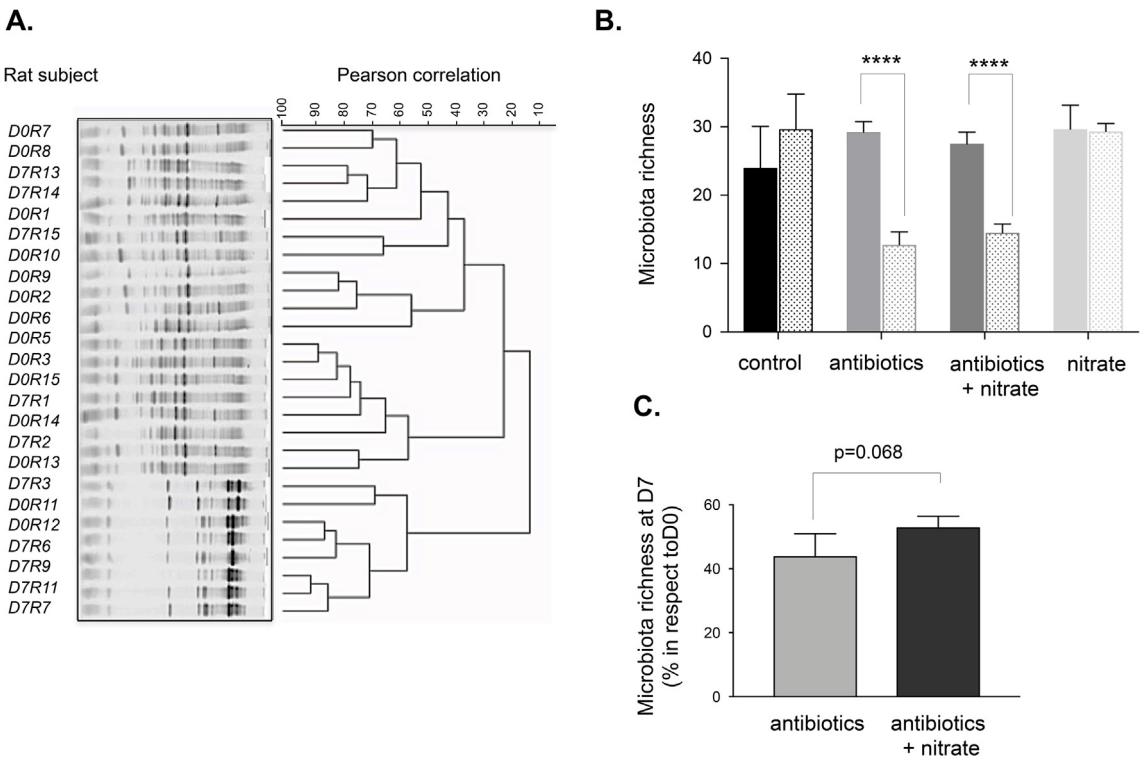

Fig. 3. Structure of gut microbiome communities of rats exposed to broad spectrum antibiotics and/or nitrate. A) DGGE gel exhibiting gut microbial richness. Bacterial richness and diversity were analysed for 4 animals *per* group and two major clusters were obtained: one corresponds to all animals before the treatment (all samples presented as D0) plus the animals exposed to control conditions for 7 days (D7R1-4) and to nitrate (D7R13-16); the other cluster corresponds to all animals exposed for 7 days to antibiotics (D7R5-8) or antibiotics plus nitrate (D7R9-12). B) Microbial richness, traduced by the number of bands obtained for each animal microbiome, before (solid columns) and after (dashed columns) treatment. C) Microbiota richness after 7 days of treatment with antibiotics with or without nitrate, calculated for each animal, in respect to their respective microbial richness at D0 (considered as 100%). ****p < 0.0001. *Sample nomenclature: D0 – day 0; D7 – day 7; R – rat; R1-4: control; R5-8: antibiotics; R9-12: antibiotics + nitrate; R13-16: nitrate.*

Table 1
Diversity metrics for fecal microbiome upon supplementation with nitrate with or without antibiotics.

Group treatment (n = 8)	Shannon diversity index ± SD	Richness (number of bands ± SD)
Control		
D0	2.74 ± 0.06	24 ± 5
D7	3.03 ± 0.02$^{\$\$}$	30 ± 4$^{\$\$}$
Antibiotics		
D0	3.00 ± 0.01	29 ± 1****
D7	2.22 ± 0.02$^{\$\$}$	13 ± 2$^{\$\$}$
Antibiotics + nitrate		
D0	2.92 ± 0.01	28 ± 2****
D7	2.33 ± 0.04$^{\$\$}$	15 ± 1$^{\$\$}$
Nitrate		
D0	2.98 ± 0.03	30 ± 3
D7	3.13 ± 0.02	29 ± 1

$^{\$\$}$ Significant differences on microbial richness and diversity between controls (at D7) and animals exposed to antibiotics or antibiotics + nitrate (at D7) ($p < 0.002$).
**** Significant differences on microbiome richness between D0 and D7 in the groups exposed to antibiotics and antibiotics + nitrate ($p < 0.0001$).
No statistical differences were observed neither in microbial richness ($p = 0.178$) nor diversity ($p = 0.316$) at D7 between animals exposed to antibiotics or antibiotics + nitrate.

therefore are not absorbed, but are instead accumulated in the intestinal lumen (reviewed in Ref. [16]). Interestingly, these metabolic and anatomic changes are accompanied by differences on the expression of tight junction proteins. In fact, many studies have reported that different models of dysbiosis disrupt gastrointestinal tight junctions skeleton and increase epithelial permeability [38,39]. Actually, this phenomenon has been associated with multiorganic disorders such as type 2 diabetes, obesity and gastrointestinal symptoms such as dyspepsia, diarrhea and heartburn [40–42]. Here, it is confirmed that broad-spectrum antibiotics tend to decrease the expression of gastric occludin and claudin-5, two major transmembrane proteins of the tight junctions, but dietary supplementation with nitrate recovers the expression of both proteins towards control levels. Apart from putative effects on gastric microbiome, nitrate has been shown to promote nitrite and $^{\bullet}$NO-derived reactions in the acidic lumen, targeting proteins for post-translational modifications [14,15,43], including occludin tyrosine nitration [22]. However, further studies are required to ascertain as to whether these differences have any functional impact. Indeed, although both claudin-5 and occludin have been shown to ensure the fence function and promote selective paracellular permeability, functional tight junctions without occludin have been reported in testis [44]. Thus, future functional studies on intestinal permeability will elucidate if nitrate, by increasing claudin-5 expression during dysbiosis, also diminishes epithelial permeability. Moreover, antibiotics have also shown to increase the expression of iNOS suggesting the activation of local inflammatory pathways beyond the constitutive and physiological *threshold* of the gut mucosa. Intriguingly, myeloperoxidase does not increase upon antibiotic exposure despite the activity of this enzyme has been shown to increase upon neomycin and bacitracin administration by other groups [45]. The different observations regarding the levels of iNOS and myeloperoxidase upon antibiotic exposure may indicate that different inflammatory pathways may be operative: while iNOS is constitutively expressed in mucosal lymphocytes, myeloperoxidase reflects the dynamic recruitment of neutrophils and other polymorphonuclear cells from the systemic circulation. Interestingly, the simultaneous exposure to the antibiotic cocktail and nitrate reduce

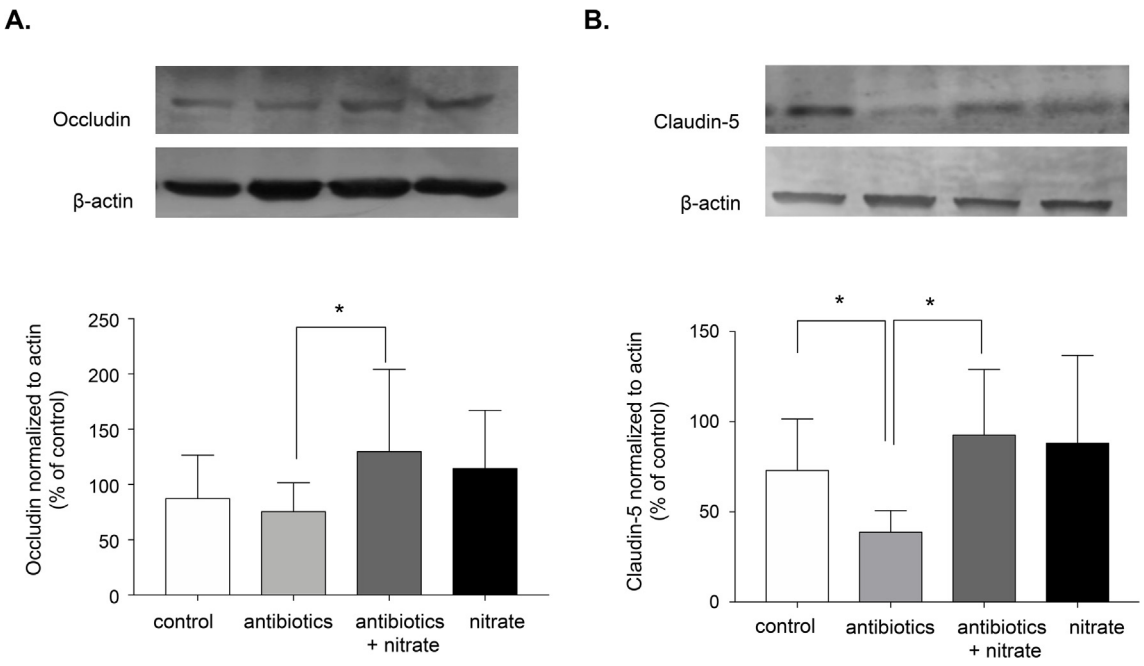

Fig. 4. Expression of tight junction proteins in the gastric epithelial lining. Occludin (A) and claudin-5 (B) immunoreactivity decrease upon a 7-day treatment with broad-spectrum antibiotics while simultaneous supplementation with sodium nitrate recovers both proteins levels to values not significantly different from controls. No significant differences were observed between controls and animals fed with antibiotics ($p = 0.946$) nor between controls and animals fed with antibiotics + nitrate ($p = 0.185$) for occludin. *$p < 0.05$, $n = 8$.

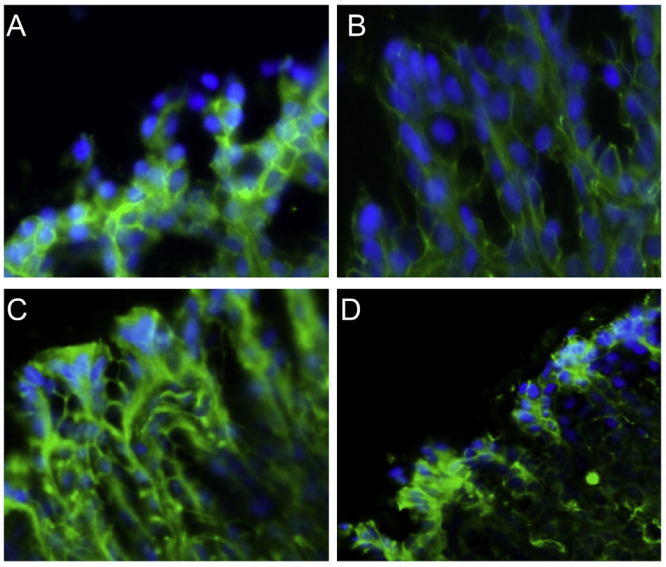

Fig. 5. Microphotographs of the gastric mucosa showing occludin (green) located at the epithelial cell membrane of the gastric pits. In all conditions (A – control, B – antibiotics, C – antibiotics + nitrate and D – nitrate) occludin is located at the cell membrane suggesting no internalization of the protein. Nuclei are stained as blue (Hoescht). Original magnification 200X.

myeloperoxidase levels bellow control. This puzzling observation may suggest that inorganic nitrate may attenuate the recruitment of the polymorphonucelar cells both under constitutive and inductive inflammatory events [12]. In agreement, nitrate has been shown to decrease the expression of adhesion molecules (ICAM, P-selectin) needed by polymorphonuclear cells to translocate from the systemic circulation into the intestinal mucosa and therefore may brake local inflammatory processes [12]. The data herein presented adds that, during antibiotherapy, nitrate is able to reduce both iNOS and myeloperoxidase expression, indicating that it may hamper local inflammation under this condition.

Surprisingly, the molecular, metabolic and anatomical alterations induced by broad spectrum antibiotics, that were reversed or attenuated by nitrate, were not paralleled by gross differences on the intestinal microbiome. When microbial richness was analysed for each animal before and after the treatment, it was observed that after 7 days of exposure to antibiotics (D7), the animals maintained 43.7 ± 3.6% of the initial (D0) microbiota richness. However, when nitrate was offered in addition to antibiotics, the microbial richness at D7 was 52.8 ± 1.8% in respect to D0. Despite this tendency suggesting that nitrate may preserve, at least partially, gut microbiota richness during antibiotherapy, no significant differences were observed ($p = 0.068$). The analysis of these results have to be done in the light of several experimental constrains. First, DGGE allows the detection of dominant microorganisms of a given ecosystem, that is, bacteria that comprise > 1% of the community. Thus, changes in non-dominant bacteria, that notwithstanding may have important physiological functions (such as nutrient harvesting bacteria) may escape to this methodology. Second, it may be possible that although no differences were seen in microbiota composition, functional changes may occur. In fact, some bacterial species may respire on nitrogen derivatives and therefore a luminal increase of nitrate may impact on bacterial metabolic pathways and not necessarily on the community structure. Of note, several lines of evidence now suggest that circulating nitrate may be secreted from the circulation into the gut lumen where it can be reduced to nitrite, $^{\bullet}$NO and ammonia [24,46]. Hence, changes in the metabolic activity of certain species, without affecting their survival and/or proliferation, may ensure the inter-kingdom crosstalk and keep epithelial fence function and inflammatory pathways under a physiological threshold. In this context, future studies on the microbial community metabolome would provide further insights concerning the influence of the nitrate-nitrite-NO pathway on gut microbiome structure and activity. Finally, one may not exclude that, as shown both in rodents [23] and humans [31], dietary nitrate induces a shift in the structure of the oral bacterial community. Physiological concentrations of nitrate significantly alter the operational taxonomic units in the mouth, by increasing species with denitrification activity and thus promoting nitrite and $^{\bullet}$NO

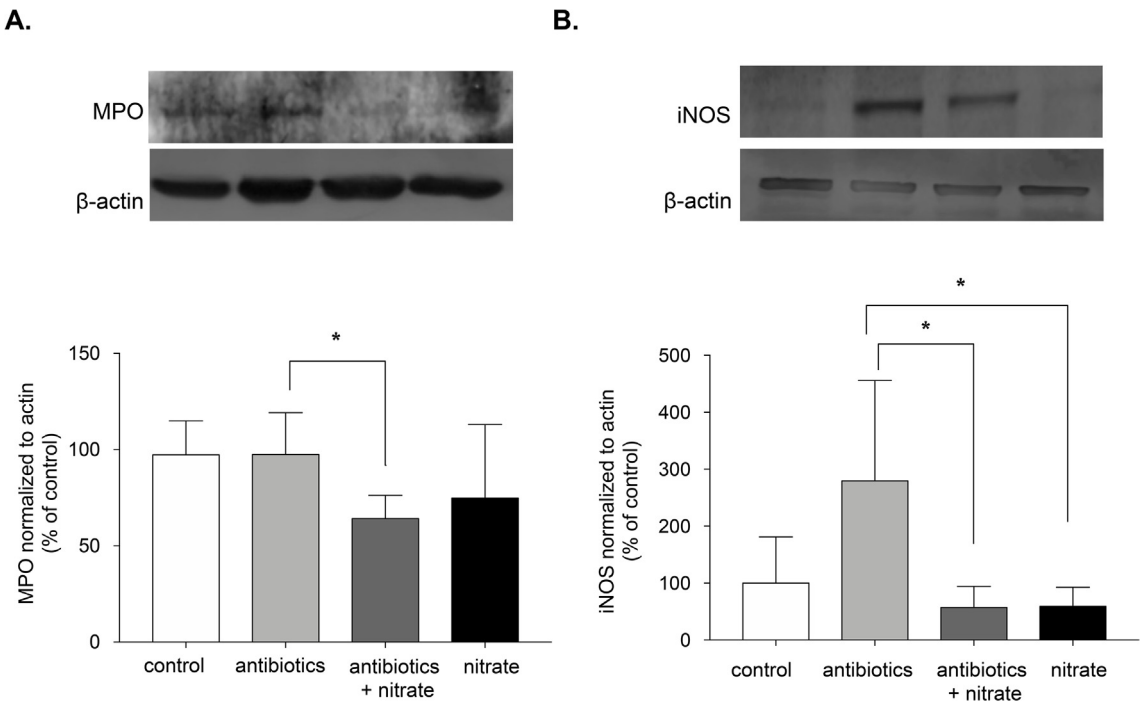

Fig. 6. Protein expression levels of inflammatory markers such as myeloperoxidase (A) and iNOS (B), are reduced in the gastric mucosa when animals exposed to antibiotics are also fed with nitrate. Also, while dysbiosis does not trigger a significant increase of myeloperoxidase levels, iNOS is strongly increased, suggesting the activation of local inflammatory pathways. However, nitrate reduced iNOS to control and myeloperoxidase to levels bellow control, which may suggest that different inflammatory pathways may operate under antibiotic-induced dysbiosis. *$p < 0.05$, $n = 8$.

synthesis [23,31]. Even though no significant differences were seen in fecal microbiome, it is possible that potential changes on oral microbiome may increase plasma nitrate and nitrite and thus fuel nitrate secretion into the intestinal lumen thereby providing nitrogen for bacterial respiration along the bowel. In this regard, one should furthermore consider that the antibiotics used in the present study may also kill some oral bacteria, such as the denitrifying strains, essential to reduce dietary nitrate to nitrite. Therefore, the measurement of plasma nitrate and nitrite is important to ascertain as to whether changes on oral microbiome would influence the enterosalivary circulation of nitrate thereby boosting or hampering the nitrate-nitrite-nitric oxide pathway. Under physiological conditions, similar experimental settings (10 mM sodium nitrate dissolved in the drinking water) yielded c.a. 50–100 μM plasma nitrate and c.a. 300–500 nM plasma nitrite [47]. Lower nitrate doses, such as 10 mg/kg, increased plasma nitrate to c.a. 400 μM after 30 min of oral intake and plasma nitrite to 300–400 nM in human volunteers [48]. However, while humans are able to actively transport nitrate from the blood into saliva due to sialin transporters [49], rodents apparently lack this ability and do not concentrate dietary nitrate as efficiently as humans in the oral cavity. This may indeed by one of the reasons why in rats and mice higher doses of nitrate are usually needed to obtain a nitric oxide-like effect in comparison with humans. In accordance, Montenegro et al. have recently shown that upon the administration of 0.1 mmol/kg of nitrate, plasma nitrite increases more than threefold in humans and only 1.3–1.5 times in rodents, to c.a. 300–400 nM [50]. Still, these *in vivo* kinetics must necessarily be addressed when the microbiome is being modulated by the administration of antibiotics, probiotics, prebiotics or any other intervention as the oral microflora may also be affected and impact on the nitrate-nitrite-nitric oxide pathway. Thus, the absence of data regarding plasma levels of nitrate and nitrite is a limitation of the present study. Moreover, in future studies it is not only mandatory to measure plasma nitrate and nitrite but also to measure gastric and intestinal $^{\bullet}$NO. If changes in oral microbiome promote of prevent nitrate reduction, changes in gastric $^{\bullet}$NO should also be expected.

In conclusion, dietary nitrate may be envisaged as a key component of functional foods with a beneficial impact on gastric mucosal integrity and likely on gut microbiota functional performance. Thus, nitrate consumption may be useful during antibiotherapy in order to prevent gastrointestinal dysfunction and possibly symptoms such as heartburn, dyspepsia and diarrhea.

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