

Research Article

Nitric Oxide Production by the Human Intestinal Microbiota by Dissimilatory Nitrate Reduction to Ammonium

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The free radical nitric oxide (NO) is an important signaling molecule in the gastrointestinal tract. Besides eukaryotic cells, gut microorganisms are also capable of producing NO. However, the exact mechanism of NO production by the gut microorganisms is unknown. Microbial NO production was examined under in vitro conditions simulating the gastrointestinal ecosystem using L-arginine or nitrate as substrates. L-arginine did not influence the microbial NO production. However, NO concentrations in the order of 90 ng NO-N per L feed medium were produced by the fecal microbiota from nitrate. ¹⁵N tracer experiments showed that nitrate was mainly reduced to ammonium by the dissimilatory nitrate reduction to ammonium (DNRA) pathway. To our knowledge, this is the first study showing that gastrointestinal microbiota can generate substantial amounts of NO by DNRA and not by the generally accepted denitrification or L-arginine pathway. Further work is needed to elucidate the exact role between NO produced by the gastrointestinal microbiota and host cells.

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1. Introduction

Nitric oxide (NO), a free radical with moderate reactivity, has emerged as an important signaling molecule in a multitude of physiological systems in the human body [1]. In the gastrointestinal tract, NO is involved in the regulation of regional blood flow, smooth muscle relaxation, secretory and immunological regulation [2]. During inflammation the production of NO is induced. Rectal NO concentrations in healthy volunteers (range 3–25 ppbv) are significantly lower than the concentrations in patients with active inflammatory bowel disease (IBD) (range 71–8978 ppbv) [3]. NO can be synthesized in eukaryotic cells through oxidation of L-arginine by NO synthase (NOS) [4, 5]. Depending on the isoform of NOS catalyzing the reaction, nanomolar (endothelial NOS and neuronal NOS) or micromolar (inducible NOS) NO concentrations are produced serving as a messenger or as an antibacterial agent. Besides eukaryotic NO production, it is well known that NO can be produced also by microorganisms.

A first way of microbial NO synthesis can be achieved from L-arginine by bacterial NOS (bNOS) with features

resembling the eukaryotic NOS (eukNOS). bNOS activity has been described in a group of Gram-positive bacteria [6–14] and genome sequencing has revealed genes coding for similar proteins that are however shorter than the eukNOS [15]. bNOS from *Bacillus subtilis*, *Bacillus anthracis*, *Deinococcus radiodurans*, and *Streptomyces* spp. display NO-forming activity in vivo dependent on arginine [14–17]. Besides L-arginine, nitrate and nitrite can also serve as an N-source for NO. Per day, between 0 and 10 mg NO₃⁻-N can reach the large intestine in healthy persons [18]. Feces were shown to contain around 0.15 mg NO₃⁻-N/L [3]. In vitro studies showed a correlation between the concentration of the added nitrate or nitrite and the concentration of NO produced by the fecal microbiota [19]. The metabolic process best known for the microbial production of NO, and extensively described in soils, sediments and water treatment plants, is denitrification [20]. This dissimilatory process uses oxidized nitrogen species as final electron acceptors when oxygen levels are limiting. During the denitrification process nitrate or nitrite is reduced to N₂ gas with NO and N₂O as intermediates [21]. At steady-state conditions, the NO concentrations range between 14 and 900 ng NO-N/L

aqueous phase depending on species and conditions [22]. Another NO producing mechanism is by the reduction of nitrite. For lactobacilli and bifidobacteria it is not clear whether NO production from nitrite occurred biologically or chemically. Hence, acidic nonenzymatic reduction of nitrite was suggested as the predominant pathway for NO production in vitro by lactic acid producing bacteria (lactobacilli and bifidobacteria) as the produced organic acids decrease the pH of the agar plates to 4 [19]. Whereas NO production by lactic acid bacteria was considered to be chemical, NO production from nitrite by *Escherichia coli* and *Salmonella typhimurium* was shown to be biological. The enzymes responsible for the NO formation from nitrite in *E. coli* and *S. typhimurium* are periplasmic and cytoplasmic nitrite reductase and nitrate reductase, respectively [23, 24].

To date, the relevance and mechanism of bacterial NO production under gastrointestinal conditions remain elusive. With NO being produced by inducible NOS in epithelial cells as a response to cytokines and bacterial products [25], it is of interest to know whether bacterial NO production can occur under gastrointestinal conditions. The objective of our study was to unravel the mechanisms by which human fecal microbiota are able to produce NO in vitro. Three possible pathways were considered (i) L-arginine as substrate for NO synthase, (ii) denitrification to nitrogen gas and (iii) dissimilatory nitrate reduction to ammonium (DNRA). The latter was considered as fermentative nitrate reduction is expected under gastrointestinal conditions however no relation with NO production has been considered [26, 27].

2. Materials and Methods

2.1. Growth Media. NO production was examined in a mineral and a complex medium. The mineral medium G4M3 is a medium optimized for denitrification [28]. Feed medium is a complex medium simulating the nutritional conditions in the colon. It is composed of arabinogalactan (1 g/L), pectin (2 g/L), xylan (1 g/L), starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L), and L-cysteine (0.5 g/L) [29]. L-cysteine was added to scavenge dissolved oxygen and to lower the initial redox potential. Analyses of the feed medium with ion chromatography indicated that very low concentrations of nitrate, that is, $110 \pm 50 \mu\text{g NO}_3^- \text{-N/L}$, were present.

2.2. Fecal Suspensions. Fecal samples were obtained from 6 healthy women with a mean age of 55.5 ranging between 53 and 59. Fecal suspensions were prepared and stored at -80°C until use [30]. The main group of organisms in fecal samples belongs to the anaerobic Gram-positive bacteria. Anaerobic populations belonging to the genera *Bacteroidetes* and *Firmicutes* dominate [31, 32].

Incubations were performed with fecal suspension of different healthy persons which all showed similar results. In the figures, the results of incubations of one representative person are shown.

2.3. Batch Experiments. Anaerobic incubations were performed in penicillin flasks with a total volume of 120 mL.

The bottles were filled with 50 mL of one of the growth media and the pH was set at 7.0 using a 1 M NaOH solution. The headspace (70 mL) was flushed for 45 minutes with N_2 -gas to create anaerobic conditions. Different parameters (growth media, concentrations of nitrate and L-arginine, pH, dilution of the fecal suspension and supernatant) were varied to examine their effect on the production of NO, as described in Figure 1. For mineral media the concentration of nitrate added was the only parameter tested. Nitrate as a possible N-source for NO was added in concentrations of 5 and 50 mg $\text{NO}_3^- \text{-N/L}$ using a 14 g $\text{NaNO}_3^- \text{-N/L}$ solution. To study L-arginine as N-source, a stock solution of 3.5 g (L-arginine-HCl)-N/L was added to final concentrations of 5 and 50 mg L-arginine-N/L. When incubations were performed in buffered feed medium, a 1 M phosphate buffer (88 g $\text{K}_2\text{HPO}_4/\text{L}$ and 68 g $\text{KH}_2\text{PO}_4/\text{L}$) was added to a final concentration of 150 mM. The pH was measured before and after incubation. Dilutions of the fecal suspension (1/2, 1/10, 1/20, 1/200, and 1/2000) were made in saline (8.5 g NaCl/L). Both for the undiluted and diluted fecal suspensions, 1 mL was inoculated in the growth medium. To study the effect of the supernatant of the fecal suspension on NO production, the fecal suspension was centrifuged at 10 000 g for 15 minutes. The supernatant was collected and 1 mL was added to the growth medium. The penicillin flasks were incubated at 37°C for 24 hours while shaking at 130 rpm.

To assess the production of NO during DNRA, *Escherichia coli* K12 (LMG 18221) (BCCM culture collection, Ghent University, Ghent, Belgium) was grown anaerobically for 24 hours at 37°C in buffered feed medium without additional nitrate.

2.4. ^{15}N Batch Experiments. To determine the reduction of nitrate to N_2 or ammonium by, respectively, denitrification or DNRA, an experiment was set up as described in the section above but using a 14 g $\text{K}^{15}\text{NO}_3^- \text{-N/L}$ (10 atom %) solution to final concentrations of 5 and 50 mg $^{15}\text{NO}_3^- \text{-N/L}$ (10 atom %). Atom % $=^{15}\text{N}/(^{14}\text{N} + ^{15}\text{N})$. The headspace was flushed with Argon gas to avoid interference during $^{15}\text{N}_2$ analyses. Fecal suspensions were added undiluted.

2.5. Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The reactor setup was adapted from the SHIME, representing the different parts of the adult human gut and consisting of a succession of 5 compartments [29]. The first 2 compartments are a fill-and-draw setup and represent the stomach and small intestine. Peristaltic pumps add a defined amount of feed medium (140 mL, 3 times/day) and pancreatic and bile liquid (60 mL, 3 times/day), respectively, to the stomach and duodenum compartments and empty the respective compartments after specified intervals. The last 3 compartments are continuously stirred reactors with constant volume and pH control, specific for each compartment. Upon inoculation with fecal microbiota, these reactors simulate the ascending, transverse, and descending colon. Inoculum preparation, retention time, pH, temperature settings, and reactor feed composition were described previously [33]. Closing off the gas phase of the different colon compartments (ascending, transverse, and descending) upon N_2 flushing

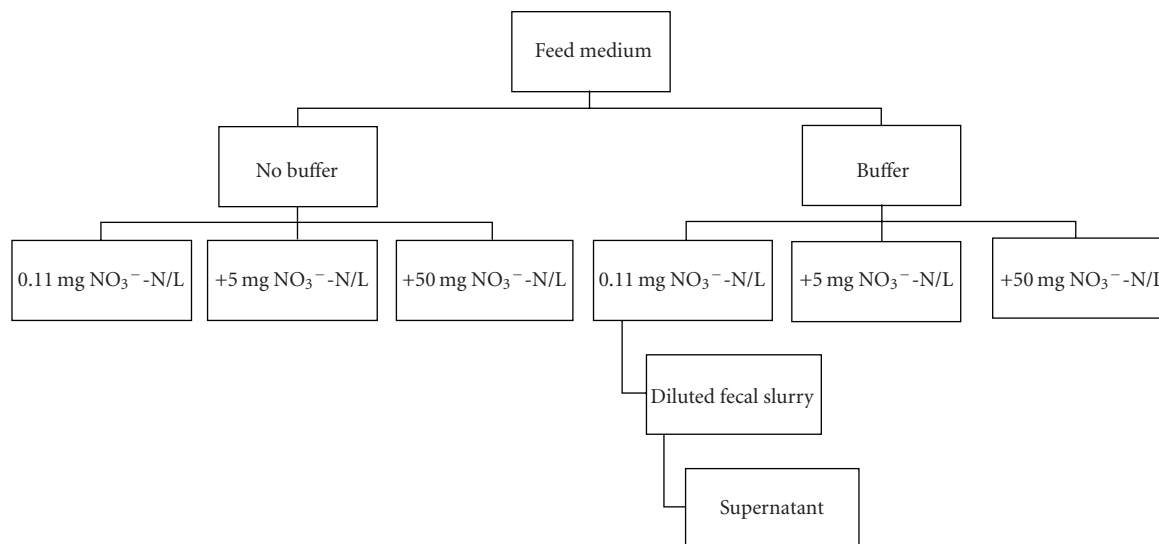


FIGURE 1: Overview of the experimental setup for the different parameters that were studied using feed medium simulating the conditions in the human colon.

allowed to study the NO production in the respective colon regions separately. Gas samples were collected and analyzed for NO. Samples of the different SHIME vessels of the 3 colon parts were collected. All the incubations were done anaerobically in penicillin flasks of 120 mL, as described previously. The suspensions were incubated as such or with 20 mL fresh feed medium. To test the effect of the biomass, the biomass of the suspensions was washed 3 times with saline and resuspended in feed medium. The supernatant was prepared by centrifuging the suspension at 10 000 g for 15 minutes. The supernatant was filtersterilized using 0.22 μm membrane filters (Millipore, NYSE:MIL, USA) or autoclaved.

2.6. Analytical Methods. Both NO_3^- and NO_2^- concentrations were measured before and after incubation of fecal suspension in growth media supplemented with NO_3^- . A Metrohm 761 Compact Ion Chromatograph (Metrohm AG, Herisau, Switzerland) equipped with a conductivity detector and a Metrosep A supp 5 column was used. The operational parameters were as follows: eluent 1.06 g $\text{Na}_2\text{CO}_3/\text{L}$; flow 0.7 mL/min; sample loop 20 μL . The samples were centrifuged at 4000 g for 3 minutes, filtered using 0.45 μm membrane filters, and diluted in milli-Q water before analysis (Millipore, NYSE:MIL, USA).

During ^{15}N experiments, NH_4^+ concentrations were determined colorimetrically by the salicylate-nitroprusside method [34]. The amount of NO_3^- and NO_2^- was measured by first reducing NO_3^- to NO_2^- by a copper-cadmium reductor coil at pH of 8.0. NO_2^- concentrations were determined colorimetrically by an imidazole buffered reaction with N-1-naphthylethylenediamine. The NO_3^- concentration was quantified by subtraction of the concentrations of NO_2^- from the concentration of $(\text{NO}_3^- + \text{NO}_2^-)$ [35]. The samples were centrifuged at 4000 g for 3 minutes, filtered using 0.45 μm membrane filters, and diluted in milli-Q

water before analysis (Millipore, NYSE:MIL, USA). $^{15}\text{NH}_4^+$ analyses were performed after conversion to N_2O using a trace gas pre-concentration unit (ANCA-TGII, SerCON, UK) coupled to an isotope ratio mass spectrometer (IRMS) (20-20, SerCON, UK) [35, 36]. To determine the $^{15}\text{N}/^{14}\text{N}$ ratio of the biomass, the samples were centrifuged at 1750 g for 15 minutes and the pellet was dried at 60°C overnight. The $^{15}\text{N}/^{14}\text{N}$ ratio was determined by an Elemental Analyzer (EA) (ANCA-SL, SerCON, UK) coupled to the IRMS (20-20, SerCON, UK). Analyses were triplicated.

NO measurements were done based on the principle of chemiluminescence using Eco Physics CLD 77 AM (Eco Physics AG, Duernten, Switzerland) with a detection limit of 1 ppbv. Gas samples of 25 mL were collected in syringes from the headspace of the penicillin flasks after 24 hours of anaerobic incubation. The concentrations were calculated by comparison with a standard curve prepared with NO standard gas (98 ± 0.5 ppmv) diluted in air.

For N_2O measurements, 6 mL of gas from the headspace was collected in a vacutainer with a total volume of 12 mL (Labco limited, Buckinghamshire, UK). One mL of this dilution was used for analysis with a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) with an electron capture detector (ECD) and two packed columns (Porapack Q, 80/100 mesh, 1 and 2 m). The operating conditions were as follows: carrier gas N_2 (55 mL/min), injector temperature 105°C, column and oven temperature 35°C, and detector temperature 250°C. The chromatograph was calibrated using N_2O standard gas (250 ± 13 ppmv or 25.3 ± 1.5 ppmv in He).

For N_2 analyses, 12 mL of gas from the headspace was collected in a vacutainer (Labco limited, Buckinghamshire, UK). The gas was analyzed with a Finnigan Trace Ultra GC (Interscience, Breda, The Netherlands) with a thermal conductivity detector (TCD), a precolumn (Hayesep Q, 80/100 mesh, 0.25m) and 2 packed columns (Hayesep Q,

80/100 mesh, 2 m and Molsieve 5Å, 80/100 mesh, 2 m). The injector temperature was 65°C and oven temperature 90°C.

2.7. Calculations. All NO and N₂O measurements were subsequently converted to concentrations present in the growth medium. The NO and N₂O concentrations measured in the gas phase (ppbv) were converted to molar concentrations using the ideal gas law $pV = nRT$ with $T = 294.65$ K. Taking into account $[\text{NO}]_g/[\text{NO}]_{\text{aq}} \cong 20$ and $[\text{N}_2\text{O}]_g/[\text{N}_2\text{O}]_{\text{aq}} \cong 1.64$ at equilibrium, the NO and N₂O concentrations in the gas phase were converted to the concentration in the liquid phase and expressed as g NO-N or N₂O-N per L.

2.8. Statistical Analysis. SPSS version 16.0 (SPSS Inc., Chicago, USA) was used to carry out all statistical analyses. Normality of the data and homogeneity of variances was assessed using the Kolmogorov-Smirnov and the Levene test, respectively. Comparison of normally distributed data was performed with ANOVA or Tamhane; when ANOVA indicated significant differences, means were compared using the Bonferroni comparison test. Comparison of means of not normally distributed data was evaluated with the nonparametric Kruskal-Wallis test; when Kruskal-Wallis indicated significant differences, means were compared using the Mann-Whitney comparison test. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. L-Arginine as N-Source for NO and N₂O Production. The biological production of NO with L-arginine as substrate was studied by supplementing mineral medium and feed medium with 0, 5, or 50 mg L-arginine-N/L. Medium supplemented with 50 mg L-arginine-N/L but not inoculated with fecal suspension, served as a negative control. The pH was set at 7 and NO and N₂O concentrations were analyzed after 24 hours. When L-arginine was supplemented to the mineral medium, only traces of NO (0.28 ng NO-N/L medium) could be detected for all the L-arginine concentrations. In feed medium, the mean NO concentration after 24 hours was for all the samples around 75 ng NO-N/L medium. No proportional effect of L-arginine on the NO production could be observed. No N₂O was detected in any of the samples.

3.2. Nitrate as N-Source for NO and N₂O in Mineral Media Optimized for Denitrification. Fecal microbiota were tested for their ability to produce NO and N₂O by denitrification. Fecal suspensions were inoculated anaerobically for 24 hours in mineral media (with succinate as electron donor) containing 0, 5, or 50 mg NO₃⁻-N/L. Nitrate was completely consumed for all biotic conditions. None of the samples contained nitrite in the beginning or at the end of the incubation and after 24 hours the mean pH was 6.53 ± 0.35 . After 24 hours of incubation, the NO concentrations in the mineral media remained below 1 ng NO-N/L medium in all experiments (Figure 2(a)). A proportional effect was observed for N₂O production and the amount of supplied nitrate (Figure 2(b)). The fecal microbiota without added

nitrate did not produce N₂O. However, addition of 5 and 50 mg NO₃⁻-N/L resulted in significant N₂O production.

3.3. Nitrate as N-Source for NO and N₂O in Feed Medium

3.3.1. Nitrate as N-Source for NO and N₂O in Nonbuffered Feed Medium. To examine whether microbiological production of NO and N₂O was possible under gastrointestinal conditions, fecal microbiota were grown in the presence of a complex medium simulating the conditions in the colon (Figure 1). The feed medium was analyzed for nitrate and a background of 0.11 mg NO₃⁻-N/L was measured. The preparation of feed medium without trace levels of nitrate was not possible. After 24 hours of incubation, the pH dropped to 4.13 ± 0.05 , 4.24 ± 0.03 and 4.47 ± 0.03 for 0.11, 5 and 50 mg NO₃⁻-N/L, respectively. The concentrations of nitrate measured after 24 hours were below 0.5 mg NO₃⁻-N/L for all biotic conditions. Nitrite at time 0 was below 0.1 mg NO₂⁻-N/L but after 24 hours concentrations of 0.1 ± 0.1 and 2.0 ± 0.2 mg NO₂⁻-N/L were detected for 5 and 50 mg NO₃⁻-N/L, respectively. Figures 2(c) and 2(d) show the concentrations of NO and N₂O measured after 24 hours. The NO concentration measured in the medium without nitrate addition was 37.0 ± 0.3 ng NO-N/L. The addition of 5 mg NO₃⁻-N/L increased the NO concentration slightly to 44.1 ± 2.5 ng NO-N/L medium. However the addition of 50 mg/L NO₃⁻-N increased the NO concentration drastically to 298 ± 33 μg NO-N/L medium. As for the mineral media, a positive effect for the N₂O production with the supplemented nitrate was observed.

3.3.2. Nitrate as N-source for NO and N₂O in Buffered Feed Medium. The same experimental setup as described above was used but under buffered conditions (measured pH was always between 6 and 6.8), thus avoiding fermentative acidification during biotic incubations. Nitrate concentrations decreased after 24 hours to 1.0 ± 1.5 and 3.1 ± 1.9 mg NO₃⁻-N/L for 5 mg and 50 mg NO₃⁻-N/L respectively. No nitrite was detected. As shown in Figures 2(e) and 2(f), the NO concentrations increased slightly when nitrate was added. The concentration of N₂O in buffered feed medium supplemented with 5 mg NO₃⁻-N/L was comparable to the concentration measured in the nonbuffered test reported above. For the highest concentration of nitrate a lower N₂O concentration was detected compared to the nonbuffered test (Figure 2).

3.4. ¹⁵N Experiment. A ¹⁵N tracer study was used to link nitrate consumption to denitrification or DNRA by the fecal microbiota under gastrointestinal conditions. The setup was the same as for the buffered feed medium but K¹⁵NO₃ (10 atom %) was used as N-source in 2 different concentrations, that is, 5 and 50 mg ¹⁵NO₃⁻-N/L (Figure 1). Table 1 gives an overview of the nitrogen concentrations measured and the recovery of ¹⁵N after 24 hours of anaerobic incubation. After 24 hours all nitrate was reduced and the control maintained 41.3 ± 4.5 mg NO₃⁻-N/L. The nitrite concentrations were stable over 24 hours of anaerobic incubation. The amount of NO and N₂O produced per volume of feed medium was

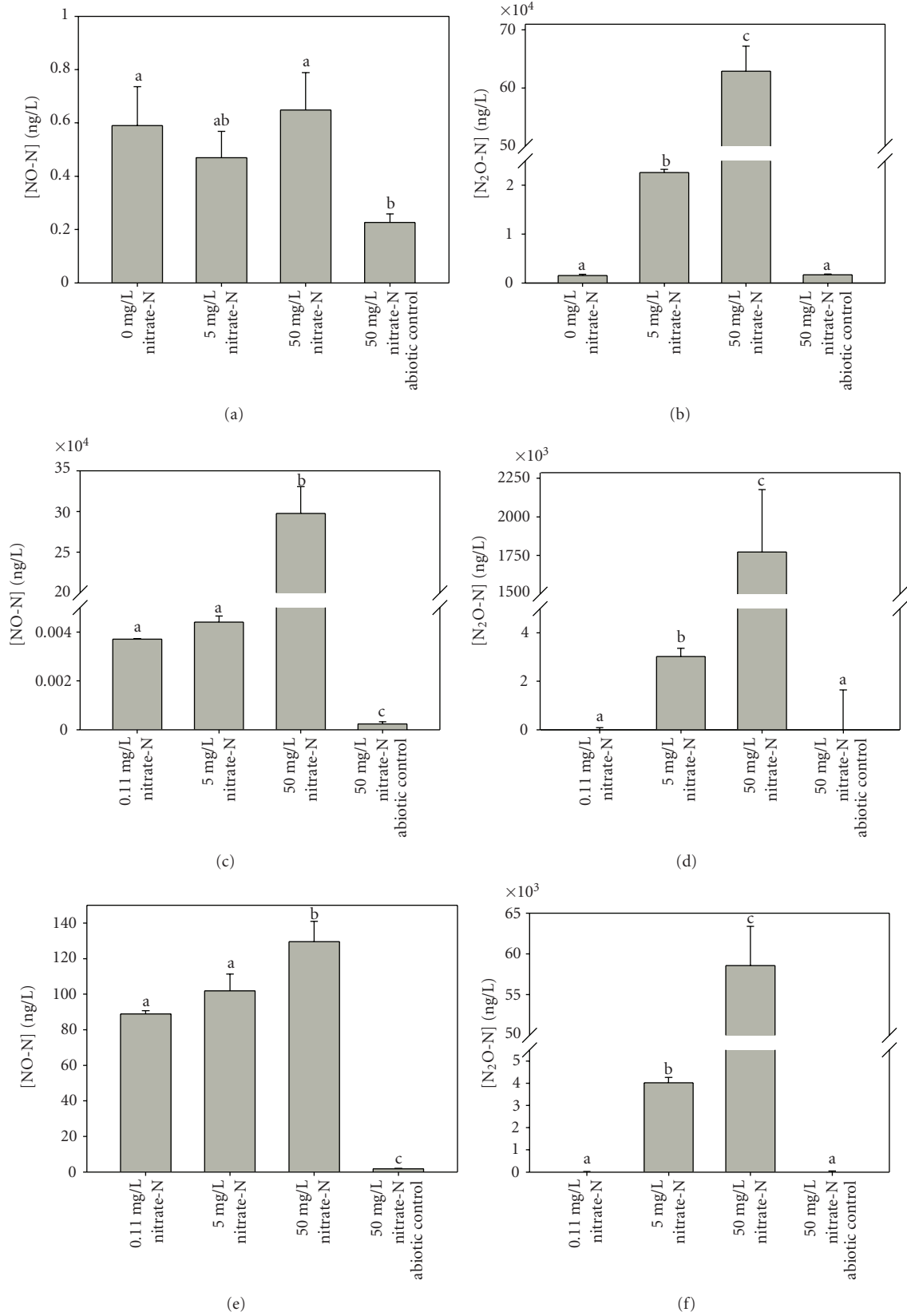


FIGURE 2: NO-N and N₂O-N concentrations produced after 24 hours of anaerobic incubation of the human fecal microbiota in mineral medium (a), (b), nonbuffered feed medium (c), (d), or buffered feed medium (e), (f) with or without the addition of nitrate (*n* = 3). Significant differences are indicated with a, b, or c (*P* < .05). Results are means ± standard deviation of 3 incubations of fecal suspension from 1 representative person.

TABLE 1: Nitrogen concentrations measured after 24 hours of anaerobic incubation of fecal microbiota in feed medium supplemented with $K^{15}NO_3$ (10 atom %). The N_2 concentration measured in the control is subtracted from the concentrations measured in the samples. $^{15}NH_4^+$ and organic ^{15}N are recovered from $K^{15}NO_3$ (10 atom %). Results are means \pm standard deviation of 3 incubations of fecal suspension from 1 representative person.

	5 mg $^{15}NO_3^-$ -N/L	50 mg $^{15}NO_3^-$ -N/L	50 mg $^{15}NO_3^-$ -N/L abiotic control
Nitrate-N (mg/L)	0	0	41.3 \pm 4.5
Nitrite-N (mg/L)	0.1 \pm 0.02	0.08 \pm 0.01	0.4 \pm 0.03
NO-N (ng/L)	111 \pm 5 ^a	215 \pm 1 ^b	2.3 \pm 0.4 ^c
N_2O -N (ng/L)	4020 \pm 246 ^a	58553 \pm 4837 ^b	0 ^c
N_2	0	0	—
$^{15}NH_4^+$ (atom %)	> 10	>10	0
Organic ^{15}N (atom %)	0.05 \pm 0.03	0.14 \pm 0.03	0

^{abc} significant differences between a, b, and c ($P < .05$).

in the same range as what was found in the buffered feed medium experiment (Table 1). High ammonium concentrations, 56.5 ± 6.1 mg NH_4^+ -N/L, were present in the feed medium at time 0. After 24 hours, concentrations reached 182 ± 4 , 210 ± 1 , 275 ± 6 , and 50.1 ± 2.6 mg NH_4^+ -N/L for no extra, 5 mg NO_3^- -N/L, 50 mg NO_3^- -N/L, and the chemical control, respectively.

3.5. NO Production in Buffered Feed Medium During DNRA. To verify if NO can be produced as a side product or as an intermediate during DNRA, *E. coli* LMG 18221, a strain capable of DNRA but not denitrification was grown anaerobically at 37°C in buffered feed medium. After 24 hours, NO concentrations of 170 ng NO-N/L medium were measured. This NO concentration was twice as high compared to NO concentrations produced by the fecal cultures.

3.6. Inhibitory Effect of Fecal Supernatant on NO Production. Figure 3(a) shows that diluting the fecal suspension 200 times before inoculation in buffered feed medium results in a 75% increase in the NO concentration. There was a difference in the NO concentration related to the fecal suspension and optimal dilution. The optimal dilution ranged between 1/20 and 1/200 and the increase of NO between 50 and 75%, respectively. Higher dilutions than 1/200 yielded lower NO concentrations. The addition of supernatant from a fecal suspension resulted in a decrease of the NO concentration (Figure 3(b)).

3.7. Production of NO in the Colon Vessels of the SHIME. A continuous model that simulates the gastrointestinal tract (SHIME) was used to measure NO production by human gastrointestinal microbiota. Feed medium containing trace levels of nitrate (110 ± 50 μ g NO_3^- -N/L) is used to simulate the nutritional conditions. The NO concentrations in the headspace of the 3 colon vessels were analyzed after an incubation period of 24 hours. In the colon ascendens, 101 ng NO-N/L SHIME suspension was measured but lower concentrations were found in the colon transversum and descendens (5.4 and 8.5 ng NO-N/L SHIME suspension, respectively). A batch setup was performed to test whether this difference was due to (i) the microbial community or

(ii) the availability of nutrients in the different colon compartments. The biomass of the different colon compartments was washed and grown anaerobically in fresh feed medium for 24 hours. As references, the microbial suspensions of the different colon vessels were incubated in parallel in penicillin bottles with or without the addition of fresh feed. To exclude a chemical process, cell-free supernatant of the SHIME suspension was filtersterilized or autoclaved and subsequently incubated. Figure 4 shows that the addition of feed increases the NO concentrations at least 3 times but the difference between the first colon vessel and the other 2 was maintained. Incubation of the washed biomass showed that even higher concentrations of NO were produced (ranging between 220 and 250 ng NO-N/L) and that the supernatant that was removed might have an inhibitory effect on the NO production as observed above. No chemical production of NO was seen when the cell-free supernatant of the SHIME suspension was incubated.

4. Discussion

Our results demonstrate that the fecal microbiota of healthy persons are able to produce NO in concentrations up to 90 ng NO-N/L medium when grown in conditions simulating the colon (Figure 2(e)). Adding L-arginine to the feed medium inoculated with fecal microbiota of a healthy person had no effect on the NO production. High amounts of nitrate in combination with low pH resulted in substantial NO and N_2O production (Figures 2(c) and 2(d)). However, NO and N_2O were also formed under pH buffered conditions (Figures 2(e) and 2(f)). These data corroborate the metabolic capacity of the fecal microbiota to reduce nitrate and produce NO. By using ^{15}N tracer experiments, it was shown that nitrate was mainly reduced to ammonium by DNRA suggesting that NO was produced as a side product or intermediate of this pathway. NO was detected not only in the batch tests but also in the in vitro SHIME model.

Indirect evidence from in vitro studies demonstrates that extracellular L-arginine increases the NO production by eukNOS (at concentrations at which the enzyme should be saturated), known as the “L-arginine paradox” [37, 38] and some prokaryotic NOS proteins have been shown to

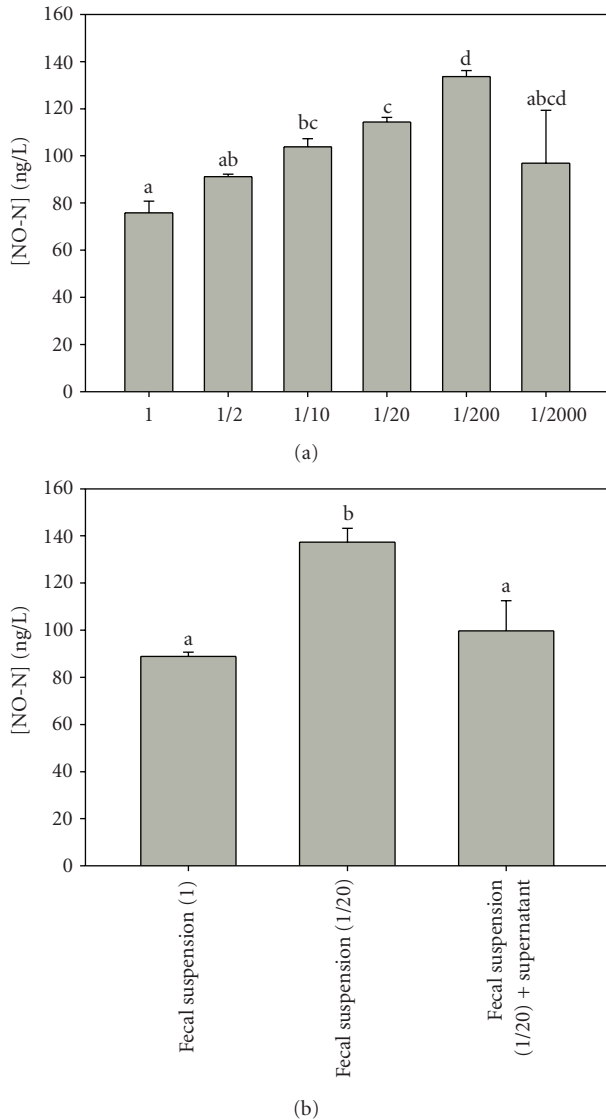


FIGURE 3: NO-N concentrations in function of the different dilutions of fecal suspension (a) and the inhibitory effect of the supernatant of the fecal slurry (b). The inoculated medium was incubated anaerobically for 24 hours and the NO-N concentration measured in the headspace ($n = 3$). Significant differences are indicated with a, b, or c ($P < .05$). Results are means \pm standard deviation of 3 incubations of fecal suspension from 1 representative person.

produce NO in vivo [14–17]. However, our results show that L-arginine does not promote NO production. The reaction from L-arginine to NO is oxygen dependent what might explain why L-arginine has no increasing effect during anaerobic growth of the fecal microbiota. Therefore, we consider the arginine pathway for bacterial NO production under gastrointestinal conditions as not important.

In the lower parts of the gastrointestinal tract, nitrate originates from dietary products rich in nitrate, like spinach, beetroot, or fennel or simply from minor levels as present in drinking water [39]. Nitrate is reduced by the bacteria in the oral cavity or is absorbed in the small intestine. In

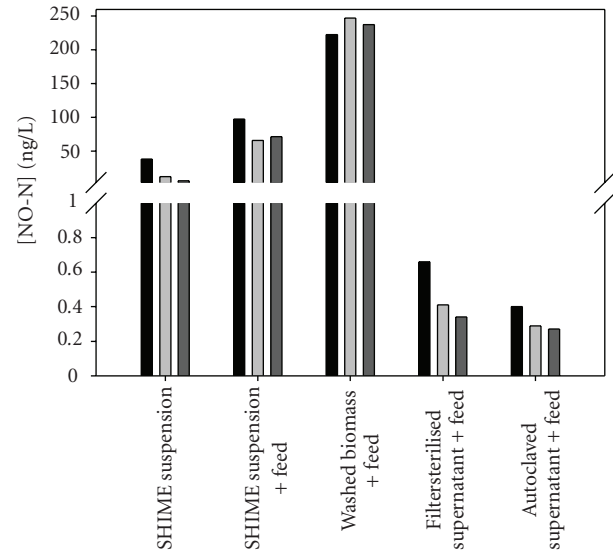


FIGURE 4: NO-N concentrations produced by the microbiota of the 3 colon vessels (black = colon ascendens (CA), light grey = colon transversum (CT), dark grey = colon descendens (CD)) of the in vitro SHIME model measured in the headspace. The microbial suspension of the 3 vessels was incubated anaerobically for 24 hours with or without fresh feed medium or washed and resuspended in fresh feed medium. The supernatant of the SHIME suspension was incubated as well in the feed medium after filtersterilisation or autoclaving.

healthy persons, levels in the order of $\text{mg NO}_3^- \text{-N/L}$ can be estimated in the large intestine [18] and feces were shown to contain around $0.15 \text{ mg NO}_3^- \text{-N/L}$ [3]. Based on these data, 2 different concentrations of nitrate (5 and $50 \text{ mg NO}_3^- \text{-N/L}$) were chosen for the in vitro experiments. Very low concentrations of nitrate were measured in the feed medium receiving no additional nitrate ($110 \pm 50 \mu\text{g NO}_3^- \text{-N/L}$), representative for the concentrations found in feces. When fecal suspensions were incubated in nonbuffered feed medium containing $50 \text{ mg NO}_3^- \text{-N/L}$ very high concentrations of NO ($29.8 \mu\text{g NO-N/L}$) were measured. During the incubations, the pH dropped to a mean value of 4.24 due to the accumulation of organic acids produced by the microorganisms. At these low pH values, a chemical reduction of nitrite to NO (pH below 5.5) cannot be excluded [19]. In vivo, the pH increases over the different colon parts. Considering a pH of 6.37 ± 0.58 in the colon ascendens, 6.61 ± 0.83 in the colon transversum and 7.04 ± 0.67 in the colon descendens [40], a chemical reduction of nitrite to NO in vivo is not likely. Because it was unclear whether NO was produced chemically or biologically, a buffer was added to stabilize the pH between 5.7 and 8. The very high NO concentrations produced at low pH in feed medium containing the highest nitrate concentration were not found at neutral pH. In a study of Sobko et al. [19] the production of NO by fecal microbiota cultivated on ISO-sensitest agar supplemented with nitrate was demonstrated. For the plates supplemented with $1.4 \text{ mg NO}_3^- \text{-N/L}$, concentrations in the range 29–3600 ppbv NO, corresponding to 1–145 ng NO-N/L in the microbial suspension, were measured but a high variability was seen between

different fecal samples. As the pH of the plates decreased from 7 to 5, a chemical reduction could not be excluded. However, it was concluded that the fecal NO generation was not solely due to acidification of the plates [19]. We can conclude that the NO concentrations found in our study, which were between 90 and 100 ng NO/L for low concentrations of nitrate, are in the same order of magnitude.

Under anaerobic conditions, bacteria can use nitrate as N-source in nitrate assimilation and 2 dissimilatory processes, denitrification and DNRA. Denitrification is assumed to be the major nitrate removal pathway in many anoxic ecosystems like soils, sediments, and water treatment plants [20] but information about the occurrence of this process in the human colon is limited [26]. Denitrifying bacteria are found in Alpha-, Beta, Gamma- and Epsilonproteobacteria, Firmicutes, and Bacteroidetes. During denitrification NO is produced in the nanomolar range, without reaching toxic levels [21, 22]. When the supply of electron acceptor is limited, it is more beneficial for the bacteria to exploit DNRA rather than denitrification because of the higher electron consumption [41]. Molecular and biochemical studies have described DNRA in some enteric bacteria [42] but information about the occurrence of this process in complex microbial cultures including the microbiota of the human gastrointestinal tract is limited [20]. Allison and Macfarlane [26] reported that DNRA is the major pathway for nitrate reduction by the fecal microbiota. Identification based on phenotype and genotype showed that *Clostridium ramosum*, *Bacteroides vulgates*, and the enterobacteriaceae were mainly capable of DNRA [27]. These species are representative for the dominant groups of the fecal microbiota [31, 32]. By adding the stable isotope ^{15}N in the form of nitrate, the occurrence of all nitrate-reducing processes under gastrointestinal conditions was studied and quantified. Maximum 0.14 atom % of the added nitrate was used for biomass production. High ammonium concentrations in the medium repress nitrate assimilation and enhance the use of nitrate for the dissimilatory processes [41]. Under gastrointestinal conditions nitrate seems to be completely reduced to ammonium and not to N_2 gas and during this reductive process, NO is produced in low concentrations. To our knowledge, no studies are available indicating NO as an intermediate in DNRA. Further proof for NO production during DNRA was given by *E. coli* K12, a strain capable of DNRA but not denitrification (data not shown) [27]. This strain produced 170 ng NO-N/L under gastrointestinal conditions with low concentrations of nitrate ($110 \mu\text{g NO}_3^-$ -N/L). Evidence for NO as an intermediate in DNRA would be even more decisive when ^{15}N -NO was measured during the ^{15}N tracer study. However, equipment for ^{15}N -NO analyses is very specialized [43] and not in the scope of this paper. Whether N_2O is an intermediate as well or the reduction product of a defense mechanism of bacteria to NO [44] is not clear.

The gaseous NO concentration measured in the colon ascendens (CA) vessel of the in vitro SHIME model was around 15 times higher than the NO concentrations measured in the colon transversum (CT) and colon descendens (CD). During the batch experiments incubation of the

microbial suspension from the different colon vessels showed that the NO concentration of the CA doubled when adding fresh medium. The addition of fresh feed medium decreased the difference in NO concentrations between the colon vessels. Remarkably, when removing the supernatant of the suspension, a 6-fold increase of NO was seen and the difference between the colon vessels disappeared, indicating that the availability of nutrients rather than a difference in the microbial community was responsible for the differences between the colon vessels. Also, a similar inhibitory effect was seen by adding the supernatant of the fecal suspension to the inoculated buffered feed medium. Diluting the fecal suspension to increase NO production confirmed our observation. A possible explanation for this inhibitory effect can be the presence of bile salts. About 5% of the bile salts secreted in the duodenum is available to the microbiota in the colon. To protect them, the microbiota tend to hydrolyze the bile salts but around 5% of the nonconverted bile salts can be found in human feces and will have toxic effects on the bacteria [45]. Diluting the fecal suspension could decrease the toxic effect and thus increase the bacterial metabolism. The same explanation might be valid for the lower NO concentrations in the nonbuffered compared to the buffered feed medium for the 2 lower concentrations of nitrate. As the pH drops during the nonbuffered incubations, the metabolism of the bacteria might be suppressed leading to a lower formation of NO.

Epithelial cells are known to produce NO through oxidation of L-arginine by NOS [4, 5]. Roediger et al. [46] demonstrated elevated luminal NO in the rectum of patients with active IBD. Although bacterial origin was considered, the NO was believed to originate from the mucosa [46]. Several years later, NO concentrations were measured in the rectum that was not cleared from luminal bacteria and again NO concentrations in patients with active IBD were elevated. A bacterial origin for the NO production was proposed [3]. Our results corroborate that the fecal microbiota actively produce substantial levels of NO, even from traces of nitrate. In vitro the fecal microbiota were shown to produce around 100 ng NO-N/L. In vivo no NO was found in the rectum of healthy volunteers but 40 ng NO-N/L was measured in the rectum of patients with active IBD [3]. Externally added NO (in the form of gas or NO donor) has been shown to alter the mucosal barrier function [47–49] and to have an effect on the metabolism of colonocytes [50]. As it was strongly suggested that several constitutive and/or inducible defense systems exist in mammalian cells that neutralize the damaging effects of NO [51], the high rectal levels of NO in IBD patients and the low levels in healthy persons might indicate that the expression of these defense mechanisms in IBD patients is ineffective.

In this work, the human fecal microbiota were shown to produce NO from trace levels of nitrate under gastrointestinal conditions. Moreover, our data indicate that NO was produced by DNRA and not by denitrification or the L-arginine pathway. Studying the interaction between bacterially produced NO and the host epithelial cells might be pivotal in the etiology of inflammatory diseases of the gastrointestinal tract.

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