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Intestinal Formation of *N*-Nitroso Compounds in the Pig Cecum Model

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ABSTRACT: *N*-Nitroso compounds (NOC) are a group of compounds including *N*-nitrosamines and *N*-nitrosamides, which are well-known for their carcinogenic, mutagenic, and teratogenic properties. Humans can be exposed to NOC through the diet and environmentally, or NOC can be formed endogenously in the stomach and intestine. In the intestine, the formation of NOC is supposed to be afforded by the gut microbiota. In this study, the formation of the *N*-nitrosamines, *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine (NPYR), and the *N*-nitrosamides, *N*-nitrosomethylurea (NMU) and *N*-nitrosoethylurea (NEU), was investigated in the pig cecum model after the incubation of the corresponding precursor amine or amide with nitrite or nitrate. Following the incubation with nitrate, the formation of NMOR, NPYR, NMU, and NEU was detectable with the microbiota being responsible for the reduction of nitrate to nitrite. After the incubation of nitrite a chemical formation of NOC was shown.

KEYWORDS: *N-nitroso compounds, N-nitrosamines, N-nitrosamides, N-nitrosopyrrolidine, N-nitrosomorpholine, N-nitrosoethylurea, N-nitrosomethylurea, intestinal formation, pig cecum model*

INTRODUCTION

N-Nitroso compounds (NOC) consist of *N*-nitrosamines and *N*-nitrosamides. Magee and Barnes identified *N*-nitrosodimethylamine (NDMA) in 1956 as a liver carcinogen in rats, [1] and for this reason NDMA was no longer used as a solvent in the rubber and chemical industry.[2] But not only does NDMA as representative of the NOC have carcinogenic potential, out of 300 tested *N*-nitrosamines, over 90% showed carcinogenic activity.[3] The International Agency for Research on Cancer (IARC) classified NDMA, *N*-nitrosodiethylamine (NDEA), *N*-nitrosomethylurea (NMU), and *N*-nitrosoethylurea (NEU) as carcinogens of the category 2A.[4]

Humans are exposed to NOC exogenously through, e.g., food, tobacco smoke, workplace conditions, and cosmetics.[5−7] Additionally, NOC can be formed endogenously mainly in the stomach and the bowel or in infected organs like the bladder.[8,9] Based on the comparison of the NOC intake and the excretion via urine, it was estimated that approximately 50% of the daily exposition of humans with NOC are formed endogenously.[2] In the stomach NOC are formed due to chemical catalysis in the acidic environment or biological catalysis by bacterial growth at higher pH levels.[10,11] Because of the neutral pH in the bowel, a biological catalysis by the microbiota is supposed. Massey et al. showed the necessity of the intestinal microbiota for the formation of NOCs during studies with germfree rats. Apparent total *N*-nitroso compounds (ATNC) were only detectable after feeding nitrate with the drinking water in the feces while bacteria colonized the bowel.[12]

During studies with human volunteers, Rowland et al. showed that the ATNC levels in feces decrease with less NOC uptake via food. If a diet poor in NOC is supplemented with nitrate, the ATNC content in feces rises again.[8]

The endogenous formation of NOC is a possible explanation for the epidemiological connection between a high intake of meat and a higher risk for colorectal cancer.[13−15] This assumption is based on the catalytical effect of the haem group on the formation of NOC. Bingham et al. showed that the ATNC content in feces increased in human volunteers with the intake of red meat, whereas after the intake of white meat no increase of the ATNC levels was observed.[16] The intake of 420 g red meat per day leads to ATNC contents in feces comparable to NOC levels in tobacco smoke.[17] Haem is nitrosated under acidic conditions and afterward can mediate the nitrosation under the conditions in the intestine.[18] All these studies showed the possibility of an endogenous NOC formation.

For different bacterial species the ability to reduce nitrate or nitrite has been shown, e.g., *Pseudomonas* sp., *Lactobacillus* sp., *Bifidobacterium* sp. and some strains of *Escherichia coli*.[19−21] During incubation studies with single strain bacteria, Calmels et al. showed that different enzymes are responsible for a nitrosation reaction. Additionally, in *Paracoccus denitrificans*, *E. coli*, and *Proteus morganii* the nitrosation reaction correlated with the ability of the bacteria to reduce nitrate to nitrite.[21] Cytochrom-cd1-nitrite reductase was identified as an enzyme able to catalyze the nitrosation reaction. It was isolated from *Pseudomonas aeruginosa* and *Neisseria mucosae*.[19] In the literature the formation of NOC in the intestine is mainly explained by enzymatic reactions of these bacteria and probably other bacteria of the microbiota.

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In the literature, mostly ATNC are analyzed during studies of NOC in feces.[8,12,16,17,22] This can lead to false positive results and to an overestimation of NOC due to the detection of *S*-nitrothiols, *O*-nitrosated compounds, and nitrolic acid in addition to *N*-nitroso compounds. Secondary, the ATNC content differs between the different analytical methods.[2] Therefore in this study, the formation of two *N*-nitrosamines (*N*-nitrosopyrrolidine NPYR and *N*-nitrosomorpholine NMOR) and two *N*-nitrosamides (*N*-nitrosomethylurea NMU and *N*-nitrosoethylurea NEU) from its precursor amines/amides and nitrate/nitrite was directly analyzed by gas chromatography coupled with mass specific detection (GC-MS) or liquid chromatography with tandem mass spectrometry (LC-MS/MS). By this, differences concerning the formation of the model NOC were detectable. In addition, the pig cecum model enables the simulation of the conditions in the intestine. Experiments were performed with a fecal suspension containing a crude bacterial mixture consisting of 400−1000 different bacterial species.[23−25] Experiments with fluorescence in situ hybridization have shown higher interindividual variations between different pigs than interspecies differences between humans and pigs.[26] Therefore, the pig cecum model simulates the human in vivo situation quite well.

MATERIALS AND METHODS

Chemicals and Reagents. NMOR, NPYR, pyrrolidine, NMU, NEU, *N*-nitrosopiperidine (NPIP), sodium nitrite, and methylurea were obtained in p.A. quality from Sigma-Aldrich (Steinheim, Germany). Morpholine for synthesis, Extrelut-NT, ammonium amido sulfamate, and anhydrous calcium chloride for synthesis were purchased from Merck (Darmstadt, Germany). Sodium nitrate was provided by Roth (Karlsruhe, Germany) and ethylurea by Alfa Aesar (Karlsruhe, Germany). Formic acid, acetic acid, and sodium hydroxide were purchased from Grüssing (Filsum, Germany).

Dichloromethane was provided by Honeywell Riedel-de Haën (Seelze, Germany). Solvents for HPLC as well as other chemicals were purchased from Merck or Sigma-Aldrich in gradient or reagent grade quality. Water was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

Sample Preparation and Analysis. *Preparation of Inoculum.* Ceca obtained from freshly slaughtered pigs (German Landrace or Angler Sattel x Pietrain) bred under biodynamic conditions were used exclusively. The pigs were 10−12 months old and weighed between 120 and 150 kg. The ceca were obtained during slaughtering and removed by ligation to maintain anaerobic conditions. In the laboratory the ceca were used directly without further storage. The time of storage was intended to be as short as possible and alike for all ceca. All preparation steps were done in an anaerobic chamber under strictly anaerobic conditions. In addition, all buffers, solutions, and glass vessels were flushed with a mixture of N_2 and CO_2 (5/1; V/V) before use to achieve total exclusion of oxygen. Sixty grams of the isolated inoculum of each cecum was diluted with 60 mL of 0.15 M PBS (pH 6.2) containing a trace element solution 0.0125% (13.2 g/100 mL $CaCl_2 \times 2H_2O$, 10.0 g/100 mL $MnCl_2 \times 4H_2O$, 1.0 g/100 mL $CoCl_2 \times 6H_2O$, and 8.0 g/100 mL $FeCl_3 \times 6H_2O$) and a Na_2S-solution 11.1% (575.9 mg/100 mL of 0.037 M NaOH).[27] Larger particles of the fecal solution were removed by filtration through net lace, and the solution was used for incubation experiments. In addition, an inactivated fecal suspension was prepared after sterilization of a cecal aliquot at 121 °C for 15 min at 1.1 bar (AMB240 autoclave, Astell, Kent, Great Britain). Each incubation experiment was performed in duplicate. The metabolic activity was tested by incubation with quercetin as control substance (data not shown).[28]

Incubation Experiments. Stock solutions of nitrite (50 mM in water) or nitrate (500 mM in water) and stock solutions of amines or amides (50 mM or 500 mM in water) were diluted 1:20 (V/V) with active or inactivated fecal suspension or reductive buffer/autoclaved water (50/50; V/V). In addition, blank samples were prepared by diluting water 1:10 (V/V) with fecal suspension or the buffer mixture. Incubation took place for 10 min up to 24 h at 37 °C under continuous shaking. The metabolism of the bacteria was stopped after incubation by deep-freezing at −80 °C, or the samples were directly prepared for the analysis.

Sample Preparation and LC-MS/MS and GC-MS Analysis. The samples were either prepared directly after incubation with an addition of 300 mg of ammonium amido sulfamate or quickly defrosted at 37 °C after storage at −80 °C. For analyzing NMOR and NPYR, gas chromatography coupled with a mass specific detector was used (7980A gas chromatograph, 5975C mass specific detector, Agilent Technologies, Böblingen, Germany; cooled injection system KAS 4, Gerstel, Mühlheim, Germany) using positive chemical ionization with methane. Quantification took place in the single ion monitoring mode (NMOR m/z 117 (Dwell 150), NPIP m/z 115 (Dwell 300), NPYR m/z 101 (Dwell 300)). Additionally, the full scan mode was measured for the mass range m/z 50−550 to identify high matrix contaminations. The samples (1 mL fecal suspension in total) were spiked with *N*-nitrosopiperidine (NPIP) (500 nM) as internal standard and transferred to glass columns filled with Extrelut-NT. The *N*-nitrosamines were eluted with dichloromethane, and the obtained extract was dried with anhydrous $CaCl_2$. The chromatographic separation took place on a VF5-MS column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies, Böblingen, Germany) with the following temperature program: 100 °C was held for 2 min, then the temperature was increased by 4 °C/min until 200 °C, and then the temperature was ascended by 100 °C/min until 280 °C, which was held for 10.2 min. Ten microliters of extract was injected in the solvent vent mode (10 °C starting temperature was increased by 12 °C/s until 280 °C, which was held for 3 min). The *N*-nitrosamines were quantified by a matrix calibration curve (0.25−200 μM). The matrix calibration was performed by adding 500 μL of fecal suspension on the Extrelut columns, eluting with dichloromethane solutions which were spiked with *N*-nitrosamines (0.25−200 μM), and drying the eluate with $CaCl_2$.

For the analysis of *N*-nitrosamides, the samples were extracted with 200 μL of formic acid in methanol (10% V/V) and sonicated for 15 min. The samples were centrifuged at 12 000 *g* for 10 min, and 10 μL of the supernatant was analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS, 1100 Series HPLC (Agilent, Böblingen, Germany) with an API 4000 QTrap system (Applied Biosystems, Darmstadt, Germany) with Autosampler Agilent 1100 ALS G1316A + ALSTherm G1330B). The analytes were separated by a gradient of methanol with 1% formic acid (V/V) (A) and water with 1% formic acid (V/V) (B) at a flow rate of 0.3 mL/min using Agilent 1100 BinPump G1312A and Agilent 1100 G1379A degasser: 0 min 10% A, 1 min 10% A, 5 min 100% A, 6 min 100% A, 7 min 10% A, 10 min 10% A. The ionization was performed in the positive mode with the following parameters: 20 psi (curtain gas), 35 psi (nebulizer gas), 45 psi (heater gas), 5×10^{-5} torr (collision gas), 310 °C (source temperature), 5500 V (ion spray voltage), 2200 V (CEM voltage). The samples were quantified by an external matrix calibration in the multireaction monitoring mode (MRM). The transitions measured for NMU were 104 → 61, 104 → 74, and 104 → 73 and for NEU 118 → 73, 118 → 75, and 118 → 87. The matrix calibration (2.5−200 μM) was performed by spiking the extract of inactivated fecal suspension with NMU and NEU dissolved in methanol. The matrix calibration was performed for each cecum.

Nitrite Analysis. The frozen samples were defrosted quickly at 37 °C. After centrifugation at 12 000 *g* for 10 min, either 50, 100, or 250 μL of the supernatant was diluted in a 10 mL volumetric flask with 400 μL of sulfanilic acid solution (0.6 g/100 mL sulfanilic acid in HCl (0.2 M)) and 3.75 mL of water. After 4 min, 400 μL of 1-naphthylamine solution (0.6 g diluted with 1 mL of HCl and 99 mL of water), 400 μL of sodium acetate solution (25 g solved in 75 mL of water), and 400 μL of glacial acetic acid were added, the mixture was vigorously shaken, and the volumetric flask was filled up with water. After 10 min reaction time the samples were measured photometrically at λ = 520 nm, and nitrite was quantified by an external calibration

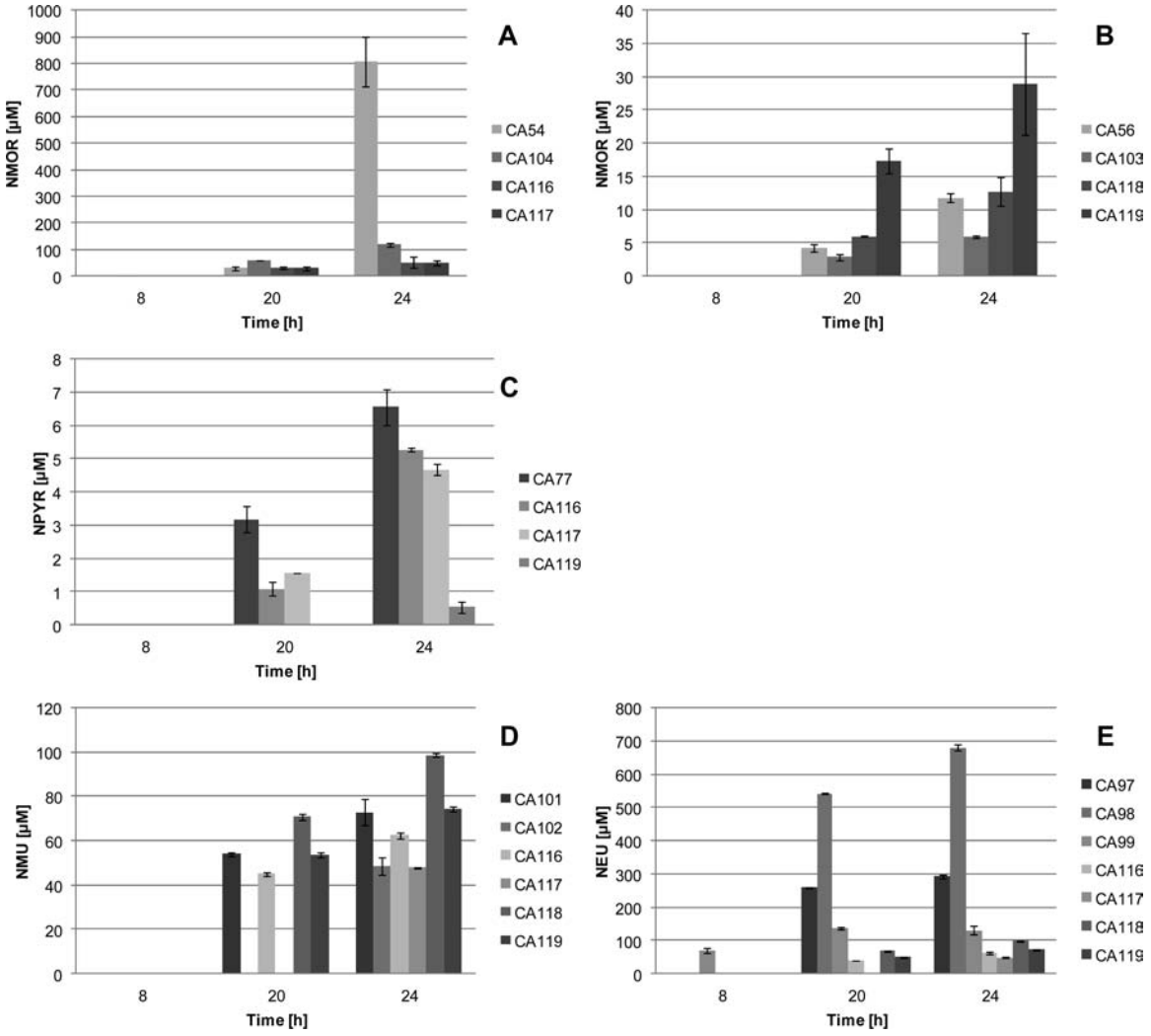

Figure 1. Incubation of 25 mM nitrate and corresponding amines or amides in the pig cecum model. *N*-Nitroso compounds (NOC) are formed with large interindividual differences. Four out of eight analyzed ceca showed high formation of *N*-nitrosomorpholine (NMOR) (A). The other four ceca showed lower formation rates (B). The formation of *N*-nitrosopyrrolidine (NPYR) is shown in (C), of *N*-nitrosomethylurea (NMU) in (D), and of *N*-nitrosoethylurea (NEU) in (E) (n = 2, mean ± SD).

curve ranging from 17.4 to 104 nM. In addition, the recovery was tested for each cecum (50.1−79.3%), and the results were corrected by the recovery.

RESULTS

Nitrate and Amines as Precursors for *N*-Nitrosamines. In order to study the formation of *N*-nitrosamines catalyzed by the cecal microbiota, 25 mM nitrate and an equimolar amount of morpholine or pyrrolidine were incubated in analogy to Calmels et al.[21] The acceptable daily intake (ADI) of nitrate is 3.65 mg/kg body weight per day, and the use of 25 mM nitrate in the incubation experiments reflects the ADI quite well.[29] If the amount of the ADI is consumed within one meal and ends up in the large bowel, this accounts for 8.1 mM (120 kg pig with a mean cecal content of 400 g). Therefore, the incubated amount was approximately four times higher compared to the ADI. Of course this value does not consider the uptake rates of nitrate, the nitrate salivary recycling, and partial reduction to nitrite in the human body which adds up to the dietary nitrate levels.[30] In addition, Tannenbaum et al. identified a de novo formation of nitrate and nitrite in the intestine.[31] Therefore, the value is just an approximation to the in vivo conditions. Figure 1A and B shows the results after the incubation of 25 mM nitrate and morpholine. Only the results of the active ceca are shown as NMOR was detected neither in the inactivated ceca nor in the blank samples incubated with water. Autoclaved feces were used in the pig cecum model as inactivated control. NMOR was detected only in the active ceca beginning at an incubation time of 20 h. Large interindividual variations were observed between the different donor animals. The concentration of NMOR varied from 5.90 to 805 μM after 24 h incubation at 37 °C. The ceca were divided in high producer and low producer. The high producer formed between 50.1 and 805 μM NMOR (Figure 1A), the low producer between 5.90 and 28.9 μM (Figure 1B). NMOR was detected in all of the eight analyzed ceca after 20 and 24 h incubation time.

After the incubation of 25 mM nitrate and pyrrolidine, large interindividual variations in the formation of NPYR were observed, too (Figure 1C). NPYR was only detectable in four out of eight active ceca. The highest content of NPYR measured after 24 h incubation time was 6.55 μM. Again no NPYR was detected in the inactivated controls and the blank samples incubated with water.

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To investigate a possible correlation between the formation of *N*-nitrosamines and the reduction of nitrate to nitrite, incubation experiments with 25 mM nitrate were performed in the pig cecum model without amines. Nitrite as one reduction product of nitrate was analyzed in the fecal suspensions using a photometric assay. Figure 2 shows the concentration of nitrite

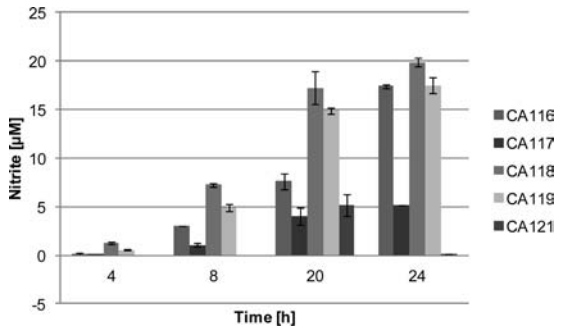

Figure 2. Incubation of 25 mM nitrate in the pig cecum model. Nitrate is reduced to nitrite with large interindividual differences. No nitrite was detectable in the blank samples or after the incubation of nitrate in the inactivated ceca (n = 2, mean ± SD).

after the incubation of 25 mM nitrate. Nitrite was detected only in the active ceca starting after 4 or 8 h incubation time. Higher levels of nitrite were observed after 20 and 24 h incubation with large interindividual differences. After 24 h incubation time, 19.9 μM nitrite was the maximal and 0.12 μM the minimal concentration.

Nitrate and Amides as Precursors for *N*-Nitrosamides. Challenging for the detection of *N*-nitrosamides in fecal suspensions was their instability in neutral aqueous solutions.[32] During the incubation of 0.5 mM NMU or NEU in inactivated fecal suspensions at pH 6.2, both *N*-nitrosamides were no longer detectable after 20 h incubation, and after 4 h incubation about half of the *N*-nitrosamides were already degraded (data not shown). Despite this instability, the formation of the *N*-nitrosamides NMU and NEU from the corresponding amides (25 mM) and nitrate (25 mM) was detectable in the active fecal suspensions with large interindividual differences (Table 1 and Figure 1D–E). NMU was detectable after 20 and 24 h incubation time, whereas the concentration was below the limit of quantification (25 μM) for one out of seven analyzed ceca (Figure 1D). The maximal NMU concentration was 98.8 μM (Table 1 and Figure 1 D). NEU was already detected after 8 h incubation in one out of seven analyzed samples. After 24 h the NEU concentrations varied from 49.0 to 679 μM (Figure 1E).

Table 1. Comparison of the Reduction of Nitrate to Nitrite and the Formation of *N*-Nitrosomorpholine (NMOR), *N*-Nitrosopyrrolidine (NPYR), *N*-Nitrosomethylurea (NMU), and *N*-Nitrosoethylurea (NEU) in Four Active Ceca (CA116–CA119) after 24 h Incubation Time[a]

		24 h incubation with 25 mM nitrate and			
	–	MOR	PYR	MU	EU
cecum	nitrite (μM)	NMOR (μM)	NPYR (μM)	NMU (μM)	NEU (μM)
CA116	17.4	51.2	5.27	62.3	63.0
CA117	5.2	50.1	4.68	47.8	49.0
CA118	19.9	12.7	n.d.	98.8	98.9
CA119	17.5	28.9	0.53	74.5	72.8

[a]In the blank samples and the inactivated samples neither nitrite nor *N*-nitroso compounds were detected. Nitrate (25 mM) alone or together with equimolar amount of corresponding amine or amide were incubated in the pig cecum model (n = 2, mean ± SD).

In Table 1 the formation of NMOR, NPYR, NMU, and NEU in the same ceca (ceca CA116-CA119) as well as the reduction of nitrate to nitrite is summarized. The minimal concentration of NMOR and NPYR was detected in CA118 (12.7 μM NMOR and NPYR < limit of detection) and was maximal in cecum CA116 (51.2 μM NMOR and 5.27 μM NPYR). The lowest concentration of NMU and NEU was analyzed in CA117 (47.8 μM NMU and 49.0 μM NEU) and the maximal one in cecum CA118 (98.8 μM NMU and 98.9 μM NEU). The nitrite content was minimal in CA117 (5.2 μM) and maximal in CA118 (19.9 μM).

Nitrite and Amines as Precursors for *N*-Nitrosamines. In this study 2.5 mM nitrite and an equimolar amount of amines (morpholine and pyrrolidine) were incubated with the fecal suspension. The ADI of nitrite is set to 60 μg/kg body weight per day.[29] If the amount of the ADI is consumed within one meal and ends up in the large bowel, this accounts for 88 μM (120 kg pig with a mean cecal content of 400 g). Therefore, the concentration chosen in the pig cecum model is about 40 times higher than the ADI, but it is still 10 times lower than the concentration used by Calmels et al.[21] In addition, it has to be considered that nitrite can be formed by reduction of nitrate endogenously. The endogenous concentration of nitrite is therefore higher than the dietary amount of nitrite. For example, the nitrite levels in saliva may increase to about 72 mg/L after a spinach rich diet which is even higher than the ADI.[33] During the incubations with nitrite and amines a high formation of *N*-nitrosamines was observed in the inactivated ceca, which was even higher in comparison to the active ceca (data not shown). A pH of 6.2 was existent in the active fecal suspension as well as in the inactivated one throughout the whole incubation period. From these results it was concluded that there is a chemical formation of *N*-nitrosamines in the presence of nitrite and a possible degradation of the formed *N*-nitrosamines by the active ceca as the amounts were much higher in the inactivated samples. Therefore, the ability of the cecal bacteria to degrade NMOR and NPYR was investigated. Figure 3 shows the results of the incubation of NMOR (A) and NPYR (B) in fecal suspension. Within 24 h incubation at 37 °C no differences between the active and the inactivated samples were observed indicating that the *N*-nitrosamines are not metabolized by the active microbiota. The vitality of the bacteria used in these experiments was proven by coincubation with quercetin as vitality control (data not shown) in order to exclude any antibacterial effects of the *N*-nitrosamines on the microbiota. To study the possibility of a chemical formation of NOC under the conditions in the large bowel, nitrite and morpholine or pyrrolidine were incubated for 6, 9, and 24 h in the reductive PBS buffer, which is used in the pig cecum model. The samples were either prepared directly after the incubation or stored for 2 or 4 days at −80 °C. Figure 4 shows the results after incubation of nitrite and morpholine (A) and nitrite and pyrrolidine (B). The content of *N*-nitrosamines rises with the incubation and storage time. Therefore, storage after the incubation of nitrite and corresponding amine was avoided, and a direct sample preparation after the incubation was performed. Furthermore, an addition of 300 mg of ammonium amido sulfamate to each sample was carried out to destroy excessive

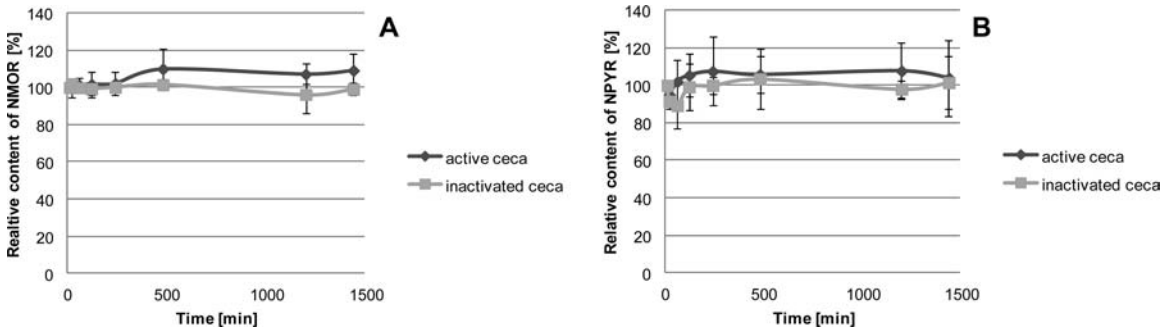

Figure 3. Incubation of 50 μM *N*-nitrosomorpholine (NMOR) (A) and *N*-nitrosopyrrolidine (NPYR) (B) in the pig cecum model (n = 8, mean ± SD).

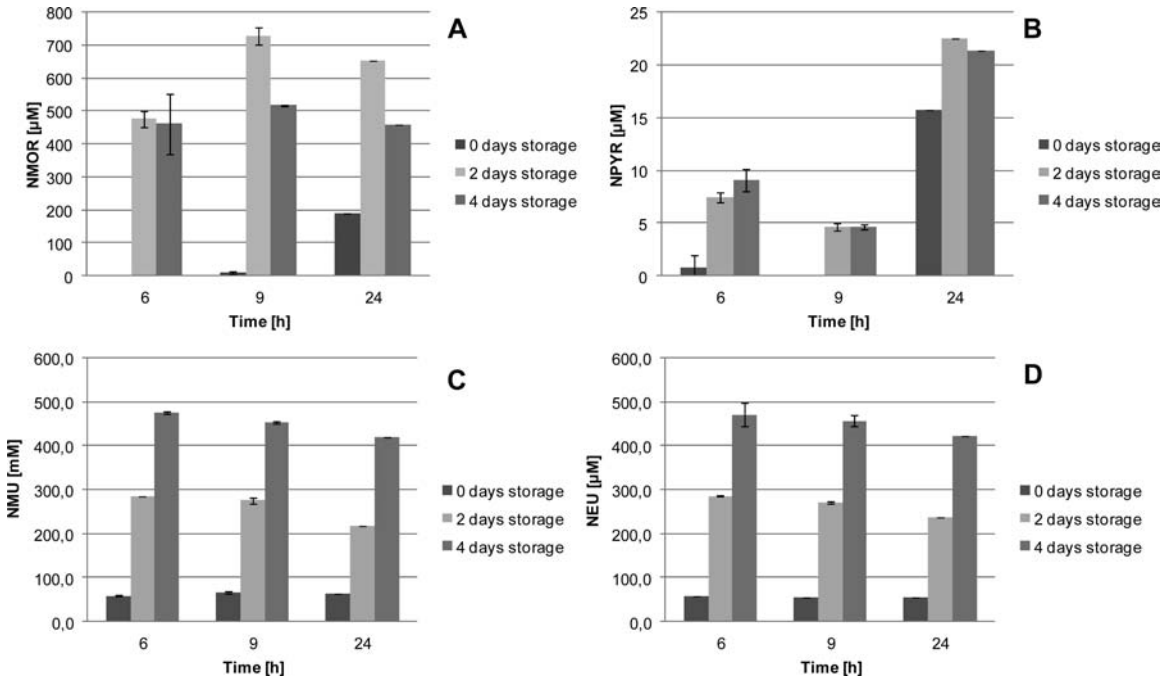

Figure 4. Incubation of 2.5 mM nitrite and morpholine (A), pyrrolidine (B), methylurea (C), or ethylurea (D) for 6, 9, and 24 h. Sample cleanup was performed either directly after incubation or after 2 or 4 days of storage at −80 °C (n = 2, mean ± SD).

nitrite. The necessary amount of ammonium amido sulfamate was tested by destroying 1 mL of 2.5 mM nitrite solution which was the highest expected concentration. The gas chromatographic method for the quantification of *N*-nitrosamines was tested after the addition of these high amounts of added salt without any changes in the performance.

Figure 5 shows the formation of NMOR after incubation of nitrite and morpholine in the pig cecum model after an immediate sample preparation following the incubation and an addition of ammonium amido sulfamate. Still the formation of NMOR in the inactivated fecal suspension was higher (14.9–48.7 μM) in comparison to the active fecal suspensions (1.56–2.54 μM). A formation of NPYR after the incubation of nitrite and pyrrolidine was detected neither in the inactivated ceca nor in the active ceca.

Nitrite and Amides as Precursors for *N*-Nitrosamides. In the studies 2.5 mM nitrite and an equimolar amount of amides were used. After the incubation of nitrite and methylurea or nitrite and ethylurea in the reductive PBS buffer, NMU (Figure 4C) and NEU (Figure 4D) were detected. Like for the *N*-nitrosamines the amount of NMU and NEU rose with the incubation time and with the time of storage at −80 °C. The samples of the *N*-nitrosamides therefore had to be prepared directly after incubation and with an addition of ammonium amido sulfamate, too, to destroy excessive nitrite. The LC-MS/MS method for quantification was checked for changes after the addition of the high amount of salt. Except for an increase of the limit of quantification

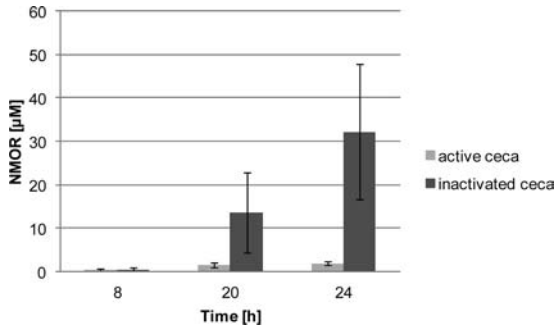

Figure 5. Incubation of 2.5 mM nitrite and morpholine in the pig cecum model. *N*-Nitrosomorpholine (NMOR) was detected after 8 h incubation time in the active and inactivated ceca (n = 8, mean ± SD).

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(25 μM without, 250 μM with ammonium amido sulfamate), no changes in the performance were detected. Using these conditions no NMU or NEU was detected in quantifiable amounts after the incubation of nitrite and corresponding amides in the pig cecum model because of the higher limit of quantification.

DISCUSSION

With the detection of up to 805 μM NMOR and 6.55 μM NPYR in the active cecal samples, we could show that the bacteria are clearly involved in the formation of *N*-nitrosamines after the incubation of nitrate and the respective amine. Concerning the formation of *N*-nitrosamines, large interindividual differences and the late time point of formation were noticeable. Furthermore, a correlation between the reduction of nitrate to nitrite by the microbiota and the formation of NOC could be observed, whereas also large interindividual differences occurred. These results are in agreement with literature data as large interindividual differences concerning the reduction of nitrate and nitrite have also been described for incubation experiments with human feces, while studying the formation of nitrogen monoxide (NO). Three out of eight human fecal samples showed no formation of NO.[20] The late time point of the formation of NOC after 20 and 24 h incubation was in parallel with the reduction of nitrate to nitrite as higher nitrite concentrations were detectable starting from 20 h incubation when incubating nitrate without amine. As can be seen from Table 1, the nitrite concentrations are lower in the presence of amines or amides as nitrite reacts with the corresponding amines to form NOC. In the literature a late formation of *N*-nitrosamines is also described. A formation of NMOR after 10 h incubation of resting cells of *E. coli* strains with nitrate and morpholine in minimal media was described by Calmels et al.[34]

When incubating amides with nitrate, a formation of NMU and NEU was detectable in the active cecal samples again with large interindividual differences. The detectable concentrations reached up to 98.8 μM for NMU and 679 μM for NEU. However, it has to be taken into account that *N*-nitrosamides are rather unstable in the fecal suspension. The instability could be overcome by lowering the pH value. However this was not done in this study as pH values below the normal range in the intestine between 5.6 and 8.0, to raise the stability of NMU and NEU, would not be consistent with the physiological conditions.[35] Thus it has to be considered that the analyzed concentrations of NMU and NEU are lower than the originally formed amounts due to the decomposition reactions.

While incubating nitrite with amines and amides, a high formation rate of NOC was observed even in the inactivated cecum samples after storage indicating a chemical formation in the presence of nitrite. NMOR was even detectable directly after the incubation without storage. This chemical formation was confirmed by the fact that NMOR and NPYR were already generated in the reductive buffer. This might be explained by the sodium sulfide in the buffer as a reduction of nitrite by hydrogensulfide under weak acidic conditions is described in the literature.[36] NMOR is formed in larger amounts in comparison to NPYR which is in agreement with the higher reactivity of morpholine concerning the nitrosation reaction because of its additional ether group.[37,38] The detection of NOC in the buffer shows the possibility of the formation under the neutral conditions of the intestine. In addition, the chemical formation of NOC in the fecal samples might be catalyzed by several other factors. For example, catalytical effects of cell wall constituents of bacteria were already described by Yang et al.[39] Furthermore, carbonyl compounds have also been described to catalyze the nitrosation reaction under neutral conditions without the formation of nitrosating agents.[40−42] Such catalytical effects have been shown for formaldehyde and even for carbon dioxide in the literature,[41,42] which are both present in the feces as carbon dioxide was used in the pig cecum model to achieve an anaerobic atmosphere in the glovebox.

As the NOC levels were even higher in the inactivated samples, a possible degradation of NMOR and NPYR by the intestinal bacteria was studied. However, NMOR and NPYR were not degraded by the microbiota within 24 h incubation. The vitality control of the bacteria by coincubation of quercetin and *N*-nitrosamines excluded an antibacterial effect of the applied NOC concentration. These results are in contrast to literature data. Rowland and Grasso showed the ability of different bacterial strains, which were isolated from the feces of Wistar rats, to degrade *N*-nitrosodiphenylamine (NDPA), NDMA, and NPYR.[43] The differences between the study of Rowland and Grasso and our study can be caused due to the different sources of the bacteria. The bacteria isolated from rat feces can possess other enzyme activities or are in general varying from the composition of the microbiota in the pig cecum. In addition, *E. coli*, as an example, was cultivated aerobically in the study of Rowland and Grasso which is not in accordance with the in vivo situation. Also the degradation was varying between the different *N*-nitrosamines with NDPA being degraded most rapidly.[43] The lack of degradation in our study could also be explained due to the different chemical structures of the used *N*-nitrosamines. The higher formation rate of NMOR in the inactivated ceca (Figure 5) might also be explained by a degradation of nitrite to non-nitrosating compounds in the active ceca by denitrifying bacteria. Because of this, less nitrite could react chemically to form NOC.

In conclusion, the intestinal formation of *N*-nitroso compounds (NOC) after the incubation of nitrate and the corresponding amine or amide was proven using the pig cecum model. The two *N*-nitrosamines NMOR and NPYR as well as the *N*-nitrosamides NMU and NEU were only formed in concentrations up to 805 μM NMOR in the presence of active microbiota after the incubation of nitrate. NOC were formed after 20 h incubation time with large interindividual differences. The time point of formation and the large interindividual differences were in parallel with the reduction of nitrate to nitrite. The formation of NOC in the intestine therefore seems to be relevant after the ingestion of a high amount of nitrate. Notable at this point are the new maximum levels of nitrate in the EU which were increased for some products. For example, the maximum levels for nitrate in rucola were set to 6000 mg/kg (summer) and 7000 mg/kg (winter).[44] After the incubation of nitrite no formation of NOC caused by the microbiota was observed. Instead a chemically catalyzed formation is possible. In addition, in the presence of a viable microbiota the NOC levels are even lower probably due to a reduction of the nitrite to not nitrosating compounds. This leads to the assumption that after the incubation of nitrate, the bacteria reduce nitrate to nitrite, followed by a chemically catalyzed formation of NOC.

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Notes

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ABBREVIATIONS USED

ADI, acceptable daily intake; ATNC, apparent total *N*-nitroso compounds; CI, chemical ionization; ESI, electrospray ionization; EU, European union; *g*, standard gravity; GC, gas chromatography; h, hour; HESI, heated electrospray ionization; HPLC, high-performance liquid chromatography; kV, kilovolt; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NDPA, *N*-nitrosodiphenylamine; NEU, *N*-nitrosoethylurea; NO, nitrogen monoxide; NMU, *N*-nitrosomethylurea; NOC, *N*-nitroso compounds; nM, nanomolar; NMOR, *N*-nitrosomorpholine; NPIP, *N*-nitrosopiperidine; NPYR, *N*-nitrosopyrrolidine

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