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Associations among dietary nitrosamine intake, fecal *N*-nitroso compounds and intestinal microbiota in adults according to intestinal mucosa damage

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Dietary intake of *N*-nitroso compounds (NOCs) has been proposed as one of the mechanisms explaining the association between the consumption of red and processed meat and the increased risk of colorectal cancer (CRC). Endogenous NOCs can also be formed along the intestine, being finally excreted in feces. The intestinal microbiota could play a role in the formation of endogenous NOCs and their impact on health. In this work, the median fecal total and heme NOC concentrations of 46 volunteers were used to split the sample and analyze discriminant differences in the relative abundance of intestinal microbiota members and fecal short-chain fatty acids (SCFAs). PERMANOVA analysis further investigated associations between dietary factors and fecal NOCs with the microbiota. Finally, the association of intestinal microbiota with fecal NOCs in relation to the degree of mucosal damage was evaluated. Shifts in the relative abundance of *Roseburia*, *Prevotella* or *Escherichia–Shigella* according to fecal NOC concentrations were detected in stool samples. PERMANOVA analysis showed that these dietary precursors could also be related to the intestinal microbiota profile of volunteers and suggested that specific intestinal bacterial genera, rather than general microbial changes, were associated with fecal NOCs. Notably, the genera *Escherichia–Shigella*, *Ruminococcus torques* group, *Subdoligranulum* or *Intestinibacter* were found to be predictors of fecal NOC concentrations, depending on intestinal mucosal lesions. Furthermore, some of these genera were positively correlated with the intake of nitrate, nitrite and nitrosamines (NAs), precursor compounds of endogenous NOCs. Our study supports a link among specific intestinal microorganisms, fecal NOCs, and intestinal mucosal damage.

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1 Introduction

Colorectal cancer (CRC) is a widespread cause of mortality, accounting for about 1 million deaths annually.[1,2] According to estimations, approximately 30% of CRC cases are driven by genetic factors, whereas environmental factors have a strong influence in sporadic CRC cases.[3–6] It has been estimated that adherence to healthy lifestyle patterns could reduce the incidence of cancer by up to 50%.[7,8] For this reason, diet is considered an important modifiable factor for the prevention of CRC.[9]

There is scientific evidence showing that the high consumption of certain food groups, such as red meat and processed meat, increases the risk of CRC.[9] The formation of potentially harmful *N*-nitroso compounds (NOCs) during the processing, cooking and digestion of red and processed meat has been proposed as a mechanism to explain these associations.[10] The most commonly found NOCs in foods are nitrosamines (NAs), which include compounds that have been positively associated with the risk of CRC, such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR).[11–13] Given that their intake has become an issue of concern, the European Food Safety Authority (EFSA) has recently launched a technical report on the risks to human health of exposure to ten carcinogenic NAs occurring in food, including NDMA, NPIP and NPYR, highlighting the need to generate more scientific evidence in this respect.[14]

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During digestion, some NAs undergo metabolization processes to acquire their carcinogenic potential, which is manifest through their ability to form DNA adducts, such as O6-alkylguanine, or to induce oxidative stress processes.[15] Notoriously, NOCs can also be formed endogenously along the gastrointestinal tract from its dietary and endogenous precursors, including protein thiols, protein amines, nitric oxide (NO) and NAs such as NDMA, NPIP and NPYR.[16] Moreover, deciphering the contribution of oral and gut microbiota to modulate the formation and transformation of endogenous NOCs is of paramount interest.[17]

Our preliminary results pointed to different fecal microbiota profiles associated with the initial stages of intestinal mucosal damage in the context of CRC development.[18] Moreover, the quantification of NOC concentrations in stools indicated that patients displaying intestinal lesions with a higher risk of turning malignant also showed higher concentrations of total and heme-derived NOCs.[19] Furthermore, both dietary NOC exposure and fecal NOC concentrations demonstrated a positive relationship with the intake of certain types of processed foods.[19] However, given the complexity of the intestinal microbiota and the wide metabolic repertoire displayed by microbiota members, it is still unknown whether these microorganisms substantially contribute to NOC metabolization and the mechanisms involved in this process.

Therefore, the present work aims to shed light on the relationships among fecal microbiota members and fecal NOC concentrations and how dietary factors may influence these variables in a sample population of adults with different stages of intestinal mucosal damage prior to the development of CRC.

2 Materials and methods

2.1 Study design and volunteers

The recruitment of volunteers was carried out from October 2019 to December 2021 among subjects attending medical consultations about clinical symptoms or as part of a CRC screening program. It was performed by Medical Doctors of the Digestive Service from the Central University Hospital of Asturias (HUCA) and the Carmen and Severo Ochoa Hospital in Cangas de Narcea, Asturias, Spain, following the same clinical protocols as previously described.[18] Volunteers aged between 40 and 79 years were informed about the objectives of the study and signed an informed consent. The exclusion criteria were: former surgery on the digestive system, immune-related disease, treatment with medical drugs, including antibiotics and corticoids, or specific cancer treatment at the time of the study or in the previous two months. A total of 46 subjects were included in the study. Before preparing patients for colonoscopy, fecal samples were collected. Necessary instructions and materials for collecting fecal samples were provided to the volunteers. After depositing fresh fecal samples in sterile plastic containers, patients transported them to the corresponding hospital within two hours. The samples were then frozen and transported to the laboratory. During colonoscopy, biopsies of the intestinal mucosa and polyps were obtained and examined at the Department of Anatomical Pathology of HUCA, as described elsewhere.[20] According to the results, individuals were classified into three histopathology groups: non-pathological controls (NP) (n = 18), hyperplastic polyps (HP) (n = 10), and conventional adenomas (CA) (n = 18).

This study was carried out within the framework of two broader projects focused on the effect of diet and dietary xenobiotics on intestinal mucosa and related gut microbiota profiles in the context of CRC (MIXED and MiToxicDiet projects), which were evaluated and approved individually by the Regional Ethics Committee of Clinical Research of Asturias (ref. 163/19 and CEImPA 2023.511, respectively) and by the Committee on Bioethics of CSIC (ref. 174/2020 and 186/2024, respectively). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in Spanish legislation on bioethics. Directive 95/46/EC of the European Parliament and the Council of October 1995, on the protection of individuals regarding the processing of personal data, was strictly followed.

2.2. DNA extraction and microbiota metataxonomic analyses

Four grams of frozen fecal samples were weighed, diluted 1/10, and homogenized with sterile phosphate-buffered saline (PBS) in a LabBlender 400 Stomacher (Seward Medical, London, UK) for 3 min at maximum speed. After 15 min of centrifugation in an Eppendorf microcentrifuge, Model 5415 R, at 4 °C and 16 100g, the supernatants and pellets were separated and kept frozen at −20 °C until use. The fecal pellets obtained after dilution and homogenization were used to extract DNA. The Q protocol for DNA extraction defined by the International Human Microbiome Standards Consortium[21] was applied using the QIAamp Fast DNA Stool Mini Kit (Cat no. 51604, Qiagen, Sussex, UK). After extraction, the 260/280 ratio was determined using a Take3 Micro-Volume plate and Gen5 microplate reader (BioTek Instrument Inc., Winooski, VT, USA). DNA was frozen at −20 °C until analysis. Sequencing and annotation of the bacterial 16S rRNA genes were performed at Novogene Bioinformatics Technology Co., Ltd. First, using specific primers connected with barcodes, the variable region V3–V4 of bacterial 16S rRNA genes was amplified by PCR, and a DNA library was prepared. The Illumina NovaSeq 6000 platform was used to sequence the libraries. After each read, they were assigned to the samples using barcodes and merged using FLASH (version 1.2.7). QIIME (version 1.7.0) was used to obtain high-quality clean tags, allowing the removal of low-quality sequences. The obtained tags were then compared with the reference SILVA 138 database, and chimeric sequences were removed using the UCHIME algorithm. To perform sequencing analysis, effective tags were utilized using Uparse software (Uparse V 7.0.1090). Sequences sharing ≥97% homology were assigned to the same OTUs, and OTU abundance was normalized. The representative sequence for each OTU was obtained against the SSU rRNA database of SILVA138 using

QIIME in the Mothur method to annotate species at each taxonomic rank.

2.3. Measurement of fecal total NOCs and heme NOCs and short-chain fatty acids

Fecal homogenates were prepared by diluting raw fecal samples (1 : 5) with ultrapure Milli-Q water (resistivity 18.2 MΩ.cm at 25 °C, Millipore, Rios30) and homogenized with T-18 Digital Ultra Turrax (IKA, Germany). Total NOCs and heme NOCs were analyzed from one replicate of the fecal homogenates using selective de-nitrosation and chemiluminescence-based detection by an Ecomedics CLD 88 Exhalyzer (Eco Medics, Switzerland) equipped with a custom-made liquid purge vessel and an NaOH (1 mol L^{-1}, kept at 4 °C) trap, as indicated in previous studies.[19,22]

The fecal concentrations of short-chain fatty acids (SCFAs), acetic, propionic, butyric, isobutyric, isovaleric, valeric, and caproic acids, were determined by gas chromatography from fecal supernatants, as previously indicated.[18]

2.4. Nutritional assessment

Detailed information about the nutritional assessment was described previously.[19,20] Briefly, dietary interviews conducted by trained personnel were performed when volunteers attended medical consultations for colonoscopy results. A 155-item semi-quantitative food-frequency questionnaire (FFQ), developed by our research group for estimating intake of dietary xenobiotics, was used.[23] This FFQ has been previously validated in an adult population sample without digestive disorders, drawn from the same geographic area and with an age profile comparable to that of the volunteers participating in the present study.[23] For this purpose, the specific type of food consumed was recorded, as well as the cooking methods used and other related questions, such as the degree of doneness or temperature, particularly in the case of meat, fried potatoes, or toasted bread.[19,20] To standardize this last point, photographs of the degree of doneness at different temperatures and the subsequent progressive increase in browning were specifically developed. Complementary questions, such as the part of food consumed and the possible consumption or cooking of the skin, were incorporated into questionnaires to improve the precision of the information recorded.[23] The content of nitrates, nitrites and the NAs NDMA, NPIP and NPYR in foods was estimated using the European Prospective Investigation into Cancer and Nutrition (EPIC) Potential Carcinogens Database and EFSA data, along with other external reference sources to complete the information.[24–29] Specific attention was paid to compilation of the concentration of NAs in processed meats typically consumed in the geographical region, such as cured ham (pork meat), chorizo (a typical Spanish sausage of minced pork meat combined with paprika and spices), and blood sausage (made from cooked blood generally obtained from pork, pork fat, onion, and spices). From the 74 foods in the final database, consisting of 258 different items regarding nitrates, nitrites, and NA concentration values, 54 foods were consumed by the study sample.[19] Dietary information was obtained for 33 volunteers from the NP (n = 11), HP (n = 8) and CA (n = 14) groups.

2.5. Statistical analyses

Results were analyzed using IBM SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, SA). GraphPad Prism 9 and RStudio version 4.3.2 software were used for graphical representation. Fisher tests were performed for categorical variables (p-value < 0.05). For continuous variables, the goodness of fit to a normal distribution was checked by means of the Kolmogorov–Smirnov test. When normality of variables was achieved, T-tests were performed; otherwise, Mann–Whitney U-tests were applied (p-value < 0.05). Values of fecal total and heme NOC concentrations were used to split the sample and group the volunteers according to median concentrations. Groups were compared using linear discriminant effect size analysis (LEfSe) from the Galaxy web tool (https://galaxy.biobakery.org/) to estimate bacterial taxa, significantly discriminating between the groups under study. LEfSe analysis consists of a Kruskal–Wallis rank sum test and a Wilcoxon test for pairwise comparison, followed by logarithmic linear discriminant analysis (LDA) to estimate the effect size at a threshold of 2.0.[30] Only microbial genera with relative abundance of at least 1% in at least two samples were considered in the analysis. To investigate the potential associations between consumption of food groups, exposure to dietary NOCs and fecal NOC concentrations against the microbiota profile at the genus level, nonparametric permutational multivariate analysis of variance (PERMANOVA) with Bray–Curtis distance was carried out using the "adonis2" function of the vegan (2.6–4) R package.[31] PERMANOVA analysis was performed in RStudio, and the results were visualized using the "ggplot2" package (3.5.0). Spearman correlations were carried out to explore the associations between the most abundant microbial families in the sample and the total and heme NOC concentrations in the feces of individuals within each histopathology group. Heatmaps were generated using the "corrplot" R package. The relative abundances of microbial genera in fecal samples were examined as predictors of fecal total and heme NOC concentrations, according to each histopathology group, by linear stepwise regression analyses.

3 Results and discussion

The sample was composed of a total of 46 volunteers, 39% men, with an average age of 61 years and a BMI of 26.43 kg m^{-2}, classified as overweight. Non-significant statistical differences among histopathology groups (NP, HP and CA) were detected for these general and anthropometrical variables.

3.1. Fecal NOC concentrations are associated with alterations in the abundance of specific members of the intestinal microbiota

Previous results from our working group on the same sample population pointed to specific changes in the composition of fecal microbiota and to increasing concentrations of fecal

NOCs as the severity of the colonic mucosal damage increased.[18,19] Therefore, we hypothesize that the fecal concentration of NOCs could be related to shifts in specific members of the fecal microbiota.

To look deeper into this, volunteers were split into two groups according to the median fecal concentration of total NOCs (5.97 pmol mg^{-1}). No statistically significant differences were observed in alpha and beta diversity between groups (data not shown). However, in the group of volunteers with higher fecal total NOC concentrations (group tNOCs-2), LEfSe analyses evidenced a differentially decreased abundance of the microbiota family *Prevotellaceae* and the genus *Roseburia* (family *Lachnospiraceae*) compared to individuals with lower fecal NOC concentrations (group tNOCs-1) (2.65% *vs.* 7.03% for *Prevotellaceae* and 0.90% *vs.* 1.78% for *Roseburia*) (Fig. 1A). The same criterion was applied to split the group of volunteers according to the median concentration of fecal heme NOCs (3.46 pmol mg^{-1}), obtaining similar findings. Concretely, in the comparison of individuals presenting higher excretion of heme NOCs (group hNOCs-2) against volunteers with lower excretion of heme NOCs (group hNOCs-1), the group hNOCs-2 was characterized by a differentially lower abundance of *Roseburia* (0.96% *vs.* 1.92%), *Prevotella* (1.60% *vs.* 5.65%) and *Prevotellaceae* (1.93% *vs.* 7.75%) (Fig. 1B). Moreover, discriminantly higher abundance of *Escherichia–Shigella* (family *Enterobacteriaceae*) was observed in the group hNOCs-2 compared to the group hNOCs-1 (2.67% *vs.* 0.31%).

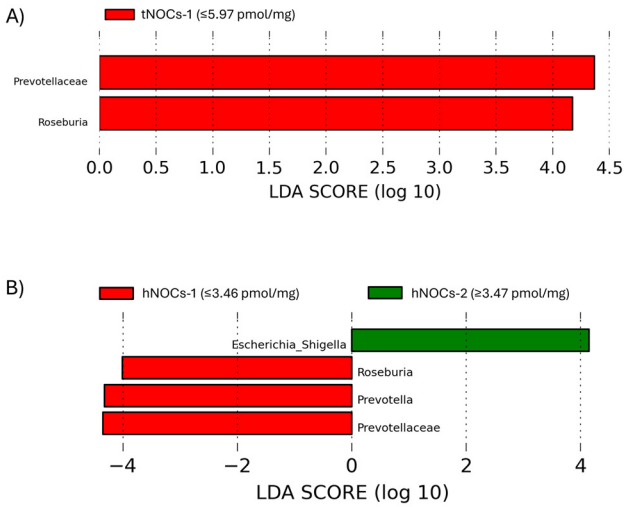

Fig. 1 Bacterial taxa showing differential linear discriminant analysis (LDA) score according to linear effect size discriminant analysis (LEfSe) for the comparison of volunteers with (A) fecal total NOC concentrations ≤ 5.97 pmol mg^{-1} (tNOCs-1) *vs.* fecal total NOC concentrations ≥ 5.98 pmol mg^{-1} (tNOCs-2) or (B) fecal heme NOC concentrations ≤ 3.46 pmol mg^{-1} (hNOCs-1) *vs.* fecal total NOC concentrations ≥ 3.47 pmol mg^{-1} (hNOCs-2). For LEfSe analyses, only those genera and the corresponding families with relative abundance higher than 1% in at least two samples were included.

Initial studies on the role of *Escherichia coli* in the formation of NOCs (concretely, NAs) and nitrites demonstrated nitrate-reducing and nitrosating activity by several strains of this species, which were able to produce NOCs from nitrate and secondary amines.[32–34] *Escherichia–Shigella* members are normal inhabitants of the human intestine. However, in altered conditions, these microorganisms have been proposed as causative agents in the CRC-associated driver–passenger model.[35] The mechanisms by which some strains of *Shigella* and *E. coli* have been associated with colorectal tumorigenesis include the production of toxins such as colibactin, Shiga toxin, hybrid polyketide-peptide (PKS) or cytolethal distending toxin (CDT), the promotion of angiogenesis, and enhanced attachment to intestinal mucosa.[36,37] In contrast, *Prevotella* and *Roseburia* genera have been positively associated with good health, as they are fermenters of dietary fiber involved in the production of SCFAs, mainly butyric acid, and are also involved in the production of several vitamins.[38–41]

In agreement with this, our findings pointed to a trend in acetic and butyric acid and significantly higher concentration of propionic acid in the feces of the group of volunteers with lower fecal total NOC concentrations (tNOCs-1) (Table 1). In this regard, it has been reported that high-protein and low-carbohydrate weight-loss diets in obese men can lead to increased concentrations of fecal NOCs and decreased *Roseburia* and SCFA levels.[42] In the present work, the group of

Table 1 Fecal SCFA concentrations according to fecal NOC concentration groups

	Fecal total NOC concentrations		Fecal heme NOC concentrations	
SCFA (mM)	tNOCs-1 (≤5.97 pmol mg^{-1} feces)	tNOCs-2 (≥5.98 pmol mg^{-1} feces)	hNOCs-1 (≤3.46 pmol mg^{-1} feces)	hNOCs-2 (≥3.47 pmol mg^{-1} feces)
Acetic acid	55.21 ± 23.20	42.20 ± 20.98	50.04 ± 18.49	47.37 ± 26.85
Propionic acid	17.20 ± 7.93[a]	12.33 ± 6.43[a]	15.85 ± 7.76	13.67 ± 7.36
Butyric acid	15.37 ± 9.68	11.17 ± 6.80	13.26 ± 7.53	13.28 ± 9.61
Isobutyric acid	0.85 ± 0.78	1.25 ± 1.36	0.92 ± 0.75	1.18 ± 1.40
Isovaleric acid	1.87 ± 1.42	2.56 ± 2.00	2.00 ± 1.35	2.43 ± 2.08
Valeric acid	2.01 ± 0.91	2.00 ± 1.71	2.02 ± 0.90	1.98 ± 1.71
Caproic acid	0.79 ± 1.25	0.53 ± 0.92	0.79 ± 01.25	0.53 ± 0.92

Values are presented as mean ± standard deviation. [a] Statistically significant differences between groups (Mann–Whitney *U*-test; $p < 0.05$). tNOCs, fecal total NOC concentrations; hNOCs, fecal heme NOC concentrations.

volunteers with higher fecal heme NOC concentration (hNOCs-2) also presented increased consumption of processed meat compared to the group hNOCs-1 (71.73 g day^{-1} *vs.* 46.98 g day^{-1}, respectively, *p*-value: 0.023). It is noteworthy that processed meats are a source of NOC precursors and other compounds that can enhance endogenous NOC formation. Various studies have related a decrease in *Roseburia* levels with intestinal tumorigenesis, whereas increased *Prevotella* levels were associated with a lower risk of CRC.[43,44]

3.2. Dietary intake of NAs contributes to the intestinal microbiota profile, while fecal NOC concentrations do not

Considering the strong influence of diet on intestinal microbiota, together with the role of dietary NAs as sources of increased fecal NOC concentrations,[19] we wanted to study the potential role of dietary factors and fecal NOCs on microbiota composition profiles in our sample population. In this regard, a PERMANOVA analysis revealed that the intake of processed meat, nitrite and related dietary compounds, such as NDMA, NPIP and NPYR, explained some variation within the microbial profiles at the genus taxonomic level (Fig. 2). In the human sample of the present study with available dietary intake records (n = 33), the mean intakes of processed meat, nitrite, NDMA, NPIP and NPYR were 66.74 g day^{-1}, 3.21 g day^{-1}, 0.20 μg day^{-1}, 0.09 μg day^{-1} and 0.14 μg day^{-1}, respectively. We also noted that fecal total and heme NOC concentrations did not contribute significantly to an explanation of the differences in the microbiota profile of the volunteers, suggesting that specific intestinal bacterial members, rather than wide microbiota profiles, were associated with the levels of NOCs in feces.

3.3. The relationship between fecal NOC concentrations and the intestinal microbiota varies depending on the histopathology groups

To assess whether the relationships among several microbiota members and fecal total and heme NOC concentrations differed depending on the type and severity of the intestinal mucosal damage in our study sample, Spearman correlation analyses were conducted within histopathology groups (Fig. 3). In the NP group, inverse associations of fecal total and heme NOC concentrations with *Prevotellaceae* and *Veillonellaceae* and positive associations with *Methanobacteriaceae* were observed. Among those individuals with HP, direct correlations were found for *Enterobacteriaceae*, *Erysipelotrichaceae* and *Clostridiaceae* with the fecal concentration of NOCs. The group of volunteers diagnosed with CA showed positive associations between the *Eubacterium coprostanoligenes* group and fecal concentration of NOCs.

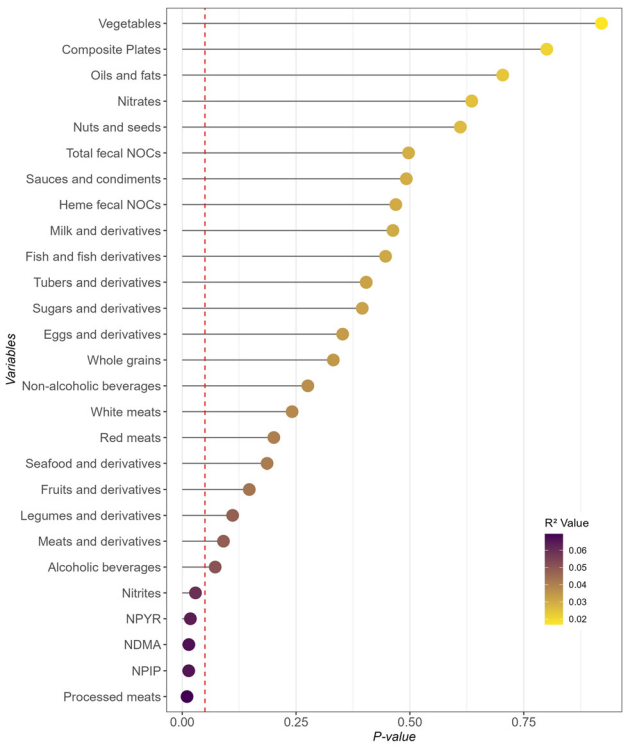

Fig. 2 PERMANOVA analysis between fecal microbiota profiles (Bray–Curtis distance) at the genus taxonomic level, and the consumption of certain foods and dietary compounds along with fecal NOC concentrations (n = 33). Analysis identified processed meat, NPIP, NDMA, NPYR and nitrite intake as variables explaining variation across taxonomic profiles. Horizontal lines connect each variable to the p-value axis, point color intensity indicates the strength of the relationship (R^2 value), and the vertical red line indicates the significance threshold (p = 0.05).

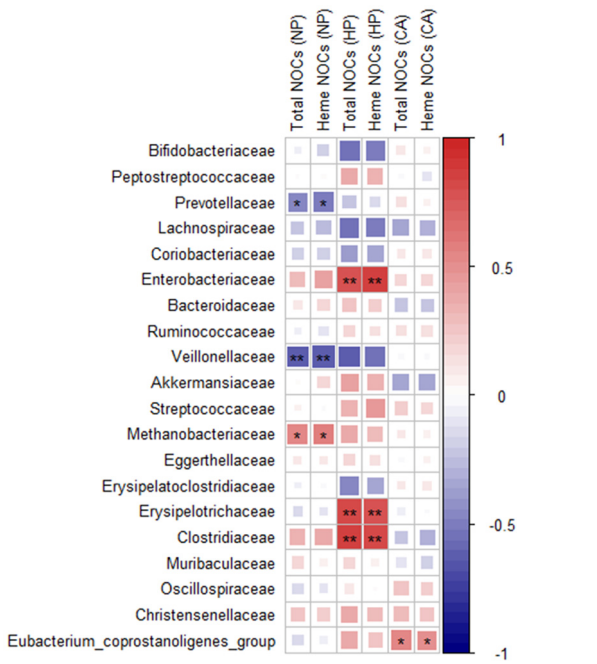

Fig. 3 Heatmap defined by Spearman correlations according to histopathology groups between fecal total and heme NOC concentrations and the most abundant microbial families in the sample (relative abundance > 1%). Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association. *p-Value < 0.05, **p-value < 0.01. NOCs, *N*-nitroso compounds; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenomas.

Members of the *Veillonellaceae* family can act as nitrate-reducing bacteria, leading to the production of nitrite, which could serve as a precursor in the formation of NOCs.[45] However, our results showed negative associations between fecal NOCs and the relative abundance of the *Veillonellaceae* family, suggesting a still unknown opposite modulation mechanism. The positive associations between *Methanobacteriaceae* and the fecal concentration of these compounds were intriguing. Members of this microbial family can use different substrates for growth, such as methanol and acetate, resulting from bacterial fermentation and H_2 produced mainly in the catabolism of proteins by hydrogenotrophs.[46–48]

The imbalanced growth of *Methanobacteriaceae* members contributes to greater production of methane, whose impact on health is still under discussion.[49] Increased methane production may slow gut transit and, therefore, may promote constipation.[50]

In this way, a longer stool transit time may contribute to increased accumulation of, and exposure to, potentially harmful molecules, such as NOCs. On the other hand, total or partial depletion of gut methanogens has been associated with adverse health effects. This is particularly noteworthy, as methanogens have a slower growth rate than the other intestinal microbial inhabitants, and their populations may therefore take longer to recover.[51,52] In this regard, lower levels of methanogens have been detected in patients with recurrent diarrhea and in those diagnosed with intestinal polyps compared with controls, with levels of such microorganisms being found to be even more reduced in high-grade dysplastic adenomas.[18,53] In the present work, among the volunteers diagnosed with HP, the positive correlation of *Enterobacteriaceae* and fecal NOC concentrations could be explained by the differential increased levels of *Escherichia–Shigella* observed in the group with higher concentrations of fecal heme NOC (Fig. 1). Regarding *Erysipelotrichaceae*, various studies have reported an enrichment of this family in intermediate steps prior to CRC development, and consistent data have evidenced an enrichment of this family in metabolic disorders such as obesity.[54–56] However, no relationships of this microbial family with NOC levels have previously been described. The positive correlation of *Clostridiaceae* with fecal NOC concentrations in HP is in agreement with a previous study reporting that in fecal cultures incubated solely with proteins and peptides (NOC precursors) as carbon and nitrogen sources, a significant growth of *Clostridiaceae* (*Clostridium sensu stricto* genus) occurred after 6 hours of fermentation.[57] Furthermore, the growing capacity of *Clostridium* spp. has been demonstrated, even in the presence of the antibiotic nitrofurantoin, through the metabolization of nitroaromatic compounds, probably mediated by the presence of nitroreductase enzymatic activity and the formation of derived nitroso compounds.[58–60] Nevertheless, the relationships between NOCs and *Clostridiaceae* are still unclear. The *Eubacterium coprostanoligenes* group was the only microbial group showing statistically significant correlations with fecal NOC concentrations in the group of patients diagnosed with CA.

Regarding individuals with intestinal polyp lesions, we have previously reported an increased relative abundance of the *Eubacterium coprostanoligenes* group in volunteers with a comparatively higher intake of nitrite (over 1.69 mg day^{-1}).[61] We have also observed a higher relative abundance of this microbial family in a sample of socially vulnerable individuals with intake of the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo (4,5,*b*) pyridine (PhIP) of over 40 ng day^{-1}.[62] In the context of CRC development, Xiang *et al.* (2023) found causal associations between the *Eubacterium coprostanoligenes* group and CRC, and Senthakumaran *et al.* (2023) identified a higher abundance of this microbial group in cancer and adenomatous polyp patients compared to healthy controls.[63,64] A possible mechanism contributing to an explanation of these associations may be related to the capacity of several bacterial genera, including *Clostridium*, *Shigella* and *Eubacterium*, to transform betaine, carnitine and choline from red meat, fish or eggs, among other things, into trimethylamine (TMA),[65] that could finally lead in different ways to the formation of endogenous NOCs.[66,67] With these considerations in mind, deciphering the role of the *Eubacterium coprostanoligenes* group in altered intestinal environments raises a future topic of interest.

3.4. Could fecal NOC concentrations be predicted by intestinal microbiota members?

To deepen the investigation into the associations between fecal NOCs and microbiota, stepwise regression analyses were carried out to determine which microbial genera could be predictors of fecal total and heme NOC concentrations in the different histopathology groups (Table 2). In NP volunteers, a statistically significant model revealed that the fecal heme NOC concentrations were predicted positively by *Senegalimassilia* (family *Eggerthellaceae*) and negatively by *Dialister* (family *Veillonellaceae*) and *Prevotella* (family *Prevotellaceae*).

In those patients showing intestinal HP, fecal total NOC concentrations were predicted directly by *Escherichia–Shigella* (family *Enterobacteriaceae*) and the *Ruminococcus torques* group (family *Lachnospiraceae*) and inversely by *Faecalitalea* (family *Ruminococcaceae*) and *Catenibacterium* (family *Erysipelatoclostridiaceae*). Furthermore, the fecal concentration of heme NOCs was predicted by *Intestinibacter* (family *Peptostreptococcaceae*). Among patients with CA, *Enterorhabdus* (family *Eggerthellaceae*) was a positive predictor of total NOC concentrations, while fecal heme NOC concentrations were predicted by a model including *Subdoligranulum* (family *Ruminococcaceae*), *Butyricicoccus* (family *Butyricicoccaceae*), and the *Lachnospiraceae-NK4A136* group (family *Lachnospiraceae*).

An as-yet unclear role for most intestinal microbial genera has been established in CRC development, and their relationships with fecal NOCs are also unknown. However, to date, the role of certain genera, such as *Escherichia–Shigella*, the *Ruminococcus torques* group, *Subdoligranulum* or *Intestinibacter*, which we have found to be predictors of fecal NOC concentrations in the presence of intestinal lesions, has been studied in depth in the CRC context. The *Ruminococcus torques* group and *Subdoligranulum* have been found to be enriched in

Table 2 Results obtained from linear stepwise regression analyses identifying microbial genera as predictors of fecal total and heme NOC concentrations according to histopathology groups

Histopathology group	Dependent variable	Independent variable	R^2	β	p
NP	Total NOCs	—	—	—	—
	Heme NOCs	*Senegalimassilia*	0.606	0.734	0.000
		Dialister		−0.378	0.036
		Prevotella		−0.386	0.042
HP	Total NOCs	*Escherichia–Shigella*	0.992	0.629	0.000
		Faecalitalea		−0.120	0.011
		Ruminococcus torques group		0.705	0.000
		Catenibacterium		−0.132	0.008
	Heme NOCs	*Intestinibacter*	0.832	0.922	0.000
CA	Total NOCs	*Enterorhabdus*	0.228	0.523	0.026
	Heme NOCs	*Subdoligranulum*	0.642	0.986	0.000
		Butyricicoccus		−0.509	0.004
		Lachnospiraceae-NK4A136 group		−0.580	0.008

Only the variables with p-value < 0.05 in each model are shown. R^2, coefficient of multiple determination; β, standardized regression coefficient; NOCs, *N*-nitroso compounds; NP, non-pathological; HP, hyperplastic polyps; CA, conventional adenomas.

altered intestinal environments and have been proposed as drivers in intestinal carcinogenesis.[68–70] More precisely, in the same population of individuals participating in the present study, we have previously found that the *Ruminococcus torques* group showed higher relative abundance in volunteers diagnosed with HP and in those individuals diagnosed with CA, in comparison with the control group.[18] In addition, *Intestinibacter* was found to be positively associated with CRC risk according to a Mendelian randomization approach.[63] Our results support the interest in these microbial groups when evaluating CRC development and suggest a link between these bacteria and fecal NOC concentrations. Finally, considering the influence of dietary factors identified in the PERMANOVA analysis, such as NPIP, NDMA, NPYR and nitrites, on the formation of endogenous NOCs,[19] we next focused on the relationships between *Escherichia–Shigella*, the *Ruminococcus torques* group, *Subdoligranulum* and *Intestinibacter*, identified as specific fecal NOCs predictors in our sample and described as CRC-associated bacteria,[18,63,68–70] and the dietary intake of NOC precursor compounds in the sample.

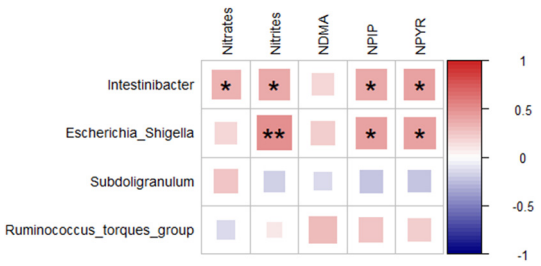

Fig. 4 Heatmap defined by Spearman correlations between intestinal microbial genera associated with CRC predictive of fecal NOC concentrations and NOC dietary precursor compounds in the sample. Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association. *p-Value < 0.05, **p-value < 0.01. NDMA, *N*-nitrosodimethylamine; NPIP, *N*-nitrosopiperidine, NPYR, *N*-nitrosopyrrolidine.

Significant positive correlations of *Intestinibacter* and *Escherichia–Shigella* with the dietary intake of nitrites, NPIP and NPYR were observed (Fig. 4), pointing to interconnections among diet, certain intestinal microbiota members and fecal NOC concentrations, as related to early intestinal mucosal damage in the context of CRC.

4 Conclusions

On the basis of our findings, we hypothesize that certain bacterial intestinal inhabitants, such as *Roseburia*, *Prevotella* or *Escherichia–Shigella* genera, were significantly shifted in relation to the concentration of NOCs detected in stool samples. In the presence of intestinal mucosal alterations prior to the development of CRC, specific gut microbial members, such as *Escherichia–Shigella*, the *Ruminococcus torques* group, *Subdoligranulum* or *Intestinibacter*, previously reported to be associated with homeostatic intestinal imbalance and CRC, may arise in an environment in which NOC formation is increased, suggesting that gut microbiota and endogenous NOCs could act as enhancers of already-existing intestinal damage. We showed that the intake of possible dietary precursors of NOCs can promote the formation of endogenous NOCs and modulate the intestinal microbial profile of volunteers. Moreover, these dietary precursors of NOCs were positively associated with the relative abundance of some of the previously mentioned specific gut microbial members. The nature of this study does not allow us to determine whether our observations regarding NOCs and gut microbiota are each other's cause or consequence; nonetheless, it provides preliminary evidence about the link among specific intestinal microorganisms, fecal NOC concentrations, and the development of mucosal damage. One of the main limitations of the present study is the modest sample size, which limits the statistical power of the analyses. Therefore, further larger and adequately powered studies are necessary to confirm these results, enabling more robust subgroup analyses, and supporting the validity of our conclusions. Another limitation of the

present work is the use of microbial relative abundances in fecal samples instead of data on absolute levels. Further large-scale studies are needed to elucidate the mechanisms explaining diet, NOCs and gut microbiota associations.

Author contributions

Sergio Ruiz-Saavedra: methodology, investigation, formal analysis, data curation, writing – original draft. Tuulia Kreetta Pietilä: methodology, formal analysis, data curation, writing – review and editing. Aida Zapico: methodology, investigation, writing – review and editing. Nuria Salazar: investigation, writing – review and editing. Silvia Arboleya: investigation, writing – review and editing. Anne-Maria Pajari: conceptualization, investigation, supervision, writing – review and editing. Sonia González: conceptualization, funding acquisition, investigation, project administration, supervision, writing – review and editing. Clara G. de los Reyes-Gavilán: conceptualization, funding acquisition, investigation, project administration, supervision, writing – review and editing.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

Datasets of fecal DNA samples were deposited in the NCBI Sequencing Read Archive PRJNA994445 (https://www.ncbi.nlm.nih.gov/bioproject/994445). Other data will be made available on reasonable request.

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