Food Research International 209 (2025) 116287

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

# *In vitro* colonic fermentation of clean label ham formulations: Gut microbiota modulation and metabolite production

Teresa Bento de Carvalho [a], Joana Bastos Barbosa [a], Nelson Mota de Carvalho [a], Norton Komora [b], Fátima Carvalho [b], Ana Raquel Madureira [a], Paula Teixeira [a,*]

[a] *Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal*

[b] *R&D Department, Primor Charcutaria Prima – S.A., Avenida Santiago de Gavião 1142, 4760-003 Vila Nova de Famalicão, Portugal*

ARTICLE INFO

*Keywords:*
Gut microbiota
Gut modulation
Natural nitrite alternatives
Cured meat

ABSTRACT

Consumer wishes for "clean label" products have prompted the rise of these products available in the market. With dietary choices directly influencing market trends, developing novel meat products with natural nitrate and nitrite alternatives is a sought-after premise. Cured meats like ham have been under scrutiny due to their potential harm to human health, having sodium nitrite been theorised to trigger dysbiosis of the gut microbiota and impair faecal short-chain fatty acids (SCFAs) production. Four novel ham formulations with a natural nitrate source coupled with nitrate-reducing starter cultures were subjected to an *in vitro* gastrointestinal digestion simulation (INFOGEST) and followed by *in vitro* colonic fermentation. The impact of each novel ham formulation on the gut microbiota and their fermentation metabolites, namely SCFAs, was assessed by quantitative Next Generation Sequencing and High-Performance Liquid Chromatography, respectively. No significant differences have been found for SCFAs levels or microbial communities throughout colonic fermentation. Further research should provide insight into how these alternatives can be associated with nitrosamine formation. The potential benefits of "clean label" alternatives need to be thoroughly demonstrated. While these solutions are often considered preferable to traditional nitrite-containing products, their implementation should be approached with caution. In addition to their antimicrobial efficacy and consumer acceptance, it is essential to assess their impact on product cost and compare their performance and health impact (positive or negative) with that of traditional nitrite formulations. Extensive research is needed to ensure that any move to "clean label" formulations is based on solid evidence rather than market trends.

## 1. Introduction

The impact of nutrition on the human gut microbiota has been a widely discussed, especially when considering innovative products that are still a novelty within consumer choices (Conlon & Bird, 2014). Dietary practices have a major influence on the human gut microbiota, which explains the variations seen in people of different ages. What we eat not only provides fuel for us, but also for the bacteria that live in our gut, regulating the diversity and composition of our gut microbiota (Carlström, Moretti, Weitzberg, & Lundberg, 2020; de Carvalho, Oliveira, Costa, Pintado, & Madureira, 2022; Nagpal, Indugu, & Singh, 2021). Even though the core gut microbiota of healthy humans may be difficult to fully establish due to several variables, it is well-known and researched that small or abnormal changes, based on dietary choices, in the composition of this ecosystem may lead to gut dysbiosis, linked to many intestinal disorders such as irritable bowel syndrome, Crohn's disease, inflammatory bowel disease, among others (Cao et al., 2020; Mansour, Moustafa, Saad, Hamed, & Moustafa, 2021). The impact of macronutrients (*i.e.*, carbohydrates, fats and proteins) and micronutrients (*i.e.*, vitamins and minerals) on the gut microbiota has been a major focus of research in recent years. However, there is a growing interest in understanding how food preservatives in modern diets affect the gut microbiota (Rinninella et al., 2019; Scott, Gratz, Sheridan, Flint, & Duncan, 2012). An increase in the consumption of food preservatives within Western cultures and in the Westernization of dietary habits over the last few decades has led to the need to study their impact on gut modulation (Rinninella et al., 2019). Sodium nitrite is the most commonly used preservative in the curing of meat products due to its

* Corresponding author.
*E-mail address:* pcteixeira@ucp.pt (P. Teixeira).

https://doi.org/10.1016/j.foodres.2025.116287
Received 30 October 2024; Received in revised form 6 February 2025; Accepted 13 March 2025
Available online 20 March 2025

ability to inhibit foodborne pathogens and spoilage microorganisms. It is considered a multifunctional food preservative because it confers cured products with their characteristic colour and odour, as well as antimicrobial and antioxidant properties, which are crucial for ascertaining their microbiological safety (Oliveira et al., 2020). Sodium nitrite has been hypothesized to induce dysbiosis of the gut microbiota and the production of faecal short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, among others, which are essential for host-microbiota interactions and directly influence the maintenance of the colonic epithelial cells by being used as an energy source (Cao et al., 2020; Oliveira et al., 2020; Rinninella et al., 2019; Singh et al., 2017). The impact of excessive nitrite intake has been widely discussed, with concerns raised regarding its effects on the human gut microbiota (Carlström et al., 2020; Oliveira et al., 2020). While processed meats like ham are known sources of dietary nitrite, it is important to recognize that vegetables, especially leafy greens such as spinach and lettuce, contribute significantly to nitrite intake due to their high nitrate content, which is converted to nitrite in the body (Hord, Tang, & Bryan, 2009). In fact, vegetables are the primary source of nitrates and nitrites in the diet, accounting for the majority of intake. This broader perspective is essential when evaluating the impact of dietary nitrite on health, as it underscores the need to consider all dietary sources, not just processed meats (Kotopoulou, Zampelas, & Magriplis, 2021).

Health trends and sustainability concerns are the main drivers of consumer awareness of the ingredients in the food they eat. The search for products with short ingredient lists, recognizable ingredients and that are perceived by the public as healthy falls under the scope of the so-called “clean label” umbrella. As the demand for these types of products continue to rise, alternative meat-curing substitutes have focused on pre-converted nitrate sources, mainly from vegetables, including celery, spinach, beet, radish, and many others (Yong et al., 2020). However, their effect on human gut microbiota is still poorly documented (Cao et al., 2020). Research suggests that antimicrobial food additives, including sodium nitrite, may alter the composition of the human gut microbiota by selectively suppressing certain gut microbes. This can potentially affect gut health and the immune system (Hrncirova, Machova, Trckova, Krejsek, & Hrncir, 2019). On the other hand, natural nitrite alternatives may have different effects on the gut microbiota than synthetic nitrites. The potential of dietary nitrates as functional foods to support gut health and mucosal integrity in dysbiosis has been suggested, highlighting their broader health benefits beyond meat preservation (Rocha et al., 2019; Xu et al., 2022). As research progresses, understanding the interplay between dietary nitrates and gut microbiome dynamics may pave the way for innovative dietary strategies aimed at improving overall well-being. The complex relationship between functional foods and gut health highlights the critical role that nutrients from vegetables play in maintaining mucosal integrity, particularly during periods of dysbiosis (Zhang, 2022). As our understanding of nitrates and the dynamics of the gut microbiome deepens, we are on the verge of developing innovative dietary strategies that could revolutionise the way we approach gut health. This holistic perspective not only emphasises the importance of a plant-rich diet but also opens up new avenues for improving overall well-being through targeted nutritional interventions.

In this context, *in vitro* batch-culture fermentation models are feasible and accurate for evaluating the impact of novel foods, as the simulation of colonic fermentation using real human faecal inoculum will help to evaluate changes in microbiota composition and SCFAs production (de Carvalho et al., 2022; Leite, Fonteles, Filho, Da Silva Oliveira, & Rodrigues, 2022). Thus, this study aimed to assess the effect of novel ham formulations with an alternative natural curing method on the modulation of the gut microbiota composition and metabolic activity. A comprehensive gastrointestinal simulation model that encompassed the phases of gastric and intestinal digestion, intestinal absorption and colonic fermentation was used for this evaluation. High-Performance Liquid Chromatography (HPLC), an ammonium ion-selective electrode, and Next-Generation Sequencing (NGS) were used to evaluate the effects of each combination on fermentation metabolites (*i.e.*, SCFAs and ammonia) and gut microbiota profile, respectively.

## 2. Material and methods

### 2.1. Experimental matrices and controls

Hams were manufactured at an industrial scale by *Primor Charcutaria - Prima, S.A.*, a Portuguese charcuterie company, as described by Bento de Carvalho et al. (2024). Briefly, the base formulation, which was the same for all treatments, consisted of 85.9% minced pork leg, 11.5% water, 1.4% sodium chloride, 0.7% sodium tripolyphosphate and 0.3% carrageenan. One formulation (E) served as the conventionally produced ham (control), while four formulations (A, B, C and D) were produced with complete replacement of sodium nitrite by natural nitrate sources coupled with commercial nitrate-reducing food cultures (Bento de Carvalho et al., 2024). Nitrate-rich powder was used for samples A and B, and the final concentration was based on manufacturer’s instructions. Vegetable blanching water with a final concentration of 6.9% was used for samples C and D. After mixing all the ingredients, the final mixture was matured for 48 h at 4 °C to allow the conversion of nitrate to nitrite and then vacuum stuffed into 3.9 kg blocks using a PA/PE plastic casing. The blocks were then pressed into ham moulds and thermally processed until the core of the block reached 70 °C and cooled overnight at 4 °C. Slicing into 1.2 mm thick ham slices took place after the stabilisation period. The slices were then packed under a modified atmosphere ($O_2 \leq 0.3\%$; $CO_2 \approx 22.5–34.5$; $N_2 \approx 70.0\%$) in thermoformed multi-layer PET/EVOH/PE cuvettes and kept under refrigerated conditions until use. Formulations are presented in Table 1.

### 2.2. Preparation of Faecal Inoculum

Human faecal samples from six healthy volunteers were collected according to internally established protocols for stool collection practices, previously accepted and validated by Universidade Católica Portuguesa Health Ethics Committee within the premises of the Alchemy project (Universidade Católica Portuguesa, Escola Superior de Biotecnologia, Porto, Portugal). Stool samples were stored in Oxoid™ AnaeroJar™ 2.5 L containers with an OxoidTM AnaeroGen™ 2.5 L sachet (Thermo Fischer Scientific, Waltham, CA, USA) and solely opened inside an anaerobic cabinet (nitrogen 80%, carbon dioxide 10%, hydrogen 10%), a Whitley A35 workstation (Don Whitley Scientific, Bingley, UK). Faecal content of each donor was weighed to obtain a pooled faecal inoculum to have a uniform and representative inoculum. The former inoculum was diluted at 10% (*w*/w) in a 0.1 M phosphate-buffered saline pH 7.3 (PBS) (Thermo Fischer Scientific, Waltham, MA, USA) solution with 30% (*v*/v) glycerol (Fisher Scientific, Loughborough, UK) and homogenised. Aliquots of the pooled faecal inoculum were stored at −20 °C to avoid repeated freezing and thawing cycles. According to de Carvalho, Oliveira, Saleh, Pintado, and Madureira (2021), inocula were subjected to two glycerol wash-out cycles to remove the glycerol, which was employed as a cryopreservative during storage.

**Table 1**
Formulations of the five ham prototypes manufactured.

| Code | Composition |
|---|---|
| A | Commercial dry blend of chard, carrot and acerola + Meat culture 1 |
| B | Commercial dry blend of chard, carrot and acerola + Meat culture 2 |
| C | Spinach blanching water + Meat culture 1 |
| D | Spinach blanching water + Meat culture 2 |
| E | Control with the nitrifying salt (150 mg/kg sodium nitrite) |

### 2.3. Information regarding the faecal donors

In accordance with the Declaration of Helsinki conduct guidelines and the regulations for the collection and processing of personal data set forth by Regulation 2016/679 of the European Parliament and the Council (EU), all subjects voluntarily completed a questionnaire about their dietary and lifestyle habits before beginning the study . The participants did not present or possess any intestinal disorders, had a typical omnivorous diet, had not used antibiotics or any other medications known to alter the microbiota for at least a year before donation, and were not regular users of probiotics or prebiotics. Furthermore, the previous hospitalisation they had was over a year before the start of this study. Volunteers were three males, and three females aged 33.33 ± 4.78 years, with a body mass index (BMI) of 26.68 ± 4.56 kg/m$^2$. Detailed information on the volunteers participating in this study is displayed in Supplementary material S1.

### 2.4. Human in vitro gastrointestinal tract (GIT) simulation model

An *in vitro* simulation of the gastrointestinal tract based on the study by Brodkorb et al. (2019), commonly referred to as INFOGEST 2.0 protocol, was performed with some modifications as previously described by de Carvalho et al. (2021), namely the colonic fermentation protocol. To simulate the oral phase, different ham formulations with different nitrate sources were homogenised in a stomacher (Seward, Worthing, UK) for 5 min and mixed with simulated salivary fluid and salivary amylase (20.0 U/mg; Sigma, St. Louis, MO, USA), with pH adjusted to 7.0, for 2 min at 37 °C. The pH was then lowered to 3.0 to begin the gastric phase, where a 1:1 simulated gastric fluid with added pepsin (571.3 U/mg; Sigma, St. Louis, MO, USA) and rabbit gastric extract (15 U/mg; Lipolytech, Marseille, France) was added to the samples and incubated at 37 °C with moderate agitation for 2 h. For the intestinal absorption phase, a pancreatin (6.10 U/mg; Sigma, St. Louis, MO, USA) and bile solution (1.77 mmol/g; Sigma, St. Louis, MO, USA) was prepared, and pH was adjusted to 7.0 and incubated for 2 h with moderate agitation at 37 °C.A dialysis step to simulate the absorption in the small intestine was performed All samples were transferred to 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing (Spectrum, New Brunswick, NJ, USA), and a dialysis was performed against 10 mM NaCl (LabChem, Zelienople, PA, USA) at room temperature with constant stirring, to remove low molecular mass digestion products. After this step, all samples were freeze-dried (Armfield SB4 model, Ringwood, UK) to obtain a powder to be used for *in vitro* faecal fermentations. For colonic fermentation, the previously prepared frozen pooled faecal inoculums were used, and six conditions (in duplicate) were tested: (I) inoculum control; (II) ham formulation A; (III) ham formulation B; (IV) ham formulation C; (V) ham formulation D; (VI) ham formulation E. Faecal batch-culture fermentation was carried out according to de Carvalho et al. (2019). Fermentation vessels, set up aseptically, were filled with sterile basal nutrient medium (peptone water (2 g/L); yeast extract (2 g/L); NaCl (0.1 g/L); $K_2HPO_4$ (0.04 g/L); $KH_2PO_4$ (0.04 g/L); $MgSO_4{\cdot}7H_2O$ (0.01 g/L); $CaCl_2{\cdot}6H_2O$ (0.01 g/L); $NaHCO_3$ (2 g/L); Tween 80 (2 mL/L); hemin (0.05 g/L); vitamin K (10 μL/L); L-cysteine HCl (0.5 g/L); bile salts (0.5 g/L) and resazurin (4 mg/L)) and gassed overnight with $O_2$ - free $N_2$ with constant shaking. Each condition was performed in duplicate, and digested ham substrates were added at 1% (*w/v*) aseptically (by flaming the entry/sampling port). Substrates were mixed with the basal media, and each vessel was inoculated with 15 mL faecal inoculum. Six stirred pH-controlled batch fermenters, FerMac 260 (Electrolab Biotech Ltd., Gloucestershire, UK), were run in parallel with each vessel between 6.7 and 6.9 (the pH of the human distal colon), and the temperature was kept at 37 °C with the help of a water bath.

A static *in vitro* batch fermentation model was conducted for 48 h. This is the recommended period to prevent nutrient depletion and metabolite accumulation that can restrict microbial fermentation and growth (Dixit, Kanojiya, Bhingardeve, Ahire, & Saroj, 2023; Isenring, Bircher, Geirnaert, & Lacroix, 2023). Samples (10 mL) were taken aseptically from each vessel, at time points 0, 24 and 48 h, and immediately put in ice to stop the reaction. After that, samples were centrifuged at 4 °C, 4696 ×*g* for 5 min, and the supernatant was collected for SCFAs and branched-chaine fatty acids (BCFAs) analysis done by HPLC and ammonium ($NH_4^+$) concentration measurement, using an ion-selective electrode 9663 of ammonium. From the centrifugation of the supernatant, the obtained pellet was resuspended in 10 mL of a 0.1 M PBS (Thermo Fischer Scientific, Waltham, MA, USA) solution, vortexed, and centrifuged once again in the same conditions (Thermo Fischer Scientific, Waltham, MA, USA). The supernatant was discarded, and the pellet was washed one more time. Pellets for NGS analysis and supernatants for HPLC from 0, 24 and 48 h were stored at −20 °C until further analysed.

### 2.5. DNA extraction and bioinformatics analysis

DNA extraction from the stored pellets from two independent colonic fermentation was carried out using the Invitrogen PureLink™ Microbiome DNA Purification Kit (Thermo Fischer Scientific, Waltham, MA, USA) protocol for stool samples. Extraction was performed according to the manufacturer's instructions.

Pooled DNA from two replicates per condition was performed and amplification of the V3-V4 region of the 16S rRNA gene using the specific 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806R (5′-GGACTACHVGGGTWTCTAAT) primers (Caporaso et al., 2010), was selected due to being regarded as the most suitable for gut microbiota analysis as well as due to being the region used on the official Illumina sequencing protocol (Kameoka et al., 2021). DNA purity and quantity were assessed by spectrophotometric measurement using NanoDrop One UV–Vis spectrophotometer (Thermo Fisher Scientific, Inc., Massachusetts, USA). Amplicons were sequenced using an Illumina paired-end platform. Raw reads were then quality filtered with PRINSEQ version 0.20.4, according to Schmieder and Edwards (2011), to remove sequencing adapters, trim low-quality bases (<Q25) and eliminate reads of less than 100 bases. Paired-end reads were overlapped using AdapterRemoval version 2.1.5. (Schubert, Lindgreen, & Orlando, 2016). Quality-controlled reads were analysed using the EZBioCloud 16S-based MTP pipeline (Yoon et al., 2016), which includes chimaera detection and removal with UCHIME and the definition of OTUs using a cut-off of 97% similarity. For alpha- and beta-diversity analysis, samples were normalised to 98,145 reads. The 16S rRNA gene sequences are available in the NCBI SRA archive under BioProject number PRJNA1179443.

### 2.6. Determination of organic acids produced throughout fermentation

Supernatants obtained after centrifugation were filtered with a 0.22 μm sterile filter and directly analysed by HPLC in duplicate, as described by de Carvalho et al. (2022), with minor adjustments. The HPLC analysis was performed using an Agilent 1260 II series system equipped with a refractive index (RI) detector and a diode array detector (DAD) set at 220 nm. The separation utilized an ion-exclusion Aminex HPX-87H column maintained at 50 °C. The mobile phase consisted of 5 mM sulfuric acid ($H_2SO_4$), with a flow rate of 0.6 mL/min, a total run time of 40 min, and an injection volume of 10 μL. Calibration curves (ranging from 2 mM to 80 mM) were used to identify and quantify lactate, acetate, propionate, butyrate, isobutyrate, and isovalerate.

### 2.7. Measurement of total ammonia nitrogen concentration

Total ammonia nitrogen concentration was measured according to de Carvalho et al. (2022). Ammonium concentration was measured using an ion-selective electrode (model 9663) under controlled conditions, including room temperature (20 °C) and a stable pH range of 6.7–6.9. Following the manufacturer's protocol, 300 μL of 1 M $MgSO_4$

(used as an ionic strength adjuster) was added to 3 mL of the supernatants obtained after centrifugation. Measurements were performed in duplicate with the electrode. Quantification was achieved using a standard calibration curve of $NH_4Cl$ ranging from 2 mM to 55 mM. The total ammonia nitrogen concentration was determined using the equation provided below:

$$\frac{[NH_4^+]}{[NH_3 + NH_4^+]} = 1 - \frac{1}{1 + 10^{pKa-pH}}$$

where ($NH_4^+$) is the ammonium ion concentration, ($NH_3^+$ $NH_4^+$) is the total ammonia nitrogen concentration, and pKa is the acid dissociation constant that can be expressed as a function of temperature (T) using the following equation:

$$pKa = 4 \times 10^{-8} T^3 + 9 \times 10^{-5}\ T^2 – 0.0356\ T + 10.072$$

A standard calibration curve of $NH_4Cl$ (Sigma, St. Louis, MO, USA) (2 mM to 55 mM) was used for quantification.

### 2.8. Statistical analysis

Statistical analysis was carried out using IBM® SPSS Statistic Analytics 28.0 (IBM, Chicago, IL, USA). The Shapiro–Wilk test was used to assess the normality of data distribution for colonic fermentations SCFAs profile, organic acid and total ammonia nitrogen concentration, at each sampling time. Since the samples exhibited normal distribution, one-way ANOVA followed by Tukey's post-hoc test was used to compare the means, with a 95% confidence interval. The Bioconductor DESeq2 package was used in R with default parameters to normalize data and identify differentially abundant genes across three food groups (Anders & Huber, 2010). *P* values were corrected for multiple testing using the Benjamini-Hochberg method, which evaluates the false discovery rate (FDR). Principal coordinate analysis (PCoA) was used to assess microbial beta diversity using Operational Taxonomy Units (OTUs) based Bray-Curtis distance matrix. Permutational multivariate analysis of variance (PERMANOVA) was used to compare microbial community differences across groups using the "Adonis" function in the R package "vegan" with 9999 permutations.

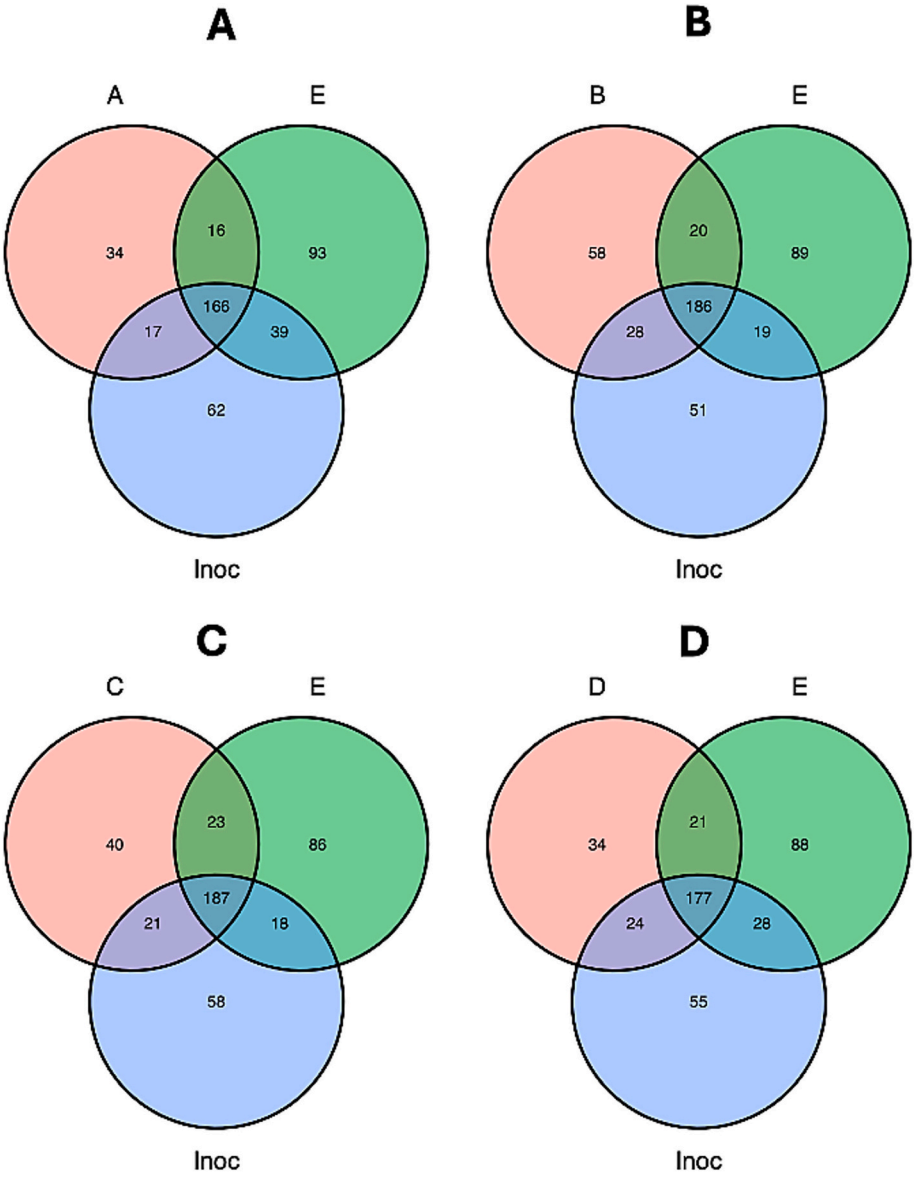


**Fig. 1.** Numbers of shared OTUs of the bacterial community: **(a)** for ham A between ham E and inoculum control (inoc); **(b)** for ham B between ham E and inoc; **(c)** for ham C between ham E and inoc; **(d)** for ham D between ham E and inoc.

## 3. Results and discussion

### 3.1. Bacterial community structure and diversity

Bacterial community structure and diversity were assessed through sequencing of the 16S rRNA gene amplicons.

To identify common OTUs between samples, comparisons were made between the composition of bacterial communities in new ham formulations (A, B, C, and D) and the control ham (E). Also, ham formulations were compared to the inoculum control (Inoc). For these purposes, the common OTUs were analysed and represented through Venn diagrams (Fig. 1).

The Venn diagram analysis illustrates the distribution of OTUs between the novel ham formulations, control ham, and inoculum control. Comparisons between ham A, control ham, and inoculum control revealed 166 common OTUs, representing 38.9% of the total number of OTUs (total OTUs = 519). For ham B, ham C and ham D, 186 OTUs (41.2%), 187 OTUs (43.2%) and 177 OTUs (41.5%) were shared, respectively. This suggests the existence of a core microbiota between the samples, as expected, since the same faecal inoculum pool was used during fermentation. Ham B had the highest number of unique OTUs (58) when compared to both control ham and inoculum control, while hams A and D presented the lowest number of unique OTUs (34 each). Overall, these findings provide valuable insights into gut microbiota diversity, showing the similarity between formulations in terms of microbial gut modulation indicators.

Alpha diversity is used to analyse the variety of microbial communities within the sample by examining the diversity of a single sample (Li et al., 2022). This analysis was conducted on a single dataset generated from a pool of two replicates per condition since this pooling approach provides a composite representation of the microbial diversity within each condition. All the samples analysed presented a Good's coverage of the library above 99.76 (Table 2, indicating whether the current sequences represent most of the bacterial community, allowing a statistically significant analysis of the bacterial diversity (Wang et al., 2012). Alpha-diversity was assessed following two parameters: the Shannon index to identify community diversity and the Chao1 estimator to identify community richness (Table 2).

Community diversity within samples was measured using the Shannon index which is calculated with the number of OTUs. The biodiversity index was observed between samples. Results show that the inoculum control (Inoc) had higher bacterial diversity than the test samples, with added digested lyophilised ham. However, no differences were observed between samples. To identify community richness, the Chao 1 index was used (Table 2), since it gives details on the different species included in the sample (Chao, 1984). Chao 1 richness differed among the ham formulations, being highest for the inoculum control sample (Inoc) and sample D, and lowest for sample C at the initial time (0 h). Higher alpha diversity in the inoculum has also been reported by Chung et al. (2016), in a study on dietary fibres. The availability of a single substrate, the lyophilised digested ham formulations, as an energy source is thought to be the primary cause of the overall decline in bacterial diversity in the test samples compared to the faecal inoculum. Conversely, since normal human diets provide more diverse nutritional properties than a single substrate, the relative stability of the environment within the fermentation vessels in comparison to the variations that happen *in vivo* as a result of regular meals is probably another significant aspect (Chung et al., 2016). Variations between formulations may be directly related to which natural nitrate alternative was used to manufacture the pilot hams, since the nitrate-rich powder and the vegetable blanching water were coupled with two different starter cultures that may rearrange the alpha-diversity of the bacterial community of the product. The results evidence the shift in microbial diversity and microbiota composition throughout the fermentation process, but do not show differences between nitrite sources, either natural or synthetic.

**Table 2**
Diversity and richness indexes obtained for 16S rRNA gene sequencing.

| Sample | Good's coverage of library | Shannon | Chao1 |
|---|---|---|---|
| **A0h** | 99.84 | 4.16 | 829.04 |
| **B0h** | 99.80 | 3.01 | 921.44 |
| **C0h** | 99.88 | 2.84 | 630.56 |
| **D0h** | 99.87 | 3.42 | 698.00 |
| **E0h** | 99.78 | 3.47 | 978.36 |
| **Inoc0h** | 99.76 | 4.43 | 1090.44 |
| **A24h** | 99.90 | 3.22 | 536.22 |
| **B24h** | 99.89 | 3.21 | 516.11 |
| **C24h** | 99.83 | 3.36 | 783.57 |
| **D24h** | 99.81 | 3.66 | 841.29 |
| **E24h** | 99.86 | 3.52 | 684.44 |
| **Inoc24h** | 99.86 | 3.98 | 755.08 |
| **A48h** | 99.87 | 3.41 | 629.72 |
| **B48h** | 99.84 | 3.66 | 766.46 |
| **C48h** | 99.86 | 3.36 | 664.00 |
| **D48h** | 99.90 | 3.19 | 529.82 |
| **E48h** | 99.87 | 3.01 | 605.18 |
| **Inoc48h** | 99.90 | 3.77 | 610.78 |

The bacteria present were identified using microbiota analysis. Taxonomic composition for all samples throughout colonic fermentation can be observed in Fig. 2 A (Phylum) and B (Family).

The microbiota community structure was determined after fermentation of digested ham samples for 48 h. The most abundant phylum present were *Bacillota* and *Pseudomonadota* for all samples (A, B, C, D, E and Inoc) and all time points (0, 24 and 48 h). One of the smallest groups was the "Unclassified" category, with individual relative abundances of less than 0.1%. A shift in bacterial communities was observed after 48 h of fermentation, with significant differences having been observed for *Bacteroidaceae*, *Desulfovibrionaceae*, *Erysipelotrichaceae*, *Ruminococcaceae, Christensenellaceae, Enterococcaceae, Fusobacteriaceae, Sphigomonadaceae, Veillonellaceae* and *Sutterellaceae* ($p < 0.05$) across all samples. No significant differences in taxonomic abundance have been observed between ham formulations A-D and B—C ($p > 0.05$). However, significant differences ($p < 0.05$) have been found for *Erysipelotrichaceae* and *Carnobacteriaceae* between samples A-B, A-C, B—D and C-D; for *Erysipelotrichaceae*for between samples B-E and C-E; and for *Carnobacteriaceae* between samples A-E and D-E. A higher relative abundance of *Enterobacteriaceae* (*Pseudomonadota*) was observed for all samples compared to the inoculum control (Inoc) for all sampling times. Nissen et al. (2023) have reported that due to the presence of fibre in the plant extracts used to produce salami, a higher relative abundance of *Bacteroidota* was observed, as well as an increase in butyrate production (Nissen et al., 2023; Pérez-Burillo et al., 2019). However, in our results, such was not observed. After fermentation, the relative abundance of *Bacteroidota* was higher for the ham control sample (added sodium nitrite) than for the samples formulated with natural plant nitrate coupled with starter cultures. Nissen et al. (2023) have also reported that an increase in the *Bacteroidota* phylum is a good indicator since species belonging to this phylum are a large contributor to propionate production, known for its anti-inflammatory properties, as well as known fibrolytic capacity (Shon et al., 2023). The abundance of this phylum after both 24 and 48 h of colonic fermentation is supported by SCFAs concentration results (Fig. 4). Our results reveal that both new ham and traditional ham formulations allow for *Bacteroidota* growth similar to the inoculum control. On the other hand, *Actinobacteria* abundance decreases after 48 h when compared to the initial time. *Actinobacteria* have been regarded as relevant microorganisms for the upkeep of gut homeostasis, mainly due to the genus *Bifidobacterium*, due to the production of acetate and maintenance of gut permeability (Binda et al., 2018). Even though acetate concentrations increased overtime (Fig. 4), it will most likely be due to the increase of *Clostridium* abundance, also known as an acetate producer (de Carvalho et al., 2022). Regarding the inoculum control (Inoc 0 h, Inoc 24 h and Inoc 48 h), a lower relative abundance of *Pseudomonadota* was observed compared to the remaining samples. *Bacillota* and *Bacteroidota* are the most abundant phyla present

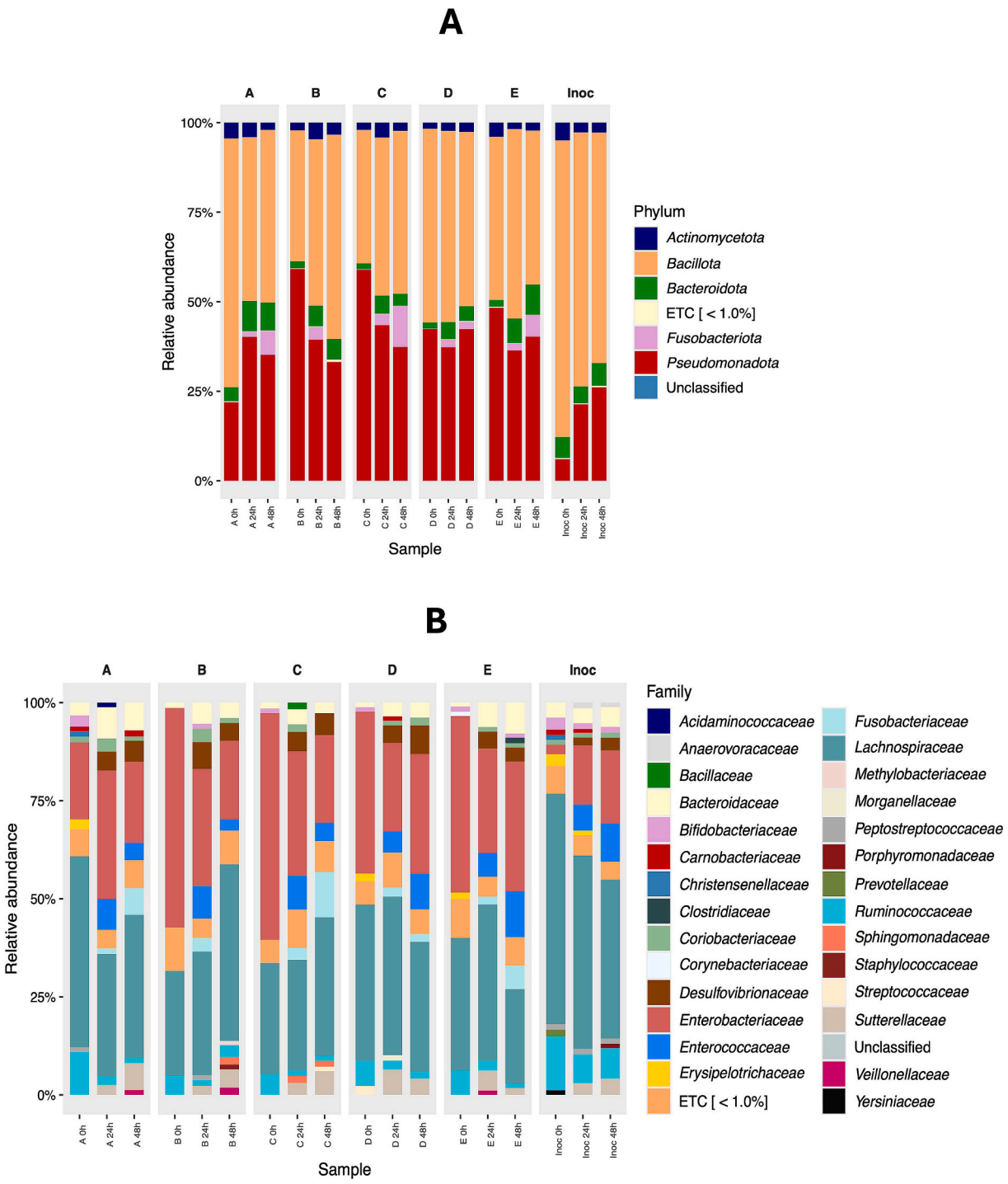


**Fig. 2.** Taxonomic composition ratio (%) for Phylum (A) and Family (B).

in the human gut (Rinninella et al., 2019). In our study, *Bacillota* and *Pseudomonadota* were found to be the most abundant phyla, with the exception of the inoculum control before fermentation, where the standard *Bacillota/Bacteroidota* ratio was observed, alluding to the donor's gut microbiota eubiosis (da Silva et al., 2023). One explanation may advent from the impact of nitrite (either from chemical or natural sources) on the taxonomic balance of the gut microbiota, resulting in dysbiosis. Although some signs of dysbiosis may be observed, it is important to emphasise that such a claim cannot be made directly since these shifts may be the result of the addition of a single substrate to the faecal inoculum, as mentioned above and not a direct consequence of nitrite consumption. Regarding the results obtained, an interesting finding lies on the fact that *Fusobacterium nucleatum* was not observed in the inoculum control but observed for all hams, new and traditional. This bacterium has been long known for its implications in oral diseases, mainly responsible for gingivitis and periodontitis, but its interactions within the gut microbiota have also been proven due to its adhesive and aggregative qualities (Allen-Vercoe, Strauss, & Chadee, 2011). *F. nucleatum* has been shown to create pro-inflammatory and tumour-promoting conditions in the digestive tract, having been correlated with colorectal cancer, being stated that its presence in healthy stool is rare (Brennan et al., 2021). Since *F. nucleatum* has not been observed for the inoculum control sample, it is possible that its proliferation in the ham samples may be due to the presence of a certain compound that may

promote its growth. It is also important to highlight that a higher taxonomic abundance of this species at the end of colonic fermentation was observed for ham C (11.50%) > ham A (6.77%) > control ham (5.95%) > ham D (1.99%) > ham B (0.00%). Cao et al. (2020) have reported that food additives like preservatives highly influence the gut microbiota. Bacterial strains such as *Bacteroides coprocola, Lacticaseibacillus paracasei* and *Clostridium tyrobutyricum*, with known anti-inflammatory benefits, were shown to be sensitive to sodium nitrite, while microorganisms that may be related to pro-inflammatory episodes and possess colitis-inducing properties such as the *Enterococcus* genus were shown to be less sensitive to these antimicrobial compounds (Cao et al., 2020; Gonza et al., 2024; Hrncirova et al., 2019; Zhou, Qiao, Wu, & Zhang, 2023). These findings have prompted the suggestion within the research community that food preservatives may induce dysbiosis of the human gut microbiota even at low doses (Cao et al., 2020). In summary, while bacterial diversity was generally lower in ham samples than in the faecal control, this was likely due to the simpler nutritional profile of the ham. No major differences in microbiota composition were found between nitrite types, though potentially harmful bacteria like *F. nucleatum*-associated with inflammation—were more prevalent in ham samples. This suggests nitrites and other additives may impact microbial balance, potentially affecting gut health. Further research is needed to assess these shifts in a more complex dietary context.

Bacterial communities' composition varied among different samples as determined by PCoA ordination analysis (Fig. 3).

In the PCoA ordination plot, a separation in the community structure can be observed mainly as a driving effect of the changes that occur during fermentation. After studying the community dissimilarity during 48 h of colonic fermentation, PERMANOVA showed significant differences in bacterial communities between times 0 h and 24 h–48 h (Adonis2, $R^2 = 0.735$, $p = 0.001$). This result suggests that the formulation variable explains a large proportion of the variation between samples, indicating a strong grouping effect, indicating that the distances between samples within the same group are much smaller than the distances between samples from different groups. The statistically significant grouping pattern is confirmed by the *p*-value (0.001). Samples A, B, C, D and E clustered together for time points 24 and 48 h, as corroborated by the alpha-diversity analysis (Table 2). Regarding the comparison between formulations, no significant differences have been found between hams (Adonis2, $R^2 = 0.09825$, $p = 0.97$), suggesting that the formulation has no significant effect on the distances between samples. For both variables (time and formulation), the homogeneity of dispersions was assessed beforehand, with the assumption of homogeneity being met ($p > 0.05$). In conclusion, the results from the PCoA ordination and PERMANOVA analysis indicate that the temporal factor, particularly the fermentation process, significantly shapes the bacterial community structure, with clear differences observed between the 0 h and 24 h–48 h time points. However, no significant effects were found between the different formulations, suggesting that the formulation variable does not substantially influence the community composition.

### 3.2. Colonic fermentation SCFAs profile

*In vitro* fermentation systems inoculated with human faecal samples were used to assess the effect of natural nitrite alternatives on the human gut microbiota. SCFAs that comprising acetate, propionate, and butyrate, are the primary metabolites of colonic fermentation, while BCFAs, namely isobutyrate and isovalerate, comprise only 5% of SCFAs concentrations found. SCFAs and BCFAs, along with ammonia, increase throughout colonic fermentation (de Carvalho et al., 2022). Concentrations of the three major SCFAs, acetate, propionate, and butyrate, and two BCFAs, isobutyrate and isovalerate, were quantified with results shown in Fig. 4 and Fig. 5, respectively.

The samples that showed higher concentrations of SCFAs (Acetate + Butyrate + Propionate) after 48 h colonic fermentation were D (65.96 mM) > A (63.36 mM) > E (59.35 mM) > C (58.64 mM) > B (57.22 mM) > Inoc (16.20 mM) (Fig. 3). Increased SCFAs production was observed for samples D and A comparatively to samples E, B and C ($p < 0.05$). Even though the presence of dietary fibres, found in vegetable extracts, has been shown to increase SCFAs production, the fact that the

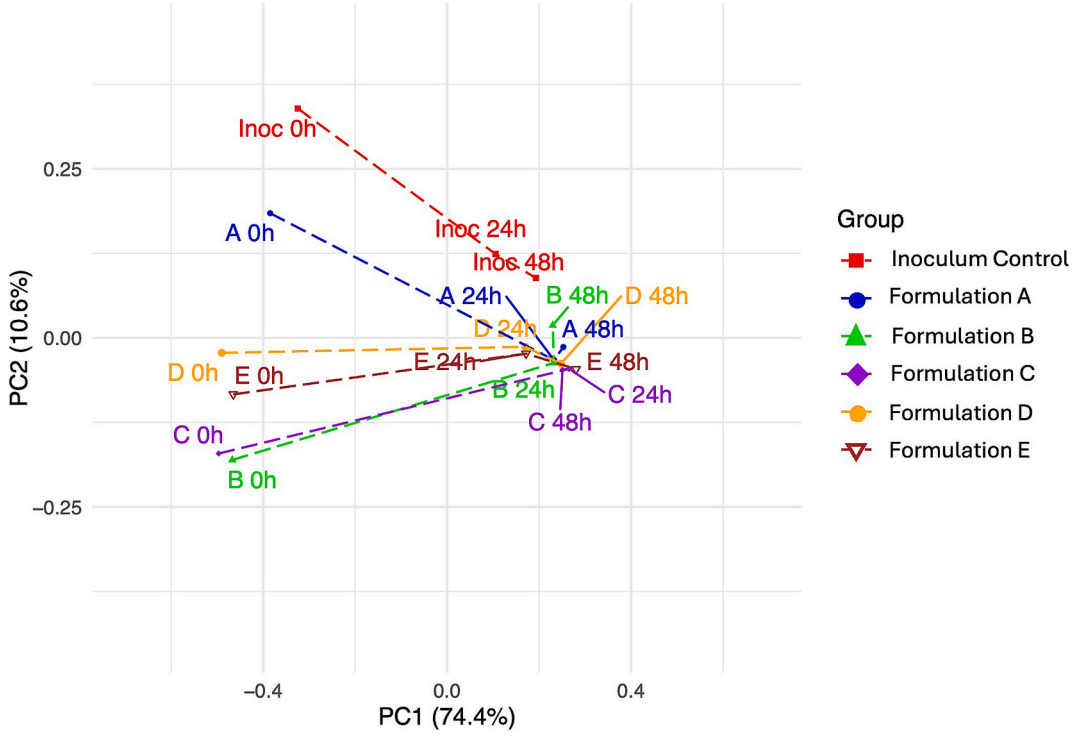


**Fig. 3.** Sample distribution according to Principal Coordinates Analysis (PCoA) of a Bray-Curtis dissimilarity matrix regarding the taxonomic annotation at the Genus level. Samples highlighted in the same colour represent the same formulation overtime.

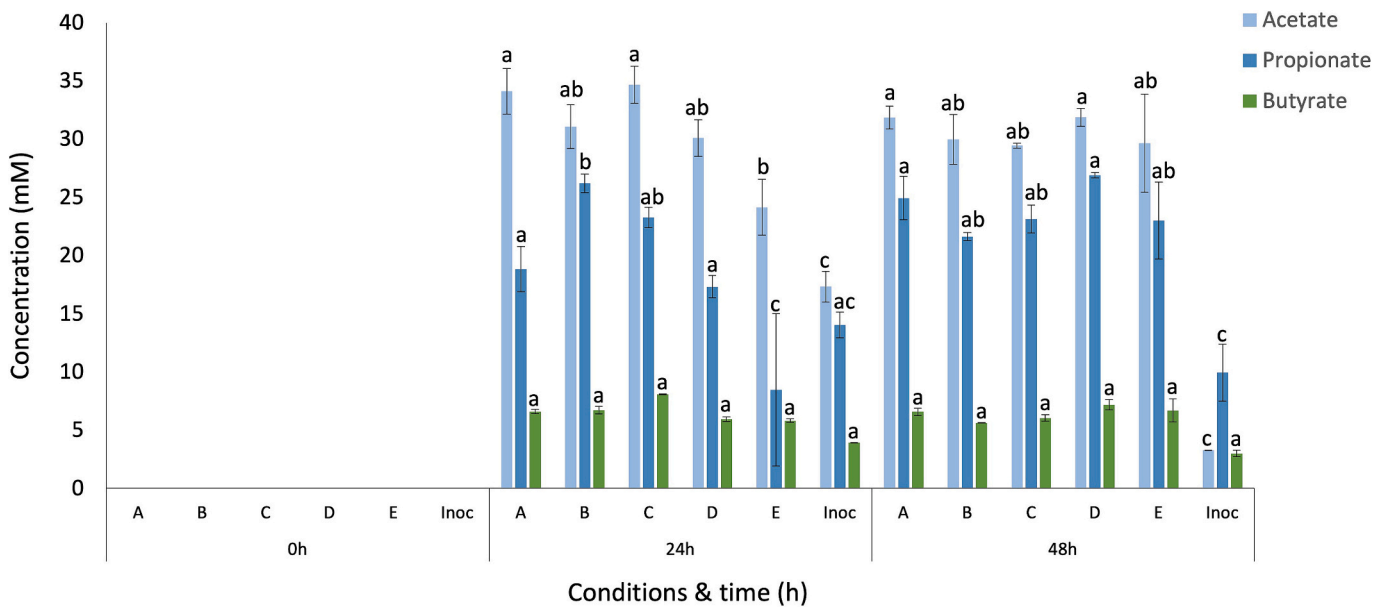


**Fig. 4.** Concentration (mM, means ± SD) of SCFAs during colonic fermentation. Equivalent lower-case letters mean no significant differences between each sample at the same sampling time ($p > 0.05$).

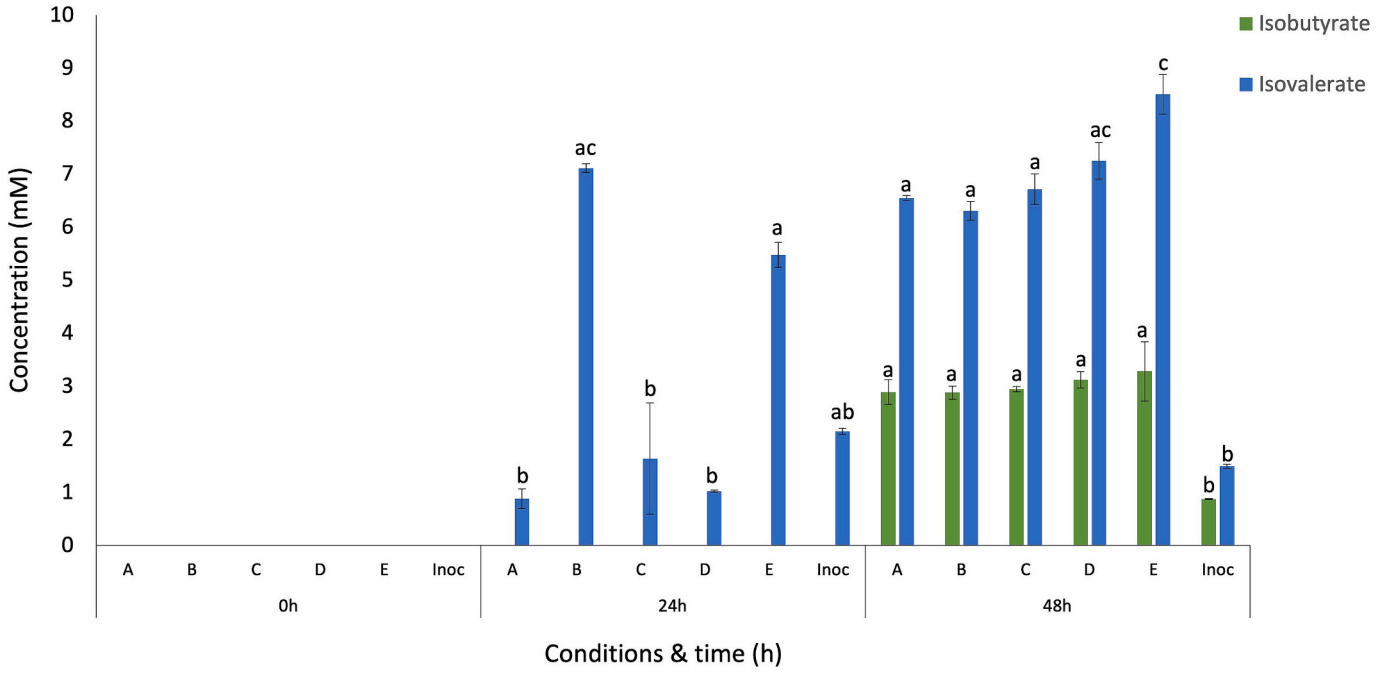


**Fig. 5.** Concentration (mM, means ± SD) of BCFAs during colonic fermentation. Equivalent lower-case letters mean no significant differences between each sample at the same sampling time ($p > 0.05$).

conventional ham formulation (E) has the third highest concentration is a good indication that different factors play a role in SCFAs production during fermentation (Pérez-Burillo et al., 2019). Lowest SCFAs concentrations were found for the inoculum control (Inoc), as expected, due to the lack of nutrients to promote bacterial fermentation over time (de Carvalho et al., 2022). As seen by our results, acetate concentration is the highest of all faecal metabolites in human faeces (Duncan, Iyer, & Russell, 2020). Acetate concentration was shown to increase overtime for all ham samples and control sample, except ham A. For propionate, a significant increase was observed ($p < 0.05$) between 24 and 48 h of fermentation for hams A, D and E. For sample B and inoculum control (Inoc), a significant ($p < 0.05$) decrease was observed between 24 and 48 h. Sample C showed no significant difference in propionate production, while a decrease in acetate and an increase in isovalerate was observed between 24 and 48 h ($p < 0.05$). The physiological functions of SCFAs are crucial for gut homeostasis by directly influencing the maintenance of epithelial cells (Cao et al., 2020). Alterations in the gut environment may lead to altered inflammatory responses and increased vulnerability to opportunistic pathogens (Leclerc et al., 2021). Although the advantages of the new formulations were not significantly greater than those of the control and the product containing sodium nitrite, as observed by Pini, Aquilani, Giovannetti, Viti, and Pugliese (2020), in pork dry-fermented sausages and by Nissen et al. (2023) in salami, the results are encouraging, since the alternative natural nitrate sources

employed in place produced outcomes equivalent to those achieved with the traditional curing process (Nissen et al., 2023). Colonic bacteria, primarily *Bacteroides* and *Clostridia* species, cleave proteins resulting in metabolites called BCFAs, mainly represented by isobutyrate and isovalerate (LaBouyer et al., 2022). During the colonic fermentation, BCFAs showed lower concentrations than SCFAs, as expected (de Carvalho et al., 2022). After 48 h of fermentation, no differences between the test ham formulations and the traditional ham recipe were found ($p > 0.05$). The pH of the luminal membrane may rise as a result of high isobutyrate concentrations, and the noxious substances produced during protein fermentation. Thus, a healthy gut microbiota is associated with a low level of isobutyrate (Leite et al., 2022). Regarding isovalerate concentration, no significant differences ($p > 0.05$) were found between novel formulations, but differences were found ($p < 0.05$) between all test hams and the traditional formulation. Regarding the additional effects of BCFAs on colonic epithelial cells, not much is known. It is therefore believed that faecal concentrations of BCFAs are not indicators of colon health but rather only of bacterial protein fermentation (Verbeke et al., 2015).

Total ammonia nitrogen production throughout 48 h of colonic fermentation is shown in Fig. 6.

At 0 h, total ammonia nitrogen concentration was not significantly different ($p > 0.05$) for all conditions except for the inoculum control sample ($p < 0.05$). After 6 h, the condition with the lowest concentration of ammonia nitrogen was also the inoculum control sample. The total ammonia nitrogen concentration of control sample was significantly different from all other samples for all time points. At 24 and 30 h, sample B showed the highest concentration ($p < 0.05$), while samples A and C were shown not to be significantly different, as well as D and E ($p < 0.05$). After 48 h, ham sample A showed the highest ammonia concentration ($p > 0.05$), followed by sample C ($p > 0.05$), B, D and E, respectively ($p < 0.05$). Ammonia is a well-known marker of protein fermentation, being a major product of amino acid catabolism (Peled & Livney, 2021). Ammonia can be found in the large intestine at concentrations usually ranging from 12 to 30 mM, being valuable as a nitrogen source for bacterial cells that compose the gut microbiota (de Carvalho et al., 2022; Scott et al., 2012). Increased values may advent from high protein intake (Duncan et al., 2020). In high concentrations, ammonia is considered a potentially toxic metabolite, leading to alterations in the intestinal tissue, and potentially carcinogenic (Duncan et al., 2020; Scott et al., 2012). In our study, total ammonia nitrogen levels were below the 30 mM range for all samples and all time points. As expected, the inoculum control had the lowest concentration of ammonia due to the lack of nutrients, while samples A through E showed an increase in concentration throughout the 48 h of colonic fermentation due to protein degradation (Xiao et al., 2020). Ammonia nitrogen concentration will be directly influenced by the gut microbiota. Sample A has the most obvious increase throughout the fermentation process. While the relative abundance of ammonia nitrogen-producing bacteria was low at 0 h, at 24 h, *Fusobacterium* and *Escherichia* genera, well-known ammonia nitrogen producers, were abundant, resulting in the observed increase (Han et al., 2020; Vince & Burridge, 1980). While a shift in microbiota composition was observed at 48 h, relative abundance was high for *Clostridium*, *Fusobacterium* and *Bacteroides*, also good representatives of ammonia nitrogen producing genera (Dandachi et al., 2021; Han et al., 2020; Vince & Burridge, 1980) On the other hand, samples B and C showed a decrease after 48 h in total ammonia nitrogen concentration. This may be explained by the increase of the *Sphingomonas*, since this genus is known to be able to reduce ammonia nitrogen levels (Wang et al., 2012) (Fig. 2). An increase in concentration was observed from 0 to 24 h for samples D and E, and a concentration plateau was observed for the remaining two time points, 30 and 48 h. Relative taxonomic abundance was the most similar for these fermentation points, possibly explaining that no significant differences were found for the 24- h (24 to 48 h) sampling period (Fig. 2).

In summary, SCFAs levels, beneficial for gut health, were highest in specific ham samples, indicating potential influences beyond nitrite source alone. Ammonia levels, a marker of protein fermentation, increased over time in all samples but stayed below toxic thresholds, with differences influenced by specific bacterial genera. No major microbiota changes were seen between traditional and natural nitrite formulations, suggesting natural alternatives might match conventional nitrites without altering microbiota balance. More research could help clarify these impacts, especially regarding ammonia and long-term gut health.

## 4. Conclusion

The addition of sodium nitrite is a widely recognised conundrum concerning cured processed meat preservation. Nitrite plays multiple

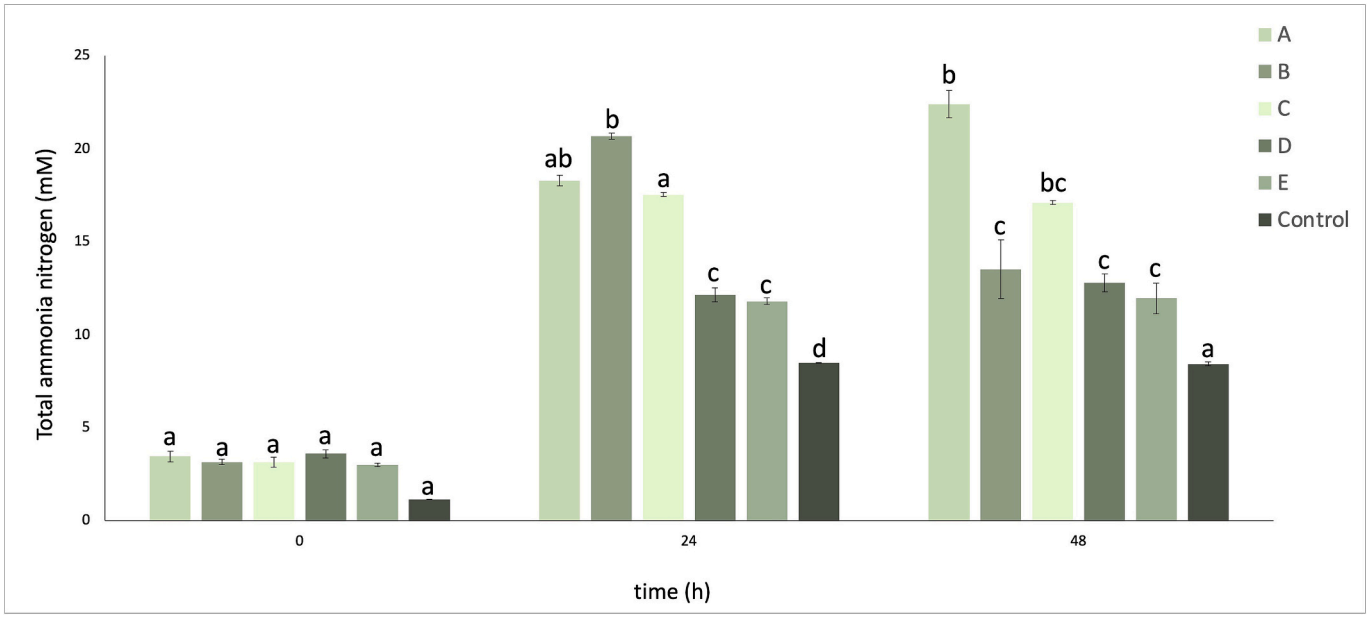


**Fig. 6.** Concentration (mM, means ± SD) of total ammonia nitrogen produced during colonic fermentation. Equivalent lower-case letters mean no significant differences between each sample at the same sampling time ($p > 0.05$).

roles, such as preventing lipid oxidation, maintaining colour, and preventing microbiological hazards by hindering the growth of microorganisms, mainly clostridial species. However, many studies have proven their ability to produce N-nitrous carcinogenic chemicals. Clean label formulations and curing strategies have surged from the need to deter this problem and as a suggested healthier option to traditional curing methods. The present study evaluated innovative ham formulations in which sodium nitrite was replaced by vegetable nitrate sources coupled with nitrate-reducing starter cultures. Results have shown that sodium nitrite and nitrite from natural sources show no differences regarding gut modulation and metabolite production, indicating these new formulations may be a feasible option when considering their impact on the human gut microbiota. Since these alternatives entail higher production costs and consequently higher prices for consumers, the implementation of these new formulations may not be seen as economically desirable by manufacturers, their potential benefits need to be thoroughly evaluated and demonstrated. For example, further studies need to investigate how this "natural" nitrite influence nitrosamine formation.

## CRediT authorship contribution statement

**Teresa Bento de Carvalho:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Joana Bastos Barbosa:** Writing – review & editing, Validation, Supervision, Investigation. **Nelson Mota de Carvalho:** Writing – review & editing, Validation, Investigation, Conceptualization. **Norton Komora:** Writing – review & editing, Validation, Supervision, Conceptualization. **Fátima Carvalho:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Ana Raquel Madureira:** Writing – review & editing, Supervision, Conceptualization. **Paula Teixeira:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors have nothing to declare.

## Acknowledgements

This work was developed in the scope of the project "cLabel+: Innovative 'clean label' natural, nutritious, and consumer-oriented foods" (POCI-01-0247-FEDER-046080), co-financed by the European Regional Development Fund (ERDF) through the Competitiveness and Internationalization Operational Program (POCI). The authors would also like to thank the scientific collaboration under the FCT project UIDB/50016/2020. Financial support for author T. Bento de Carvalho was provided by a doctoral fellowship 2023.03709.BD (FCT).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116287.

## Data availability

The data that support the findings of this study are available from the corresponding author, P.T, upon reasonable request.

## References

Allen-Vercoe, E., Strauss, J., & Chadee, K. (2011). Fusobacterium nucleatum. *Gut Microbes, 2*(5), 294–298. https://doi.org/10.4161/gmic.2.5.18603

Bento de Carvalho, T., Oliveira, M., Gomes, A. M., Monteiro, M. J., Pintado, M., Komora, N., … Teixeira, P. (2024). Clean labelling sodium nitrite at pilot scale: In-situ reduction of nitrate from plant sources and its effects on the overall quality and safety of restructured cooked ham. *Meat Science, 216*, Article 109572. https://doi.org/10.1016/j.meatsci.2024.109572

Binda, C., Lopetuso, L. R., Rizzatti, G., Gibiino, G., Cennamo, V., & Gasbarrini, A. (2018). Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Digestive and Liver Disease, 50*(5), 421–428. https://doi.org/10.1016/j.dld.2018.02.012

Brennan, C. A., Clay, S. L., Lavoie, S. L., Bae, S., Lang, J. K., Fonseca-Pereira, D., … Garrett, W. S. (2021). *Fusobacterium nucleatum* drives a pro-inflammatory intestinal microenvironment through metabolite receptor-dependent modulation of IL-17 expression. *Gut Microbes, 13*(1). https://doi.org/10.1080/19490976.2021.1987780

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Cao, Y., Liu, H., Qin, N., Ren, X., Zhu, B., & Xia, X. (2020). Impact of food additives on the composition and function of gut microbiota: A review. *Trends in Food Science and Technology, 99*, 295–310. https://doi.org/10.1016/j.tifs.2020.03.006

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2010). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences, 108*(supplement_1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Carlström, M., Moretti, C. H., Weitzberg, E., & Lundberg, J. O. (2020). Microbiota, diet and the generation of reactive nitrogen compounds. *Free Radical Biology & Medicine, 161*, 321–325. https://doi.org/10.1016/j.freeradbiomed.2020.10.025

de Carvalho, N. M., Oliveira, D. L., Costa, C. M., Pintado, M., & Madureira, A. R. (2022). Can supplemented skim milk (SKM) boost your gut health? *Fermentation, 8*(3), 126. https://doi.org/10.3390/fermentation8030126

de Carvalho, N. M., Oliveira, D. L., Saleh, M. A. D., Pintado, M., & Madureira, A. R. (2021). Preservation of human gut microbiota inoculums for in vitro fermentations studies. *Fermentation, 7*(1), 14. https://doi.org/10.3390/fermentation7010014

de Carvalho, N. M., Walton, G., Poveda, C., Silva, S., Amorim, M., Madureira, A., … Jauregi, P. (2019). Study of in vitro digestion of *Tenebrio molitor* flour for evaluation of its impact on the human gut microbiota. *Journal of Functional Foods, 59*, 101–109. https://doi.org/10.1016/j.jff.2019.05.024

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics, 11*(4), 265–270. https://www.jstor.org/stable/4615964.

Chung, W. S. F., Walker, A. W., Louis, P., Parkhill, J., Vermeiren, J., Bosscher, D., … Flint, H. J. (2016). Modulation of the human gut microbiota by dietary fibres occurs at the species level. *BMC Biology, 14*(1). https://doi.org/10.1186/s12915-015-0224-3

Conlon, M., & Bird, A. (2014). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients, 7*(1), 17–44. https://doi.org/10.3390/nu7010017

Dandachi, I., Anani, H., Hadjadj, L., Brahimi, S., Lagier, J., Daoud, Z., & Rolain, J. (2021). Genome analysis of *Lachnoclostridium phocaeense* isolated from a patient after kidney transplantation in Marseille. *New Microbes New Infections, 41*, Article 100863. https://doi.org/10.1016/j.nmni.2021.100863

Dixit, Y., Kanojiya, K., Bhingardeve, N., Ahire, J. J., & Saroj, D. (2023). In vitro human gastrointestinal tract simulation systems: A panoramic review. *Probiotics and Antimicrobial Proteins, 16*(2), 501–518. https://doi.org/10.1007/s12602-023-10052-y

Duncan, S. H., Iyer, A., & Russell, W. R. (2020). Impact of protein on the composition and metabolism of the human gut microbiota and health. *The Proceedings of the Nutrition Society, 80*(2), 173–185. https://doi.org/10.1017/s0029665120008022

Gonza, I., Goya-Jorge, E., Douny, C., Boutaleb, S., Taminiau, B., Daube, G., Scippo, M., Louis, E., & Delcenserie, V. (2024). Food additives impair gut microbiota from healthy individuals and IBD patients in a colonic in vitro fermentation model. *Food Research International, 182*, Article 114157. https://doi.org/10.1016/j.foodres.2024.114157

Han, K., Kim, J., Lee, K. C., Eom, M. K., Suh, M. K., Kim, H. S., … Lee, J. (2020). *Senegalimassilia faecalis* sp. nov., an anaerobic actinobacterium isolated from human faeces, and emended description of the genus *Senegalimassilia*. *International Journal of Systematic and Evolutionary Microbiology, 70*(3), 1684–1690. https://doi.org/10.1099/ijsem.0.003958

Hord, N. G., Tang, Y., & Bryan, N. S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition, 90*(1), 1–10. https://doi.org/10.3945/ajcn.2008.27131

Hrncirova, L., Machova, V., Trckova, E., Krejsek, J., & Hrncir, T. (2019). Food preservatives induce proteobacteria dysbiosis in human-microbiota associated NoD2-deficient mice. *Microorganisms, 7*(10), 383. https://doi.org/10.3390/microorganisms7100383

Isenring, J., Bircher, L., Geirnaert, A., & Lacroix, C. (2023). In vitro human gut microbiota fermentation models: Opportunities, challenges, and pitfalls. *Microbiome Research Reports, 2*(1), 2. https://doi.org/10.20517/mrr.2022.15

Kameoka, S., Motooka, D., Watanabe, S., Kubo, R., Jung, N., Midorikawa, Y., … Nakamura, S. (2021). Benchmark of 16S rRNA gene amplicon sequencing using Japanese gut microbiome data from the V1–V2 and V3–V4 primer sets. *BMC Genomics, 22*(1). https://doi.org/10.1186/s12864-021-07746-4

Kotopoulou, S., Zampelas, A., & Magriplis, E. (2021). Dietary nitrate and nitrite and human health: a narrative review by intake source. *Nutrition Reviews, 80*(4), 762–773. https://doi.org/10.1093/nutrit/nuab113

LaBouyer, M., Holtrop, G., Horgan, G., Gratz, S. W., Belenguer, A., Smith, N., … Scott, K. P. (2022). Higher total faecal short-chain fatty acid concentrations correlate with increasing proportions of butyrate and decreasing proportions of branched-chain fatty acids across multiple human studies. *Gut Microbiome, 3*. https://doi.org/10.1017/gmb.2022.1

Leclerc, M., Bedu-Ferrari, C., Etienne-Mesmin, L., Mariadassou, M., Lebreuilly, L., Tran, S., Brazeau, L., Mayeur, C., Delmas, J., Rué, O., Denis, S., Blanquet-Diot, S., &

Ramarao, N. (2021). Nitric oxide impacts human gut microbiota diversity and functionalities. *mSystems, 6*(5). https://doi.org/10.1128/msystems.00558-21

Leite, A. K. F., Fonteles, T. V., Filho, E. G. A., Da Silva Oliveira, F. A., & Rodrigues, S. (2022). Impact of orange juice containing potentially prebiotic ingredients on human gut microbiota composition and its metabolites. *Food Chemistry, 405*, Article 134706. https://doi.org/10.1016/j.foodchem.2022.134706

Li, Z., Zhou, J., Liang, H., Ye, L., Lan, L., Lu, F., Wang, Q., Lei, T., Yang, X., Cui, P., & Huang, J. (2022). Differences in alpha diversity of gut microbiota in neurological diseases. *Frontiers in Neuroscience, 16*. https://doi.org/10.3389/fnins.2022.879318

Mansour, S., Moustafa, M., Saad, B., Hamed, R., & Moustafa, A. (2021). Impact of diet on human gut microbiome and disease risk. *New Microbes New Infections, 41*, Article 100845. https://doi.org/10.1016/j.nmni.2021.100845

Nagpal, R., Indugu, N., & Singh, P. (2021). Distinct gut microbiota signatures in mice treated with commonly used food preservatives. *Microorganisms, 9*(11), 2311. https://doi.org/10.3390/microorganisms9112311

Nissen, L., Casciano, F., Di Nunzio, M., Galaverna, G., Bordoni, A., & Gianotti, A. (2023). Effects of the replacement of nitrates/nitrites in salami by plant extracts on colon microbiota. *Food Bioscience, 53*, Article 102568. https://doi.org/10.1016/j.fbio.2023.102568

Oliveira, W. A., Rodrigues, A. R., Oliveira, F. A., Oliveira, V. S., Laureano-Melo, R., Stutz, E. T., … Guerra, A. F. (2020). Potentially probiotic or postbiotic pre-converted nitrite from celery produced by an axenic culture system with probiotic lacticaseibacilli strain. *Meat Science, 174*, Article 108408. https://doi.org/10.1016/j.meatsci.2020.108408

Peled, S., & Livney, Y. D. (2021). The role of dietary proteins and carbohydrates in gut microbiome composition and activity: A review. *Food Hydrocolloids, 120*, Article 106911. https://doi.org/10.1016/j.foodhyd.2021.106911

Pérez-Burillo, S., Mehta, T., Pastoriza, S., Kramer, D. L., Paliy, O., & Rufián-Henares, J.Á. (2019). Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure. *LWT, 105*, 355–362. https://doi.org/10.1016/j.lwt.2019.02.006

Pini, F., Aquilani, C., Giovannetti, L., Viti, C., & Pugliese, C. (2020). Characterization of the microbial community composition in Italian Cinta Senese sausages dry-fermented with natural extracts as alternatives to sodium nitrite. *Food Microbiology, 89*, Article 103417. https://doi.org/10.1016/j.fm.2020.103417

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms, 7*(1), 14. https://doi.org/10.3390/microorganisms7010014

Rocha, B. S., Correia, M. G., Pereira, A., Henriques, I., Da Silva, G. J., & Laranjinha, J. (2019). Inorganic nitrate prevents the loss of tight junction proteins and modulates inflammatory events induced by broad-spectrum antibiotics: A role for intestinal microbiota? *Nitric Oxide, 88*, 27–34. https://doi.org/10.1016/j.niox.2019.04.001

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics, 27*(6), 863–864. https://doi.org/10.1093/bioinformatics/btr026

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. *BMC Research Notes, 9*(1). https://doi.org/10.1186/s13104-016-1900-2

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2012). The influence of diet on the gut microbiota. *Pharmacological Research, 69*(1), 52–60. https://doi.org/10.1016/j.phrs.2012.10.020

Shon, H., Kim, Y., Kim, K. S., Choi, J., Cho, S., An, S., … Kim, D. (2023). Protective role of colitis in inflammatory arthritis via propionate-producing Bacteroides in the gut. *Frontiers in Immunology, 14*. https://doi.org/10.3389/fimmu.2023.1064900

da Silva, R. M., Santos, B. N., Da Silva Oliveira, F. A., Filho, E. G. A., Fonteles, T. V., Campelo, P. H., & Rodrigues, S. (2023). Synbiotic Sapota-do-Solimões (*Quararibea cordata* Vischer) juice improves gut microbiota and short-chain fatty acid production in an in vitro model. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-023-10178-z

Singh, R. K., Chang, H., Yan, D., Lee, K. M., Ucmak, D., Wong, K., … Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine, 15*(1). https://doi.org/10.1186/s12967-017-1175-y

Verbeke, K. A., Boobis, A. R., Chiodini, A., Edwards, C. A., Franck, A., Kleerebezem, M., … Tuohy, K. M. (2015). Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutrition Research Reviews, 28*(1), 42–66. https://doi.org/10.1017/s0954422415000037

Vince, A. J., & Burridge, S. M. (1980). Ammonia production by intestinal bacteria: The effects of lactose, lactulose and glucose. *Journal of Medical Microbiology, 13*(2), 177–191. https://doi.org/10.1099/00222615-13-2-177

Wang, F., Jiang, H., Shi, K., Ren, Y., Zhang, P., & Cheng, S. (2012). Gut bacterial translocation is associated with microinflammation in end-stage renal disease patients. *Nephrology, 17*(8), 733–738. https://doi.org/10.1111/j.1440-1797.2012.01647.x

Xiao, T., Liang, T., Geng, D., Wang, L., Liu, L., Zhou, X., Pu, H., Huang, J., Zhou, S., & Tong, L. (2020). Dietary proteins alter fermentation characteristics of human gut microbiota in vitro. *Plant Foods for Human Nutrition*. https://doi.org/10.1007/s11130-020-00836-w

Xu, J., Wang, M., Liu, Q., Lin, X., Pu, K., & He, Z. (2022). Gut microbiota mediated the toxicity of high concentration of dietary nitrite in C57BL/6 mice. *Ecotoxicology and Environmental Safety, 231*, Article 113224. https://doi.org/10.1016/j.ecoenv.2022.113224

Yong, H. I., Kim, T., Choi, H., Jang, H. W., Jung, S., & Choi, Y. (2020). Clean label meat technology: Pre-converted nitrite as a natural curing. *Food Sci Animal Resources, 41*(2), 173–184. https://doi.org/10.5851/kosfa.2020.e96

Yoon, S., Ha, S., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2016). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology, 67*(5), 1613–1617. https://doi.org/10.1099/ijsem.0.001755

Zhang, P. (2022). Influence of foods and nutrition on the gut microbiome and implications for intestinal health. *International Journal of Molecular Sciences, 23*(17), 9588. https://doi.org/10.3390/ijms23179588

Zhou, X., Qiao, K., Wu, H., & Zhang, Y. (2023). The impact of food additives on the abundance and composition of gut microbiota. *Molecules, 28*(2), 631. https://doi.org/10.3390/molecules28020631