Food Bioscience 53 (2023) 102568

Contents lists available at ScienceDirect

Food Bioscience

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

Effects of the replacement of nitrates/nitrites in salami by plant extracts on colon microbiota

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ARTICLE INFO

Keywords:
Processed meat
Nitrates
Gut microbiota
Colon fermentation
Proteolysis
Clean-label

ABSTRACT

Salami is a cured sausage consisting of fermented and air-dried meat obtained from a mixture of meat and fat with spices and other ingredients. Excessive processed meat consumption is negatively considered because of its high fat and salt contents and few bioactive molecules. Notwithstanding, salami is largely consumed, and there is a strong interest to produce better and healthier products by substituting nitrites and nitrates with natural extracts. This work produced four different salami, two controls including nitrates and two alternative preparations where nitrates were substituted with plant extract and ascorbic acid. The products were in vitro digested with the INFOGEST protocol to simulate the oro-gastro-duodenal phase and in vitro fermented with MICODE model to simulate the colon phase. Samples were analyzed by microbiomics and metabolomics approaches to study the changes in bacterial populations and in metabolites production. The results showed that the clean-label formulations promote a general eubiosis of the intestinal microbiota, including favorable F/B ratio, the proliferation of beneficial microbial taxa (*Bifidobacteriaceae*), and reduction of negative microbial populations (*Enterobacteriaceae*). Volatilome analysis highlighted a marked production of beneficial molecules, including acetate, propionate and butyrate, and a reduction in host negative molecules such as phenol and p-cresol. Our results tell that the plant extracts could be used to replace nitrates, because the features obtained are comparable to those of controls. This work could represent an encouraging starting point for the processed meat industry for the development of clean-label formulations aimed at reducing the negative impact of these products on consumers.

1. Introduction

Salami is a cured sausage consisting of fermented and air-dried meat, typically pork, obtained from a mixture of meat and fat with spices and other ingredients. Salami is largely consumed around all the world (Blaiotta et al., 2018), butexcessive consumption of processed meat is negatively considered because of its high contents in fat and salt and low contents in bioactive molecules, such as phenolic compounds (Martínez et al., 2014). Additionally, a well-known negative characteristic of processed meat is the presence of nitrates and nitrites. The functions of these additives are many, e.g. prevention of lipid oxidation, color maintenance, and microbiological safety by inhibiting pathogens (Majou e Christieans, 2018), but in recent years have been under attack for their capacity to form N-nitrous carcinogenic compounds. In fact, in the human colon amines and amides are deriving from the bacterial metabolism of amino acids. These could be N-nitrousated in the presence of nitrosylated heme derived from not absorbed residual of red meat (Herrmann et al., 2015; Johnson, 2017; Meurillon e Engel, 2016). Due to these reasons, there is a common interest by food technologists to improve the nutritional and health properties, and reduce the negative

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https://doi.org/10.1016/j.fbio.2023.102568
Received 6 January 2023; Received in revised form 23 February 2023; Accepted 13 March 2023
Available online 20 March 2023

features. Several attempts were tried, for example, by using probiotic as starter for the fermentation process (Giello et al., 2018), by adding bioactive compounds (dos Santos et al., 2021), by substituting nitrites and nitrates with natural extract (Pini et al., 2020) or by simple nitrites and nitrates withdrawal (Tabanelli et al., 2022). For example, in a study by Pérez-Burillo et al. (2020), the authors added different types of fiber to salami (citrus fiber, arabinogalactans, and inulin) and evaluated the effects. The results showed that all samples had a higher prevalence of *Bacteroides* in respect to the control, and that the addition of fibers resulted in a reduction of some human intestinal pathogens (Pérez-Burillo et al., 2020).

The request from consumers of foods with "clean label" defined by minimal process, few additives, and no artificial ingredients or synthetic chemicals is growing (Majou e Christieans, 2018). The first research on ingredients alternative to nitrates and nitrites resulted in a product with low organoleptic and microbiological quality (Hammes, 2012).

On the other hand, many studies tested some vegetal extracts as a substitute of nitrates and nitrites thanks to their high polyphenol content, known for their antioxidant and antimicrobial properties (Jiang & Xiong, 2016; Pini et al., 2020; Shah et al., 2014; Shan et al., 2009). Recently, new salami formulations with nitrate-reducing microbial starter cultures and vegetal extracts bringing 0.4 g/kg of bioactive polyphenols to the meat mixture were developed, without affecting negatively the release of fatty acids and the hydrolysis of proteins during digestion (Di Nunzio et al., 2022)., Additionally, these latter salami digestates were even tested for their effect on HT29 cell lines of the human colon, showing no difference in respect to controls (Di Nunzio et al., 2022). Nonetheless, none of these studies focused on the effect of such alternative formulations on gut microbiota perturbations.

For this purpose, in this work, an in vitro model of the proximal colon (MICODE – Multi-unit in vitro colon model), was used to mimic the effect of colon microbiota fermentation. The whole pipeline, including protocols, equipment, and data management, previously demonstrated high reliability as resulted by a very high level of control of ecosystem conditions, the maintenance of the original diversity, rarity and richness of the human gut microbiota such as some *Archaea* and more than 400 different OTUs (Nissen et al., 2021, 2021a, 2022). Therefore, the effects of the replacement of nitrates/nitrites with plant extracts in salami on gut microbiota were evaluated in MICODE through shifts of the microbial populations by qPCR and their volatile metabolites (VOCs) by SPME-GC-MS, while data management approach allowed to explore the correlations among bacterial taxa and beneficial or detrimental metabolites.

2. Materials and methods

2.1. Experimental samples and controls

Four different salami formulations were tested. For all the formulations, the salami mixture consisted of lean muscle tissue (75%) and minced bacon (25%). The meat was weighed, cut into small pieces, ground in a meat mincer (∅ = 6 mm plate), and then mixed with salt (2.5%), dextrose (0.2%), ascorbate (0.05%) and natural flavours. The positive control formulation (CNO_2) was added with sodium nitrite, potassium nitrate and nitrate-reducing microbial starter cultures (MSC). MSC (Chr. Hansen, S.p.A., Parma, Italy) contained lactic acid bacteria and nitrate-reducing coagulase negative *Staphylococcaceae* and was inoculated as common manufacturing practices to properly drive the fermentation phase and to promote the development of aroma during the ripening phase.

Two innovative formulations not containing nitrites were prepared: the first (SA) was added with MSC and sodium ascorbate (0.3%); the second (SMA) was added with MSC, sodium ascorbate (0.3%), and plant extracts from grapeseed, green tea and, olive (Indena S.p.A., Milan, Italy), characterized according to their total polyphenols content to provide 0.4 g/kg of bioactive polyphenols to the meat mixture. Finally, the negative control (CO) was prepared with neither MSC nor additives (nitrite, polyphenols and, ascorbate). The formulations of salami and a detailed description of processing are reported elsewhere (Di Nunzio et al., 2022; Saccani et al., 2023).

2.2. Experimental workflow

Briefly, salami samples were processed for gastro-duodenal digestion as described in Di Nunzio et al. (2022), then the digestates were transferred in MICODE in vitro colon model for proximal colonic fermentation, using human colon microbiota (HCM). The shifts of the colon microbiota and its metabolites that occurred with fermentation were then studied.

2.3. Human colon microbiota

HCM was obtained from the stools of three lean healthy individuals (either male and female). The number and type of volunteers was in accordance with previous protocols. Specifically, volunteers were adults (between 25 and 50 years old), not consuming antibiotics, pre- or probiotic supplements in the 3 months prior to the experiment, non-smokers, and with no history of chronic gastrointestinal disorders (Connolly et al., 2012; Nissen, Casciano, et al., 2021; Arnal et al., 2021). Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.

Human stools were collected by volunteers in a dedicated sterile container, placed in an anaerobic jar with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA), transferred to the laboratory, and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 54 mL of pre-reduced phosphate buffered saline (PBS) (Nissen, Cattivelli, et al., 2022; Wang et al., 2020). Two biological experiments were performed within one week, employing fresh donations from the same donors.

2.4. In vitro intestinal model

The in vitro gastro-intestinal digestion was carried out on salami samples by applying the INFOGEST protocol (Minekus et al., 2014). At the end of the intestinal phase, an aliquot of sample was withdrawn, centrifuged (10,000*g*, 10 min, 4 °C) and the supernatant stored at −80 °C for further analysis, whereas the remaining material (the denser emulsion) was subjected to the in vitro colonic fermentation trials as described below. Digestions were carried out in triplicate.

Proximal colonic fermentations were conducted for 24 h in independent vessels using an in vitro colon model, MICODE (Nissen et al., 2021, 2021a). The preparation of the experiments was made according to published procedures (Connolly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen, Valerii, et al. (2021). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connolly et al., 2012; Nissen, Casciano, et al., 2021). Once the proximal colon condition was reached, each vessel was aseptically loaded with 10 mL of independent mixtures including fecal slurry (10% w/v of human feces in O_2 reduced PBS) and 1 g of in vitro digested Salami with ascorbate (SA), Salami with ascorbate and plant extracts (SMA), control with no nitrite (CO), commercial control with nitrate (CNO_2) at a final concentration of 1% (w/v). A fourth vessel was set as blank control (BC) (basal medium and 10% faecal slurry with 1% of digestive enzymes). Batch cultures were run under controlled conditions for a period of 25.52 h including the baseline (BL) (for these experiments set at 1.52 ± 0.18 h) as described in Nissen, Valerii, et al. (2021). Sampling was performed as reported in Nissen, Casciano, et al. (2021).

2.5. Experimental set up and pipeline of activities

Parallel and independent vessels for SA, SMA, CNO2, CO, and a blank

control (BC) were run for 24 h after the adaptation of the faecal inoculum, defined as the baseline (BL). The entire experiment consisted of 5 cases biologically duplicated (SA, SMA, CNO_2, CO, and BC) (n = 10), 3 time points (BL = 1.52 ± 0.18 h, T1 = 18 h, and EP = 24 h) (n = 30) in technical duplicates for GC-MS (n = 60) and technical triplicates (n = 90). Samples of the different time points were used for qPCR and SPME GC-MS analyses. After sterile sampling of 4.2 mL of bioreactor contents, samples were centrifuged at 17,000×*g* for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis, respectively. Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce *Firmicutes* content. After, separation of the pellets from the supernatants, the pellets were washed twice in O_2 reduced PBS to increase the cleaning. DNAs for microbiomics and supernatants for SPME-GC-MS were then stored at −80 °C.

2.6. Microbiomics

2.6.1. DNA extraction

Bacterial DNA was extracted from the MICODE eluates at each time points, just after sampling; at the baseline, at T1, and EP using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

2.6.2. Absolute enumeration of bacterial groups by qPCR

Enumeration of bacterial groups was made by qPCR to evidence changes in the microbiota after fermentation (Nissen, Aniballi, et al., 2022; Tamargo et al., 2022; Tanner et al., 2014; Tsitko et al., 2019; Westfall et al., 2018) following previous protocols (Modesto et al., 2011; Nissen, Cattivelli, et al., 2022; Nissen, Aniballi, et al., 2022). Specifically, the bacterial groups were selected as generally accepted indicators of eubiotic or dysbiotic state of colon microbiota; thereafter, their perturbations may be considered closely correlated (directly or inversely) to the prebiotic potential of foods. 16 different bacterial taxa, (Table S1), were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA).

2.7. Metabolomics

2.7.1. Volatilome analysis

Volatile organic compound (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The Solid Phase Micro-Extraction (SPME) GC-MS protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Casciano et al., 2021; Di Cagno et al., 2011; Guerzoni et al., 2007; Nissen, Casciano, et al., 2021). Identification of molecules was carried out by searching mass spectra in the available databases (NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). Each VOC was relatively quantified in percentage (LOD = 0.001 mg/kg) (Bonfrate et al., 2020).

2.7.2. Quantification of main microbial VOCs

In samples before in vitro colonic fermentation (BL) (Table S2) the main microbial metabolites related to fermentation of foods were also absolutely quantified in mg/kg with the aforementioned SPME GC-MS approach and the internal standard, but with different cutoffs (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Casciano et al., 2021; Di Cagno et al., 2011; Nissen, Casciano, et al., 2021). For these compounds, samples at T1 and EP were compared to the BL and values were expressed as shifts. Values were computed as follows; i) each single compound was normalized (mean centering method) within its dataset, which included cases from SA, SMA, CNO_2, CO, and BC at different time points; ii) the BL dataset (Table S2) was then subtracted to the fermentation time points; iii) all values from any time points and replicates were used to populate a dataset, which serves to generate a ANOVA model. iv) To compare a single molecule among samples a Tukey's *post-hoc* analysis was performed. v) The changes are then represented as box-plots, where each box plot is generated either from intermediate time points and endpoints values.

2.8. Data processing and statistical analysis

For metabolomics, one-way ANOVA model ($p < 0.05$) was used to determine significant VOCs among the raw data of peak's area of the GC-MS chromatograms. The significant VOCs (n = 69) representing the total volatilome of the experiments were analyzed differently; i) the volatilome was relatively quantified, sorted for main chemical classes, and super-normalized, then each dataset was computed for Principal Component Analysis (PCA) to distribute the results on a plane and coupled to Multivariate ANOVA (MANOVA) ($p < 0.01$) (Tables S3 and S4) to address specific contributes by categorical predictors; ii) 9 main VOCs related to microbial fermentation of foods were absolutely quantified and normalized and their BL values were subtracted from T1 and EP values and represented as box plots, including *post hoc* Tukey HSD test ($p < 0.05$).

For microbiomics, MANOVA ($p < 0.05$) model (categorized for the time points and the treatments) was used to study the shifts in abundance of qPCR values, calculated as Log_2(F/C) (Love et al., 2014). Then, *post hoc* Tukey HSD test on the raw data ($p < 0.05$) was performed to define differences among treatments or time points. The baselines of values for the volatilome and for the microbiota were that obtained sampling just after adaptation of the microbiota to the bioreactor condition (Nissen, Valerii, et al., 2021). Normalization of datasets was performed with the mean centering method.

3. Results

3.1. Volatilome analysis through SPME GC/MS

To characterize colon fermentation through SPME GC-MS, among 30 duplicated cases (n = 60), 108 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA) and 69 significant VOCs were picked (ANOVA $p < 0.05$). The syntax for the name of molecules adopted in the present work is that of NIST database (NIST, USA), that are reported with initial capital letters (e.g. 1H-Indole, 3-methyl), while synonyms are reported with initial lowercase letter (e.g. skatole) (Casciano et al., 2021). 56 were relatively quantified at the baseline, while 69 were quantified during the 24 h of experiments at different timepoints. The 69 significant VOCS were then sorted and super-normalized for respective chemical identities, i.e., organic acids, aldehydes, ketones, alcohols, and aromatics (alkenes and amines). Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing different chemical classes (Nissen et al., 2020).

3.1.1. Organic acids

A PCA of 11 statistically significant organic acids distributed cases on the plot, separating the BL from time points of fermentation of the substrates, but not from those of the BC, and discriminating the controls CNO_2 and CO from the alternative formulations SA and SMA (Fig. 1A). The main descriptor of fermentation with SA and SMA was Cyanic Acid methyl (by MANOVA, approximately 42% and 49% of production, respectively) (Table S3). The descriptor of CO was principally Oxalic acid (approx. 98% of production), while those of CNO_2 were mainly pentanoic and hexanoic acids (approx. 58% and 67%, respectively). The contribution on short chain organic acids was not discriminated

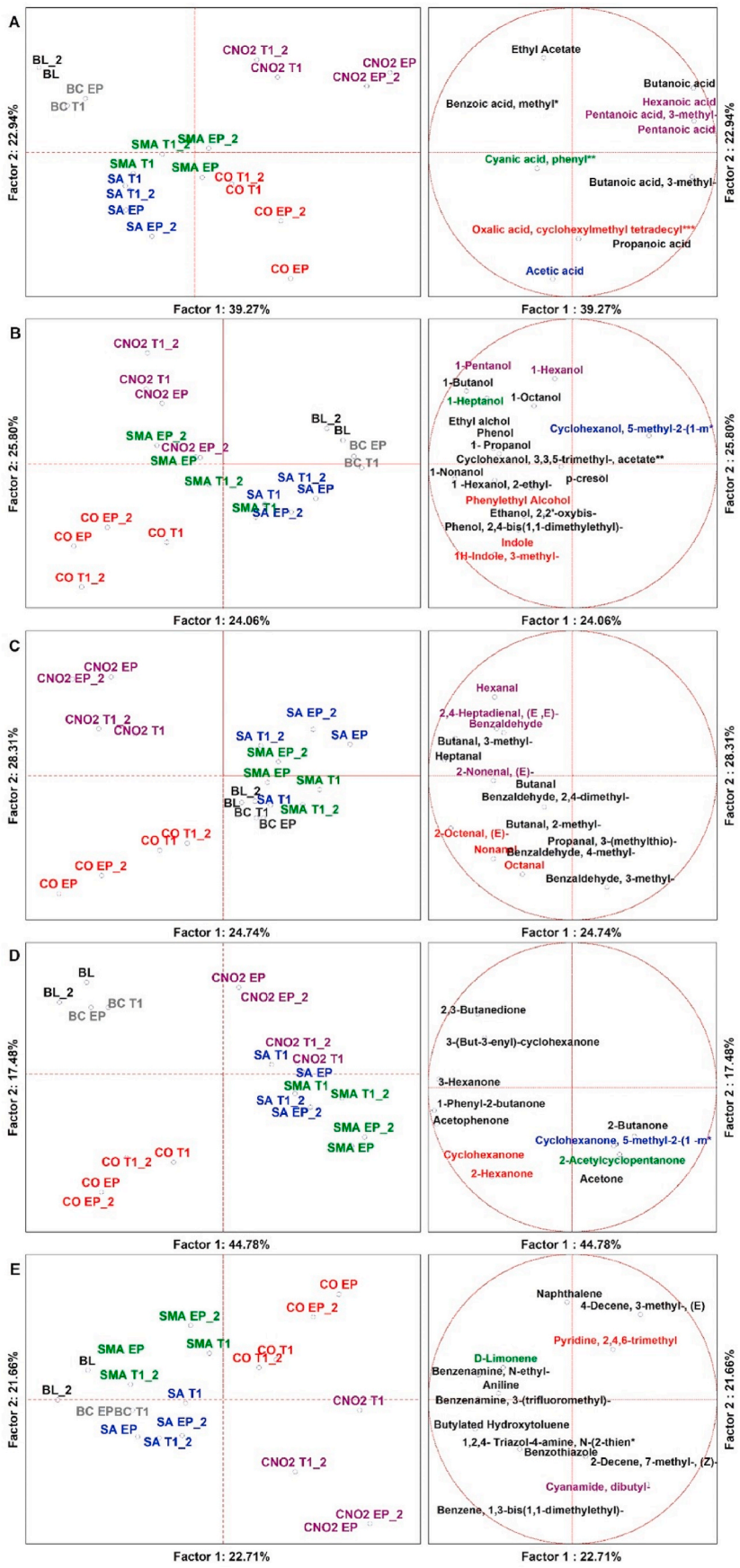

Fig. 1. PCAs of the volatilome sorted by chemical classes of significant VOCs (ANOVA $p < 0.05$). The original dataset included the biological replicas of SA, SMA, CNO_2, CO, BC, and the baseline (BL) and different time points (T1 = 18 h and EP = 24 h). A) Acids; B) Alcohols; C) Aldehydes; D) Ketones; E) Other aromatic VOCs. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of the respective group of cases. SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate. *Oxalic acid, cyclohexylmethyl = Oxalic acid, cyclohexylmethyl tetradecyl ester; **Cyclohexanol, 5-methyl-2-(1-m ac = Cyclohexanol, 5-methyl-2-(1-methylethyl) acetate; ***Cyanic acid phenyl = Cyanic acid phenyl ester; * Benzoic acid, methyl = Benzoic acid, methyl ester; *Cyclohexanone, 5-methyl-2-(1-m = Cyclohexanone, 5-methyl-2-(1 -methylethyl); *1,2,4- Triazol-4-amine, N-(2-thien = 1,2,4-Triazol-4-amine, N-(2-thienethyl); *Benzene, 1,3-bis(1,1-dim = Benzene, 1,3-bis(1,1-dimethylethyl). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

depending on the matrix (except for butanoic acids produced for the 45% by CNO_2), but it was on a time dependence (Table S4). Another interesting feature is that the branched-chain organic acids were all pushing to the quadrant relative to CNO_2, reaching high contribution ratio, as that of Pentanoic acid, 3-methyl, accounting for the 67% and only present at the EP (Table S4).

3.1.2. Alcohols

A PCA of 19 statistically significant alcohols distributed cases on the plot, separating the BL from time points of fermentation of the substrates, but not from those of the BC, and discriminating the controls CNO_2 and CO from each other and from the alternative formulations SA and SMA (Fig. 1B). The descriptors of the controls were 1-Pentanol and 1-Hexanol for CNO_2 (both around the 36% of contribution in production) and Phenylethyl alcohol, Indole, and 1H-Indole, 3-methyl for CO (around 70%, 44%, and 92%, respectively). The descriptors of the alternative formulations were 1-Heptanol for SMA (around 40%) and Cyclohexanol, 3,3,5-trimethyl-, acetate, *cis*-for SA (around 32%) (Table S3).

3.1.3. Aldehydes

A PCA of 15 statistically significant aldehydes distributed cases on the plot, separating the BL from time points of fermentation of the controls but not completely from the time points of fermentation of alternative formulations. Also, CNO_2 and CO were discriminated from each other and from the alternative formulations SA and SMA (Fig. 1C). SA and SMA did not have any specific descriptor. The aldehydes that described CNO_2 were Hexanal, Heptanal, and 2,4-Heptadienal, (E,E)- (53%, 42%, and 100% of contribution to total production, respectively) (Table S3) produced during fermentation (67%, 81% and 100%) (Table S4). Those that described CO were Octanal, 2-Octenal, and Nonanal (48%, 42%, and 47%, respectively) (Table S3), although partially present at the BL (68%, 42%, and 46%) (Table S4).

3.1.4. Ketones

A PCA of 11 statistically significant ketones distributed cases on the plot, separating the BL from time points of fermentation of any substrates but not of the BC. Also, CNO_2 and CO were discriminated from each other, but CNO_2 was partially separated from the alternative formulations. Moreover, SA and SMA did not discriminate much one to each other but had their specific descriptor (Fig. 1D). CNO_2 did not have any specific descriptor. The ketones that described CO were 2-Hexanone and Cyclohexanone (around 87% and 56% of contribution on total production) ($p < 0.05$) (Table S3); the former was produced at the EP (100%) ($p > 0.05$), but the latter was also ascribed to be already present at the BL (around 43%) ($p < 0.05$) (Table S4). The descriptors of the alternative formulations were instead Acetylcyclopentanone for SMA (around 67%) ($p < 0.05$) and Cyclohexanone, 5-methyl-2-(1 -methylethyl)-, *cis*-for SA (around 39%) (Table S3), that were both absent at the BL and whose production was spread over the process either at T1 or EP ($p < 0.05$) (Table S4).

3.1.5. Amines and alkenes

A PCA of 13 statistically significant aromatic VOCs not previously sorted (accounting mainly for amines and alkenes) distributed cases on the plot, separating the BL from time points of fermentation of any substrates but not of the BC. Also, CNO_2 and CO were discriminated from each other, but CNO_2 was partially separated from the alternative formulations. Lastly, SA and SMA did not discriminate much one to each other, and only SMA had a specific descriptor (Fig. 1E). The VOCs that defined the controls were Cyanamide dibutyl for CNO_2 (around 48.9%) and Pyridine, 2,4,6-trimethyl for CO (around 94%) ($p < 0.05$) (Table S3). The former was absent at BL and was produced for the most at T1 (around 74%) while the latter accounted to contribute for around 12% at BL and around 84% at EP (Table S4). Interestingly, even considering chemical bias due to the high volatility of D-Limonene, this bioactive was a unique descriptor of SMA fermentation. In this case, D-Limonene enrichment was achieved during the whole process (around 33% and 49% at T1 and EP, respectively), starting from an initial amount (around 18%) ($p < 0.05$) (Table S4).

3.2. Shift of microbial VOCs with functional properties

For their beneficial role, more specific attention was addressed to SCFAs (short chain fatty acids) and MCFAs (medium chain fatty acids) (from fibrinolytic and saccharolytic metabolism). On the other hand, some VOCs (mainly coming from proteolytic metabolism) were considered detrimental. Indeed, they are in general related to proteolytic fermentation and are harmful to the mucosa and the host and thereafter negatively correlated to prebiotic potential of foods.

3.2.1. VOCs related to beneficial and prebiotic effect

Three short chain and two medium chain organic acids were considered: acetic, propanoic, butanoic, pentanoic, and hexanoic. The absolute quantifications at the baseline (Table S2) were compared to that at the two time points, T1 and EP (18 and 24 h of fermentation respectively), and the difference was measured and normalized (Fig. 2). Considering the shift of fermentations compared to the BL, the results of the recipient analyses demonstrated that any fermentation tested produced low molecular organic acids. In particular, the control sample with nitrites (CNO_2) generated the best fermentation outputs. Among the alternative formulation, SA fermentation produced almost 7 times more acetic and 4 times more either pentanoic or hexanoic acids, than the BL, while fermentation of SMA produced few amounts of the five VOCs. So far, the trend in beneficial postbiotic VOCs production was CNO_2 > SA > SMA > CO.

3.2.2. Detrimental VOCs

The fermentation of any salami has produced potentially detrimental VOCs derived from lipid oxidation and amino acids (tyrosine, tryptophan, phenylaniline) fermentation. In particular, Phenol, Phenol, 2-methyl (a.k.a. p-cresol), Indole, and 1H-Indole, 3-methyl (a.k.a.

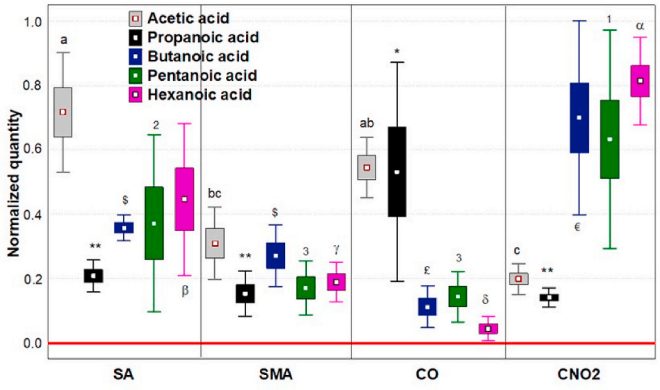

Fig. 2. Changes in the abundance of beneficial microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S2). Changes were recorded after 18, and 24 h (T1 and EP respectively) of in vitro fecal batch fermentations with SA, SMA, and controls CO and CNO_2. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = Confidence Interval 0.95. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

skatole). Starting from the concentrations of these VOCs at the baseline (Table S2), the sample (Fig. 3) that generated the top amount was the control with no nitrite (CO). In respect to this control, SA, SMA and CNO_2 produced similar overall amounts of any compounds ($p > 0.05$), except Indole ($p < 0.05$) and skatole (not detected in SA), approximately 6 times less than CO ($p < 0.05$). The trend of production of these VOCs was: CO > CNO_2 > SMA > SA.

3.3. Microbiota analyses of colonic fermentations

qPCR absolute quantifications were targeted to 16 different bacterial taxa related to the core microbiota of the human colon, including total Eubacteria, *Bacteroidetes* and *Firmicutes* to describe the large picture; *Lactobacillales*, *Bifidobacteriaceae, Clostridium* group IV, *Bifidobacterium longum, Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila* to describe the commensal beneficial part of the core colon microbiota; and *Enterobacteriaceae*, *Clostridium* group I, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Escherichia coli* (total), *Escherichia coli* (toxigenic), *Desulfovibrio*. spp., to describe the commensal opportunistic part of the core colon microbiota (Table S1).

3.3.1. Shift in taxa relative to the core microbiota

Considering the total Eubacteria, with respect to the abundances at the BL and apart from the values of the blank control (BC) (Table 1), the alternative formulations SA and SMA at EP significantly fostered the growth of Eubacteria ($p < 0.05$), alike the commercial control CNO_2, and almost thrice than the negative control CO. The quantifications of *Bacteroidetes* phylum (Table 1) have shown changes principally at EP, when any samples, but SA, had significant differences in respect to BL ($p <$ 0,05). In particular, fermentations of CO and CNO_2 triggered a reduction, while that of salami fostered growth. SMA was the best performer, able to significantly increase at the EP the loads of this taxon around 1.7 and 4 times more than SA and CNO_2, respectively. Considering *Firmicutes* (Table 1), significant increases were observed at EP for any sample, but SMA ($p > 0.05$). Although, the surges after fermentation with both the controls were quite the double in respect to those of the alternative

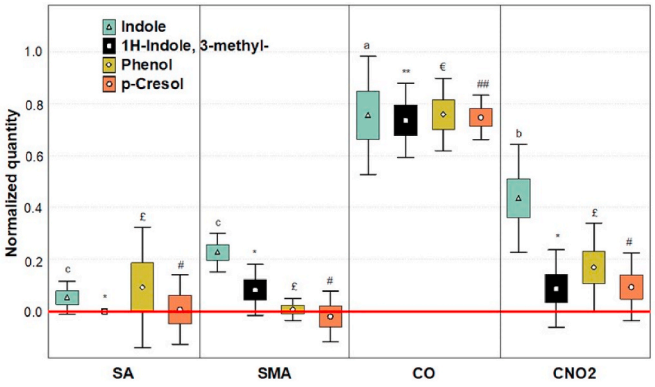

Fig. 3. Changes in the abundance of detrimental microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S2). Changes were recorded after 18, and 24 h of in vitro fecal batch fermentations with SA, SMA, CO, and CNO_2. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = Confidence Interval 0.95. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Quantification and changes of *Eubacteria*, *Bacteroidetes*, *Firmicutes* and the *Firmicutes* to *Bacteroidetes* ratio of colonic fermentation of salami measured by qPCR.

qPCR Targets & Samples	Quantification cells/mL	Changes log_2(F/C)		MANOVA (Time)	
	Baseline	T1 (18 h)	EP (24 h)	*p*	*-log₁₀(p)**
Eubacteria					
CO	2.77E+10 ± 8.47E+09	0.02	0.58^{AB}	0.810581	0.091203
SA	2.77E+10 ± 8.47E+09^{b}	0.75^{ab}	1.63^{aA}	0.023662	1.625956
SMA	2.77E+10 ± 8.47E+09^{b}	0.87^{ab}	1.38^{aA}	0.009666	2.014750
CNO_2	2.77E+10 ± 8.47E+09^{b}	0.94^{a}	1.44^{aA}	0.019003	1.721188
BC	2.77E+10 ± 8.47E+09^{a}	-0.15^{a}	-1.74^{bB}	0.015644	0.052740
MANOVA (Matrix) *p*		0.107124	0.000950		
Firmicutes					
CO	2.46E+09 ± 2.08E+08^{b}	1.43^{aA}	1.44^{aA}	0.000003	5.503136
SA	2.46E+09 ± 2.08E+08	-0.02^{B}	0.49^{B}	0.064013	3.946631
SMA	2.46E+09 ± 2.08E+08	0.50^{AB}	0.71^{AB}	0.098002	5.816437
CNO_2	2.46E+09 ± 2.08E+08^{b}	-0.39^{bB}	1.42^{aA}	0.000457	3.339918
BC	2.46E+09 ± 2.08E+08^{b}	0.91^{abAB}	1.28^{aA}	0.000134	3.872407
MANOVA (Matrix) *p*		0.000012	0.002652		
Bacteroidetes					
CO	4.80E+09 ± 1.84E+09^{a}	-0.22^{ab}	-1.16^{bB}	0.049707	1.303583
SA	4.80E+09 ± 1.84E+09	0.15	0.59^{A}	0.070006	5.202209
SMA	4.80E+09 ± 1.84E+09^{b}	0.11^{b}	1.38^{aA}	0.005644	2.248411
CNO_2	4.80E+09 ± 1.84E+09^{a}	-0.11^{ab}	-0.62^{bB}	0.000008	5.071174
BC	4.80E+09 ± 1.84E+09^{a}	0.11^{a}	-2.62^{bC}	0.006603	2.180278
MANOVA (Matrix) *p*		0.080616	0.000444		
Firmicutes/Bacteroidetes					
CO	0.51 ± 0.22^{c}	1.61^{bA}	3.10^{aB}	0.000003	5.522878
SA	0.51 ± 0.22	0.46^{B}	0.48^{C}	0.160113	0.795573
SMA	0.51 ± 0.22	0.67^{B}	0.32^{C}	0.076002	1.119174
CNO_2	0.51 ± 0.22^{b}	0.42^{bB}	2.10^{aB}	0.000457	3.340083
BC	0.51 ± 0.22^{b}	0.95^{bA}	7.64^{aA}	0.000134	3.872895
MANOVA (Matrix) *p*		0.000011	0.002652		

Quantifications are expressed as cells/mL, obtained from gene copy number/ng of DNA. Changes are expressed as Log_2(F/C). F/C = timepoint/baseline. ^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower-case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to Time effect" on rows and to "Matrix effect" on columns; *$-Log_{10}(p)$ = Significance of Log_2 (F/C). SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate; BC = Blank Control; T1 = 18 h of fermentation; EP = 24 h of fermentation. F/B = *Firmicutes* to *Bacteroidetes* ratio. All values are expressed as the mean of sextuplicates (3 technical and 2 biological replicates). Baseline values for each qPCR target are obtained from sextuplicates and are equal for the fermentation of any sample, because indicate the quantification after adaptation of the colon microbiota to in vitro colon ecology and prior in vitro colonic fermentation.

formulations. For example, at the end point CNO_2 had level of this taxon at 6.56 E+09 ± 1.71 E+09 cells/mL, which was 1.90 times higher than SA. Based on the values of quantifications relative to *Firmicutes* and *Bacteroidetes*, the ratios F/B (Table 1) was calculated to consider changes in the condition of eubiosis defined at the BL. After colonic fermentation, SMA and SA were able to keep the ratio similar to the BL ($p > 0.05$), but the controls were not ($p > 0.05$). In particular, the ratio of CO was higher

than 3, indicating a microbiota dysbiosis due to the overrepresentation of *Firmicutes*. So far, the intensity of the capacity to maintain the microbiota eubiosis among the salami tested was: SMA > SA > CNO_2 > CO.

3.3.2. Commensals beneficial taxa

Amongst the beneficial bacteria (Table 2) that were targeted, *Lactobacillales* and *Bifidobacteriaceae* had different trend during fermentation. The former taxon increased after fermentation with any substrate but significantly just for SA ($p < 0.05$), while the latter increased significantly just for SA and SMA and decreased significantly for CNO_2 ($p < 0.05$). After SA fermentation, the EP *Lactobacillales* loads was 2.10E+07 ± 3.92E+06 cells/mL, more than CNO_2. After SMA fermentations, at the EP *Bifidobacteriaceae* accounted for 8.63E+08 ± 2.97E+08 cells/mL, 7.3 times more than CNO_2. Within this family, *B. longum* was particularly affected by the controls recording dramatic losses after their fermentations but surged significantly ($p < 0.05$) and similarly ($p > 0.05$) in abundances after SA and SMA fermentations (Table 2). Between *Clostridiales*, the *Clostridium* group IV and the recipient *Faecalibacterium prausnitzii* were underrepresented after any fermentation, but SMA, that anyhow scored no shift in respect to the BL ($p > 0.05$). The group BPP (*Bacteroides-Prevotella-Porphyromonas)*, which mainly targets the *Bacteroides* genus, increased significantly at the EP just for SMA ($p < 0.05$), as we previously have observed at the phylum level. Lastly, *Akkermansia muciniphila* was not fostered by any substrates.

3.3.3. Commensals opportunistic taxa

To evaluate the shifts during colonic fermentation of a portion of the opportunistic part of the microbiota, we have selected specific taxa that have strong proteolysis activity and are also associated with western diet enterotype, namely *Enterobacteriaceae*, *Clostridium* group I, ATOP (*Atopobium – Collinsella – Eggerthella*) group, *Escherichia coli* and *Desulfovibrio* spp. (Table 3). Any substrate fermentation was able to foster *Enterobacteriaceae*, although SA did not significantly ($p > 0.05$). The same trend was observed within this family for total *E. coli*. For both these taxa, the increment at EP was anyhow minor in SA and SMA than in the controls and differently significant when compared to CO ($p < 0.05$). From the total population of *E. coli* (5.04E+05 ± 2.08E+04 cells/mL), a small portion potentially pathogenic (2.94E+02 ± 5.10E+01 cells/mL) harbored the Cytolethal Distending Toxin. Interestingly, this taxon was reduced by the alternative salami and fostered by the controls, although both not significant ($p > 0.05$). In particular, the top reduction was obtained after fermentations of SMA. The fermentation of SA made the ATOP group grow less than CO ($p < 0.05$). Any fermented substrate made *Clostridum* group I grew significantly (for SMA the growth was less), but the results were similar among the samples ($p > 0.05$). Lastly, significant shifts were observed for the genus *Desulfovibrio*, increased by CNO_2 and decreased due to SA fermentation, with the control 7.4 times higher than the clean label salami.

4. Discussion

4.1. Volatilome

Considering the VOCs grouped as organic acids, the role of short and medium chain organic acids is explained in section 4.2 by the shift observed later as absolute quantifications, but it is out of that discussion the role of branched chain organic acids. From the results of the volatilome, the alternative formulations with no nitrates/nitrites did not contribute to their production, but the controls did. For example, CNO_2 left a firm signature of Pentanoic acid, 3-methyl, also known as a mucosal pro-inflammatory agent, derived from protein fermentation (Wang et al., 2020). From our results this is another feature of quality and safety that characterize the alternative formulations.

Pondering whether the clean label formulations were better in terms

Table 2
Quantification and changes of commensal beneficial taxa of colonic fermentation of salami measured by qPCR.

qPCR Targets & Samples	Quantifications cells/mL	Changes log_2(F/C)		MANOVA (Time)	
	Baseline	T1 (18 h)	EP (24 h)	*p*	$-log_{10}(p)$*
Lactobacillales					
CO	4.80E+06 ± 3.64E+05	0.56	0.82^{AB}	0.681632	0.166450
SA	4.80E+06 ± 3.64E+05^{b}	0.72^{ab}	2.13^{aA}	0.001842	2.734769
SMA	4.80E+06 ± 3.64E+05	0.94	1.74^{A}	0.165766	0.780505
CNO_2	4.80E+06 ± 3.64E+05	0.72	1.10^{AB}	0.418668	0.378129
BC	4.80E+06 ± 3.64E+05^{a}	0.44^{ab}	-1.34^{bB}	0.048217	0.175082
MANOVA (Matrix) *p*		0.932608	0.043956		
Bifidobacteriaceae					
CO	4.10E+08 ± 4.77E+07	-0.11^{B}	0.48^{AB}	0.056606	1.247137
SA	4.10E+08 ± 4.77E+07^{b}	0.08^{abAB}	1.04^{aA}	0.003197	2.495257
SMA	4.10E+08 ± 4.77E+07^{c}	1.01^{bA}	2.04^{aA}	0.013858	1.858299
CNO_2	4.10E+08 ± 4.77E+07	-0.52^{B}	-0.83^{B}	0.058814	1.230519
BC	4.10E+08 ± 4.77E+07^{a}	-0.32^{bB}	-2.43^{cC}	0.046692	0.706213
MANOVA (Matrix) *p*		0.0003459	0.000315		
***Clostridium* Group IV**					
CO	1.36E+08 ± 1.83E+07^{a}	-1.44^{bB}	-1.38^{bB}	0.000121	3.917073
SA	1.36E+08 ± 1.83E+07	0.11^{A}	-0.46^{AB}	0.054040	2.393648
SMA	1.36E+08 ± 1.83E+07	0.02^{A}	-0.02^{A}	0.902609	0.044500
CNO_2	1.36E+08 ± 1.83E+07^{a}	-0.64^{bAB}	-1.19^{bB}	0.000436	3.360957
BC	1.36E+08 ± 1.83E+07^{a}	0.28^{aA}	-1.81^{bB}	0.000024	4.625508
MANOVA (Matrix) *p*		0.000021	0.000011		
Bifidobacterium longum					
CO	1.08E+08 ± 1.52E+07^{a}	-1.70^{bB}	-1.41^{bB}	0.043768	1.269477
SA	1.08E+08 ± 1.52E+07^{b}	0.03^{bA}	0.96^{aA}	0.023466	1.629565
SMA	1.08E+08 ± 1.52E+07^{b}	0.44^{bA}	1.37^{aA}	0.000166	3.779631
CNO_2	1.08E+08 ± 1.52E+07^{a}	-3.63^{bC}	-3.86^{bC}	0.000001	6.044062
BC	1.08E+08 ± 1.52E+07^{a}	-2.02^{bBC}	-3.38^{cC}	0.000003	5.600852
MANOVA (Matrix) *p*		0.000429	0.000001		
Akkermansia muciniphila					
CO	4.03E+05 ± 7.74E+04	−0.15	-0.07^{A}	0.063285	2.483438
SA	4.03E+05 ± 7.74E+04^{a}	-1.19^{b}	-1.52^{bB}	0.000062	9.353854
SMA	4.03E+05 ± 7.74E+04^{a}	-0.91^{ab}	-1.08^{bAB}	0.000034	10.82114
CNO_2	4.03E+05 ± 7.74E+04	−0.27	-0.79^{AB}	0.055117	3.930131
BC	4.03E+05 ± 7.74E+04^{a}	-0.51^{a}	-3.06^{bC}	0.000004	5.447605
MANOVA (Matrix) *p*		0.1051502	0.000001		
Faecalibacterium prausnitzii					
CO	1.26E+04 ± 3.18E+03^{a}	-0.66^{b}	-1.41^{bB}	0.001359	2.866780
SA	1.26E+04 ± 3.18E+03	0.42	-0.77^{AB}	0.061564	1.210673
SMA	1.26E+04 ± 3.18E+03	0.05	-0.41^{A}	0.191359	0.718151
CNO_2	1.26E+04 ± 3.18E+03	−0.15	-0.83^{AB}	0.072828	1.137701

(*continued on next page*)

Table 2 (*continued*)

qPCR Targets & Samples	Quantifications cells/mL	Changes log_2(F/C)		MANOVA (Time)	
	Baseline	T1 (18 h)	EP (24 h)	*p*	*-log$_{10}$(p)**
BC	1.26E+04 ± 3.18E+03^{a}	0.28^{a}	−1.82^{bB}	0.001090	2.962573
MANOVA (Matrix) *p*		0.090691	0.023811		
***Bacteroides – Prevotella – Porphyromonas* (BPP) group**					
CO	6.82E+09 ± 3.05E+08	−0.27	−0.29^{B}	0.918863	0.036749
SA	6.82E+09 ± 3.05E+08	−0.13	−0.47^{B}	0.512972	0.289906
SMA	6.82E+09 ± 3.05E+08^{b}	0.03^{b}	1.16^{aA}	0.005472	2.261853
CNO_2	6.82E+09 ± 3.05E+08^{a}	−0.68^{a}	−2.23^{bC}	0.025051	1.601174
BC	6.82E+09 ± 3.05E+08^{a}	−1.70^{b}	−2.54^{bC}	0.009275	2.032686
MANOVA (Matrix) *p*		0.244794	0.000006		

Quantifications are expressed as cells/mL, obtained from gene copy number/ng of DNA. Changes are expressed as log_2(F/C). F/C = timepoint/baseline. ^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower-case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to "Time effect" on rows and to "Matrix effect" on columns; **-log$_{10}$(p)* = Significance of log_2 (F/C). SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate; BC = Blank Control; T1 = 18 h of fermentation; EP = 24 h of fermentation. BPP = *Bacteroides-Prevotella-Porphyromonas*. All values are obtained as the mean of sextuplicate (3 technical and 2 biological replicates). Baseline values for each qPCR target are obtained from sextuplicate and are equal for the fermentation of any sample, because indicate the quantification after adaptation of the colon microbiota to in vitro colon ecology and prior in vitro colonic fermentation.

of a healthier alcoholic fermentation, from the volatilome results, the control with no nitrite produced skatole, which is highly toxic for the host intestinal mucosa (Wang et al., 2020). Considering beneficial fermentation alcohols, their production was similar either by CNO_2 or SMA. Among these alcohols, a robust descriptor is 1-Pentanol, that has antioxidant and prebiotic potentials (Taneyo-Saa et al., 2014) also linked to *Akkermansia muciniphila* in healthy volunteers (Vernocchi et al., 2020). This characteristic suggests that SMA substrate fermentation could compete with CNO_2, because is capable of producing positive alcohols and less detrimental alcohols than the control with no nitrite.

The fermentation metabolite profile of the alternative formulations had a unique signature in the production of ketones, such as Acetylcyclopentanone for SMA and Cyclohexanone, 5-methyl-2-(1 -methylethyl)-, *cis*-for SA. More than oxidation products, these two VOCs seemed linked to fermentation. These two VOC were also unique signature of fermentation (absent at its beginning) and they were then produced homogeneously at the different time points of the process. Interestingly, Acetylcyclopentanone is capable to protect cell cultures from oxidative stress-induced toxicity and at a very low dosage of being able to prevent lethality in acetaminophen hepatotoxicity mouse model (Zhang et al., 2013).

By multivariate analysis, alternative formulations were not discriminated by typical oxidative aldehydes, which were instead specific descriptors of the controls. In particular, CNO_2 marked a unique signature of 2,4-Heptadienal, (E,E)- and, along with CO was described by at least six other oxidative aldehydes, such as Hexanal, Octanal, Nonanal, 2-Nonenal, (E)-, 2-Octenal, (E)-. All these aldehydes are derived from lipid oxidation of food, and in a recent paper, the effect of the vegetal extract was efficaciously tested to be a deterrent of their formation when added to roasted food in a similar in vitro model (Hu et al., 2022). Analogously we could explain the effect of the vegetal extract in SMA. The higher content of oxidative aldehydes in the controls reflects their higher content in fatty acids, as previously reported (Di Nunzio et al., 2022). Also, the action of vegetal extract brought a higher antioxidant potential to salami formulation than the controls, which could have influenced the minor number of oxidative aldehydes.

D-Limonene increase after SMA fermentation was probably due to the changes of structure occurring in the fermentation. Indeed, this VOC sourced from plant extracts is delivered in SMA food matrix, increasing its bioaccessibility. The positive impact that this VOC could generate on the host mucosa has been extensively studied, and its retainment and enrichment during colonic fermentation within in vitro model have been similarly assessed in the past (Nissen, Casciano, Valerii, Spisni, Gianotti, 2021). D-Limonene is a bioactive with a potent antioxidant activity (Valerii et al., 2021). Another remarkable attribute to address to the alternative formulations is the lack of the negative impact that could bring the exposure to Cyanamide dibutyl, which instead described the control with nitrite. In fact, nitrites have the capacity to form N-nitrous carcinogenic compounds, as in the human colon there are amines and amides derived from the bacterial metabolism of amino acids, that could be N-nitrousated in the presence of nitrosylated heme derived from not absorbed residual of red meat (Herrmann et al., 2015; Johnson, 2017; Meurillon & Engel, 2016).

4.2. Changes of main microbial VOCs after colonic salami fermentation

Considering the production of volatile organic acids, the clean label formulations were able to compete with the commercial products for the production of short chain fatty acids, but not for that of medium chain fatty acids. Eventually, the formulation SA generated more organic acids of any kind regarding SMA. Other authors have found that adding plant extract and fibers to sausages can produce a higher amount of SCFA in respect to a commercial control, but no influence was ascribed to the presence or not of nitrates (Perez-Burillo et al., 2019). From our results, the production of detrimental compounds derived from protein or specifically from tryptophan and tyrosine fermentation (Agus et al., 2018) was reduced in the formulation with no or was similar to the control with nitrite. For example, CO was the top producer of skatole and indole, while SMA and CNO_2 fermentation liberated similar level of phenol and p-cresol, but less than the control with no nitrites. Skatole is a toxic product of the bacterial decarboxylation of tryptophan by *E. coli* and *Clostridium* group I, which affect the mucosa and causes the production of inflammatory cytokines (Roager & Licht, 2018). Phenol and p-cresol are shown to impair epithelial barrier function in vitro and may be targeted for carcinogens (Wang et al., 2020). p-cresol and Indole, for example, would be transformed into p-cresyl sulphate and indoxyl sulphate, which after conjugation, accumulates in the liver leading to complications and pathologies such as chronic kidney diseases and cardiovascular diseases (Arcidiacono et al., 2022; Wu et al., 2011).

4.3. Shift in the colonic microbiota after colonic salami fermentation

From our results, *Bacteroidetes* showed increases just after fermentation of the alternative formulations, expressing a positive feature since many species between this main phylum are important commensals and fibrolytic specialist. Also in this view, the higher increase in *Bacteroidetes* taxon scored by SMA in respect to SA and other salami, could be due to the higher presence of vegetal fiber brought by the plant extract included in this formulation. Other authors have reported that adding a fiber supplement in sausages, once fermented in a similar in vitro model increased the quantity of *Bacteroidetes* of comparable levels (Perez-Burillo et al., 2020). The trends of the shifts observed concerning this taxon were telling of increases in any substrates, but this outcome has to be differently considered in the view of trends that happened at lower taxonomic levels.

Firmicutes and *Bacteroidetes* are the two principal bacterial phyla that live the adult human colon. The ratio of their abundances is an index of microbiota eubiosis and values higher than 2 are commonly associated with *in vivo* microbiota dysbiosis (Koliada et al., 2017; Zhou et al.,

Table 3
Quantification and changes of commensal opportunistic taxa of colonic fermentation of salami measured by qPCR.

qPCR Targets & Samples	Quantifications cells/mL	Changes log_2(F/C)		MANOVA (Time)	
	Baseline	T1 (18 h)	EP (24 h)	*p*	*-log₁₀(p)**
Enterobacteriaceae					
CO	7.85E+07 ± 4.58E+05^{b}	0.84^{abB}	2.56^{aA}	0.049707	0.468239
SA	7.85E+07 ± 4.58E+05	0.56^{B}	0.70^{B}	0.052006	0.026239
SMA	7.85E+07 ± 4.58E+05^{b}	0.94^{abB}	1.21^{aB}	0.005644	0.155755
CNO_2	7.85E+07 ± 4.58E+05^{b}	1.09^{aB}	1.86^{aB}	0.000008	0.171091
BC	7.85E+07 ± 4.58E+05^{c}	2.32^{bA}	3.93^{aA}	0.006603	0.309035
MANOVA (Matrix) *p*		0.000616	0.000444		
***Atopobium* – *Collinsella* – *Eggerthella* (ATOP) group**					
CO	5.29E+05 ± 1.09E+05^{b}	0.16^{b}	1.21^{aA}	0.024434	1.612005
SA	5.29E+05 ± 1.09E+05	0.08	0.21^{B}	0.880294	0.055372
SMA	5.29E+05 ± 1.09E+05	0.27	0.40^{AB}	0.574153	0.240972
CNO_2	5.29E+05 ± 1.09E+05^{b}	0.72^{ab}	1.07^{aAB}	0.049402	0.070886
BC	5.29E+05 ± 1.09E+05^{b}	0.28^{b}	1.91^{aA}	0.042082	1.375903
MANOVA (Matrix) *p*		0.852626	0.026102		
***Clostridium* group I**					
CO	1.54E+04 ± 3.06E+03^{b}	1.68^{a}	2.49^{aAB}	0.000208	3.681965
SA	1.54E+04 ± 3.06E+03^{b}	1.24^{a}	2.48^{aAB}	0.000308	3.511522
SMA	1.54E+04 ± 3.06E+03	0.93	1.27^{B}	0.245233	0.610421
CNO_2	1.54E+04 ± 3.06E+03^{b}	1.80^{a}	2.03^{aAB}	0.022968	0.580097
BC	1.54E+04 ± 3.06E+03^{b}	1.67^{a}	3.05^{aA}	0.000087	4.060083
MANOVA (Matrix) *p*		0.453844	0.018590		
Escherichia coli* (total)*					
CO	5.04E+05 ± 2.08E+04^{b}	0.62^{b}	2.28^{aA}	0.031012	1.508470
SA	5.04E+05 ± 2.08E+04	0.52	0.69^{B}	0.072121	1.141938
SMA	5.04E+05 ± 2.08E+04	0.74	1.03^{B}	0.080023	1.096785
CNO_2	5.04E+05 ± 2.08E+04^{b}	0.89^{a}	1.36^{aB}	0.034346	1.464123
BC	5.04E+05 ± 2.08E+04^{b}	1.89^{a}	3.79^{aA}	0.000019	4.721246
MANOVA (Matrix) *p*		0.082102	0.035284		
***Escherichia coli* (potentially toxigenic)** *§					
CO	2.94E+02 ± 5.10E+01	0.55	1.45	0.340221	0.468239
SA	2.94E+02 ± 5.10E+01	0.20	−0.17	0.941371	0.026239
SMA	2.94E+02 ± 5.10E+01	−0.43	−1.01	0.698634	0.155749
CNO_2	2.94E+02 ± 5.10E+01	1.08	1.09	0.674387	0.171090
BC	2.94E+02 ± 5.10E+01	0.85	0.29	0.490869	0.309034
MANOVA (Matrix) *p*		0.745796	0.250029		
***Desulfovibrio* spp.**					
CO	1.35E+06 ± 1.59E+05	1.33^{A}	1.25^{A}	0.090244	1.044607
SA	1.35E+06 ± 1.59E+05^{a}	−0.12^{aC}	−1.12^{bC}	0.000012	5.705370
SMA	1.35E+06 ± 1.59E+05	0.19^{B}	0.21^{B}	0.065024	5.984299
CNO_2	1.35E+06 ± 1.59E+05^{b}	0.90^{aA}	1.77^{aA}	0.000001	5.968434
BC	1.35E+06 ± 1.59E+05	0.13^{B}	0.65^{B}	0.080410	3.981657
MANOVA (Matrix) *p*		0.000012	0.000005		

Quantifications are expressed as cells/mL, obtained from gene copy number/ng of DNA. Changes are expressed as log_2(F/C). F/C = timepoint/baseline. ^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower-case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to "Time effect" on rows and to "Matrix effect" on columns; *$-log_{10}(p)$ = Significance of log_2 (F/C). -**This taxon was amplified by targeting cell division protein (FtsZ) rRNA; *§This taxon was amplified by targeting cytolethal distending toxin (CDT IV) rRNA; SA = Salami with ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate; BC = Blank Control; T1 = 18 h of fermentation; EP = 24 h of fermentation. All values are obtained as the mean of sextuplicate (3 technical and 2 biological replicates). Baseline values for each qPCR target are obtained from sextuplicate and are equal for the fermentation of any sample, because indicate the quantification after adaptation of the colon microbiota to in vitro colon ecology and prior in vitro colonic fermentation.

2017). In our samples, starting from an eubiosis condition relative to the well-being of the donors, SA and SMA fermentation was able to keep it up, in contrast with the results of the controls. Other authors showed that the addition of fiber to sausages can increase the abundance of *Bacteroidetes* and reduce that of *Firmicutes*, eventually repealing unbalances in F/B (Perez-Burillo et al., 2020).

4.3.1. Commensals beneficial taxa

Salami substrate generally fostered classes or families with beneficial microbes (except for CNO_2 for *Bifidobacteriaceae*). However, a deeper analysis revealed a general abundance reduction at genus or species levels, with few exceptions. At the higher levels, *Lactobacillales* were fostered by any samples, but the alternative formulations were better. This feature previously observed could be due to the starter consortium's contribution and the indigenous species present in salami, of which many are part of *Lactobacillales* (Pini et al., 2020). Also, the *Bifidobacteriaceae* were increased just by the alternative formulations. SMA was the sole able to significantly increase *B. longum* and the BPP group. Within the BBP group, the most representative members are *Bacteroides* and *Prevotella*, which are important commensal genera highly saccharolytic and fibrolytic, that are fundamental in colonic fermentation processes (Oba et al., 2020). *Bifidobacteriaceae* and minorly *Lactobacillaceae* represent the most known and studied beneficial bacterial group within the intestinal microbiota, which are able to exert potent benefits to the host, as augmented immune response, improved epithelial permeability, and protection against pathogens (Nissen et al., 2009). The beneficial *Clostridia* were reduced in any sample, but the alternative formulations did smaller reductions. Clostridial species belonging to the taxonomic group IV are beneficial microbes especially known for the beneficial production of butyrate, which is a potent prebiotic that induce microbiota eubiosis (Wang et al., 2020). In a recent work, the in vitro fermentation of salami including inulin promoted *Bacteroides* and *Bifidobacterium* (Perez-Burillo et al., 2020). It is important to notice that the discussion on the role of the beneficial

taxa in salami ecosystem is also based on the ability of each formulation to limit their loss of abundance. That fact validates the choice of selected taxa also in such complex food matrices.

4.3.2. Commensals opportunistic taxa

Analogously to beneficial taxa, a similar approach was adopted for opportunistic microbes. The effects of food formulations are discussed in terms of ability to limit the growth of the selected opportunistic microbes. From our results, SA and SMA always limited more the growth of opportunistic in comparison to the controls. SA was more potent than SMA because these taxa grew less in four out of six cases. SMA was able to reduce the content of potentially toxigenic *E. coli*, although the ANOVA model was not significant. Toxigenic *E. coli* harbors cyclomodulins, like CDT (Cytholethal Distending Toxin), which take parts in the onset of colorectal cancer (Wassenaar, 2018). In contrast to our findings, in a recent paper done with similar methodologies but a different in vitro model, the authors found that adding citrus fibers to salami also reduced the prevalence of *Escherichia/Shigella* group (Perez-Burillo et al., 2020).

From our results, SA was also able to reduce the content of *Desulfovibrio*, that is a sulfurate-reducer genus able to affect and shrink the mucin barrier and thereof the integral structure of the colon mucosa by production of dimethyl sulphate and also inducer of colitis (Rowan et al., 2010), was reduced by fermentation with SA. This feature gives evidence that the absence of nitrite in formulation, which uses to be a substrate for this harmful taxon (Warren et al., 2005), results in its containment and reduction due principally to the inhibitory and antioxidant action of ascorbate.

Lastly, the results of the ecological competition between *Enterobacteriaceae* and *Bifidobacteriaceae* is an index of bifidogenic capacity of the substrates unveiling possible prebiotic features. The *Bifidobacteriaceae* competition had shown to be higher when the alternative formulations were fermented in respect to the controls. In this context, SMA was more potent than SA; maybe because, even if ascorbate of SA can limit more *Enterobacteriaceae* than SMA, the plant extract of SMA induced a higher growth of *Bifidobacteriaceae*, as it is reported that vegetal fibers use to foster this health-related family (Wang et al., 2019).

5. Conclusions

The onset of pathologies in the intestinal tract due to the excessive consumption of red meat has recently prompted the food industries to seek alternative strategies. In particular, the processed meat industry is studying alternative formulations in salami production. One of the main strategies is to replace nitrites, which in the host can lead to the formation of toxic compounds (e.g. nitrosamines).

The following study evaluated innovative formulations in which the nitrites were replaced by ascorbic acid and/or a mix of plant extracts. The results obtained show that the clean label formulations promote a general eubiosis of the intestinal microbiota, in the face of those preselected indices, including favorable F/B ratio, the proliferation of beneficial microbial taxa including *Lactobacillales*, *Bifidobacteriaceae,* and reduction of negative microbial populations, including *Enterobacteriaceae* and ATOP group. Furthermore, the volatilome analysis highlighted a marked production of beneficial molecules, including SCFA such as acetate, propionate and butyrate, and a reduction in host negative molecules such as phenol and p-cresol from the fermentation of proteins. Although the innovative formulations have not given benefits dramatically superior to those of the control and the product with nitrites, the results are promising, as the plant extracts used in place have given results comparable to those obtained with the traditional formulation. These results may represent an encouraging starting point for the processed meat industry for the development of clean-label formulations aimed at reducing the negative impact of these products on consumer health.

Funding

This study was funded by Italian POR-FESR 2014–2020 (Innovative Milk and Meat products for Consumer's Health—MiMe4Health). Call for strategic industrial research projects aimed at the priority areas of the Intelligent Specialization Strategy (DGR n. 986/2018). Project nr. PG/2018/632,065.

Institutional review board statement

The study was conducted according to the guidelines of the ethics procedures required at the University of Bologna.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authors' contributions

Conceptualization = L.N., A.B., and A.G.; methodology = L.N., M.D.N., A.B., and A.G.; software = L.N., F.C., and A.G.; validation = L.N., A.B., and A.G.; formal analysis = L.N., F.C., and M.D.N.; investigation = L.N., F.C., M.D.N., A.B., and A.G.; resources = G.G., A.B., and A.G.; data curation = L.N., F.C., M.D.N., A.B., and A.G.; writing—original draft preparation = L.N., F.C., and A.G.; writing—review and editing = L.N., F.C., M.D.N., G.G., A.B., and A.G.; visualization = L.N., F.C.; supervision = L.N., G.G., A.B., and A.G.; project administration = G.G., A.B., and A.G.; funding acquisition = G.G., A.B., and A.G. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors are grateful to Roberta Virgili (Stazione Sperimentale per l'Industria delle Conserve Alimentari, Italy) for supplying salami. The authors also acknowledge the personnel of Centre for Applied Biomedical Research – CRBA, *Alma Mater Studiorum* - Università di Bologna, Policlinico di Sant'Orsola, Bologna, Italy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102568.

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