Food Bioscience

Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota

--Manuscript Draft--

Manuscript Number:	FBIO-D-23-00074R1
Article Type:	Research Paper
Keywords:	processed meat; nitrates; gut microbiota; Colon fermentation; proteolysis; clean-label
Corresponding Author:	Lorenzo Nissen, Ph.D University of Bologna Department of Agri-Food Sciences and Technologies ITALY
First Author:	Lorenzo Nissen, Ph.D
Order of Authors:	Lorenzo Nissen, Ph.D
	Flavia Casciano, MsC
	Mattia Di Nunzio, PhD
	Gianni Galaverna, PhD
	Alessandra Bordoni, PhD
	Andrea Gianotti, PhD
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.
Suggested Reviewers:	Carlo Viti, PhD Prof, University of Florence Department of Agricultural Food Environment and Forestry Sciences and Technologies carlo.viti@unifi.it Expert in cured meat
	José Ángel Rufián-Henares, PhD Prof, University of Granada jarufian@ugr.es Expert in colon metabolims
	Christel Bera-Maillet, PhD Prof, Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France christel.maillet@jouy.inra.fr

	Expert in food fermentation
Opposed Reviewers:	
Response to Reviewers:	Editor and Reviewer comments: Editor comments: -Please, an Institutional email address for the corresponding author is welcome. An Institutional email address for the CA was given in page 1 andrea.gianotti@unibo.it; also lorenzo.nissen@unibo.it was added as CA. -Line 91. Please specify if volunteer were male or female. What does mean "adults"?....(e.g., between 25-35 age or....). Please specify if replication were performed. Was the number of volunteer (3) sufficient to generalise the finding? We thank the Editor for the comments. We have included the information required in paragraph 2.3.…(either male and female). The number and type of volunteers was in accordance with previous protocols. Specifically, volunteers were adults (between 25-50 years old)... Two biological experiments were performed within one week, employing fresh donations from the same donors. - Line 282...the term "prebiotic VOCs" is probably unappropriate. Example of "prebiotics" VOCs? We got rid of that term. We were looking to introduce this new concept, based on the observations we have collected and published in recent years. An example of prebiotic VOCs are the SCFAs and MCFAs themselves, or bioactives, such as terpenes. The title of subparagraph 3.2.1 was revised and changed in “VOCs related to beneficial and prebiotic effect”. Minor Please, language editing is suggested because grammatical errors (e.g., the highest...instead of the higher..) and/or unclear sentences. We thank the Editor and apologize for typos. We made corrections on grammatical errors and reformulated unclear sentences. Reviewer #1: This paper deals with replacing traditionally used nitrated/nitrites with plants extracts and ascorbic acid in salmi. Currently, knowledge regarding this topic is limited and has just recently been a research priority. So the paper deserves to be published. However, the results need to be presented more clearly and readily. Tables are overly complex, and captions sometimes need to carry essential information. For example, Tables 1, 2, and 3 do not state what F/C is. This part should be revised entirely to make it more immediately understandable to the reader. A comparison with a colleague who is not part of the research group would be constructive to clarify this part. We thank the Reviewer for the comments, in the revised version we have included the lines below as footnotes of the tables to give more details. “Quantifications are expressed as cells/mL, obtained from gene copy number/ng of DNA. Changes are expressed as log2(F/C). F/C = timepoint/baseline. …*-log10(p) = Significance of log2 (F/C). ….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”. In several parts of the manuscript the authors mention VOCs with functional properties or harmful products. But, in my opinion, authors do not clarify in a right way what these are and do. The same attention should be paid to commensal or opportunistic microorganisms. We thank the Reviewer, we made some amendments for the molecules (example at Line 400), and principally we strengthen the part related to microbes. For example: in paragraph 4.3: ….. 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). ….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 eubioisis (Wang et al., 2020). ….Toxigenic E. coli harbors cyclomodulins, like CDT (Cytholethal Distending Toxin), which take parts in the onset of colorectal cancer (Wassenaar, 2018). Some specific comments: 1) please clarify what "clean label formulations" mean. From lines 54 to 55, we originally gave the referenced definition of “clean label formulations”. In the revised version we have extended such definition in this way: “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).” 2) the introduction should begin by discussing risks associated with nitrates/nitrates and then focus on nitrate/nitrite replacement. Introduction has been rearranged accordingly. 3) Lines 82-83. Please report some information. Unfortunately, all the data are published with other editorial companies different to Elsevier, and reporting some in the present paper could be a plagiarism. 4) Line 106, write correctly and always the same way in the text. Corrections done on 10,000g 5) Line 276, write down what the acronyms mean. They have never been mentioned before. The acronyms of SCFAs and MCFAs are now reported in the revised version. 6) The first two sentences of the introduction should be rewritten or deleted. They cannot be the beginning of the discussion. Introduction has been rearranged accordingly. 7) In Tables 1, 2, and 3, values for BL reported six times, could be a better way to show data. I prefer to put values and not ratios. Also, why -Log10(p) value was put in? I do not understand. However, I am not a statistician. As mentioned earlier, this table should be made more understandable. We thank the Reviewer for the comments. We have tried to ameliorate the tables and provided new styled versions, with less acronyms. We understood the motions moved, but we repute Log2(F/C) the right way to discuss shifts of the microbial populations, especially for qPCR. This approach is diffused in the scientific community, and we have already published some papers with this output. Additionally, we used to report values in the MS to come along and discuss with table’s ratios. For example, at lines 357-358 the sentence says: “…After SMA fermentations, at the EP Bifidobacteriaceae accounted for 8.63E+08 ± 2.97E+08 cells/mL, 7.3 times more than CNO2”. The meaning of -Log10(p) is explained in the footnotes and is an additional parameter to extend the significance to the Log2 values and not to the MANOVA. The lower are the values and the higher is the significance. 8) Figures 2 e 3, The caption says that the changes are reported for the two sampling times. But in the figure, the two results for the two sampling times I do not see them. I need clarification on how the two sampling times were treated. In these figures the box plot of a given sample represents the whole data set of that samples, including intermediate time point (T1) and the endpoint (EP), and also including every replicate. From such data we have discounted the value of the baseline, in order to evidence the shifts. Additionally, we have improved the original indications given on paragraph 2.7.2. Quantification of main microbial VOCs, as follows: …. 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 boxplots, where each boxplot is generated either from intermediate time points and endpoints values.

Dear Editor,

we have prepared for your Journal the present paper "Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota" by Lorenzo Nissen, Flavia Casciano, Mattia Di Nunzio, Gianni Galaverna, Alessandra Bordoni and Andrea Gianotti.

The content is about the study of the impact on the host of new preparations of salami with NOx replacement by plant extract and ascorbate.

We recently read interesting research articles published in Food Bioscience based on an approach similar to the research that we have just conducted (https://doi.org/10.1016/j.fbio.2022.101754; https://doi.org/10.1016/j.fbio.2022.101682). Also, by the results obtained with the application proposed by Elsevier, Food Bioscience was on the first positions (https://journalfinder.elsevier.com/).

Thus, we choose to prepared the article for your consideration. We hope that the text could interest and satisfy the Editor's demand, before peer-review.

This is briefly the article's content:

Salami were processed in an in vitro gut model that coupled oro-gastro-duodenal digestion with colonic fermentation in order to describe the impact of such food towards in vitro healthy human colon ecology, studying the shifts of colon microbes and colon metabolites.

Sampling was performed prior, during and after the colonic fermentation and the composition of the colon core microbiota and the colon volatilome were assessed by microbiomics (qPCR) and metabolomics (GC/MS) and analysed with robust statistical multivariate models.

Despite the health issues acknowledged for processed meat consumption, mainly in relation to NOx presence, this work tested how new formulation of salami without NOx can better impact on eubiosis indicators of an healthy colon ecology.

Our results indicated that salami with the alternative preparations can mitigate the *Enterobacteriaceae* induced dysbiosis by the NOx preparations, fostering some beneficial taxa and suggesting a better profile of organic acids (SCFA and MCFAa) production during colonic fermentation of salami.

The recipient results of this work are promising for the exploitation of this alternative food products and could serve to reduce the amount of animal testing, and also could be basics for food intervention trials.

Bologna, 06/01/2023

Sincerely yours.

Prof. Andrea Gianotti

Andrea Gianotti, Ph.D.
https://www.unibo.it/sitoweb/andrea.gianotti
Dipartimento di Scienze e Tecnologie Agro-Alimentari (DISTAL)
Department of Agricultural and Food Science
Alma Mater Studiorum, Università di Bologna,Via Fanin, 42 40127 Bologna, tel +39 051 2096577
Unità Organizzativa di Cesena, Piazza Goidanich 60, 47521 Cesena (FC), tel. : +39 0547 338134
Mob.: +39 338 7868519, e-mail: andrea.gianotti@unibo.it
https://orcid.org/0000-0002-8847-6251

Reply to Reviewers

Manuscript Number: FBIO-D-23-00074

Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota

Dear Editor,

thanks for the precious work done on revisions. We have addressed them all, and we see that now the revised version has improved much and looks clearer. While hoping that the revised MS shall satisfy your demands, we send you our best regards.

Sincerely yours,

Lorenzo Nissen and Andrea Gianotti.

Editor and Reviewer comments:

Editor comments:

-Please, an Institutional email address for the corresponding author is welcome.

An Institutional email address for the CA was given in page 1 andrea.gianotti@unibo.it; also lorenzo.nissen@unibo.it was added as CA.

-Line 91. Please specify if volunteer were male or female. What does mean "adults"?....(e.g., between 25-35 age or....). Please specify if replication were performed. Was the number of volunteer (3) sufficient to generalise the finding?

We thank the Editor for the comments. We have included the information required in paragraph 2.3.*…(either male and female). The number and type of volunteers was in accordance with previous protocols. Specifically, volunteers were adults (between 25-50 years old)... Two biological experiments were performed within one week, employing fresh donations from the same donors.*

- Line 282...the term "prebiotic VOCs" is probably unappropriate. Example of "prebiotics" VOCs?

We got rid of that term. We were looking to introduce this new concept, based on the observations we have collected and published in recent years. An example of prebiotic VOCs are the SCFAs and MCFAs themselves, or bioactives, such as terpenes.

The title of subparagraph 3.2.1 was revised and changed in "VOCs related to beneficial and prebiotic effect".

Minor

Please, language editing is suggested because grammatical errors (e.g., the highest...instead of the higher..) and/or unclear sentences.

We thank the Editor and apologize for typos. We made corrections on grammatical errors and reformulated unclear sentences.

Reviewer #1: This paper deals with replacing traditionally used nitrated/nitrites with plants extracts and ascorbic acid in salmi. Currently, knowledge regarding this topic is limited and has just recently been a research priority. So the paper deserves to be published. However, the results need to be presented more clearly and readily.

Tables are overly complex, and captions sometimes need to carry essential information. For example, Tables 1, 2, and 3 do not state what F/C is.

This part should be revised entirely to make it more immediately understandable to the reader. A comparison with a colleague who is not part of the research group would be constructive to clarify this part.

We thank the Reviewer for the comments, in the revised version we have included the lines below as footnotes of the tables to give more details. "Quantifications are expressed as cells/mL, obtained from gene copy number/ng of DNA. Changes are expressed as log2(F/C). F/C = timepoint/baseline. …*-log10(p) = Significance of log2 (F/C). ….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".

In several parts of the manuscript the authors mention VOCs with functional properties or harmful products. But, in my opinion, authors do not clarify in a right way what these are and do. The same attention should be paid to commensal or opportunistic microorganisms.

We thank the Reviewer, we made some amendments for the molecules (example at Line 400), and principally we strengthen the part related to microbes. For example: in paragraph 4.3:

….. 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).

….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 eubioisis (Wang et al., 2020).

….Toxigenic E. coli harbors cyclomodulins, like CDT (Cytholethal Distending Toxin), which take parts in the onset of colorectal cancer (Wassenaar, 2018).

Some specific comments:
1) please clarify what "clean label formulations" mean.

From lines 54 to 55, we originally gave the referenced definition of "clean label formulations". In the revised version we have extended such definition in this way: "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)."

2) the introduction should begin by discussing risks associated with nitrates/nitrates and then focus on nitrate/nitrite replacement.

Introduction has been rearranged accordingly.

3) Lines 82-83. Please report some information.

Unfortunately, all the data are published with other editorial companies different to Elsevier, and reporting some in the present paper could be a plagiarism.

4) Line 106, write correctly and always the same way in the text.

Corrections done on 10,000*g*

5) Line 276, write down what the acronyms mean. They have never been mentioned before.

The acronyms of SCFAs and MCFAs are now reported in the revised version.

6) The first two sentences of the introduction should be rewritten or deleted. They cannot be the beginning of the discussion.

Introduction has been rearranged accordingly.

7) In Tables 1, 2, and 3, values for BL reported six times, could be a better way to show data. I prefer to put values and not ratios. Also, why -Log10(p) value was put in? I do not understand. However, I am not a statistician. As mentioned earlier, this table should be made more understandable.

We thank the Reviewer for the comments. We have tried to ameliorate the tables and provided new styled versions, with less acronyms. We understood the motions moved, but we repute Log2(F/C) the right way to discuss shifts of the microbial populations, especially for qPCR. This approach is diffused in the scientific community, and we have already published some papers with this output. Additionally, we used to report values in the MS to come along and discuss with table's ratios. For example, at lines 357-358 the sentence says: *"…After SMA fermentations, at the EP Bifidobacteriaceae accounted for 8.63E+08 ± 2.97E+08 cells/mL, 7.3 times more than CNO2".* The meaning of -Log10(p) is explained in the footnotes and is an additional parameter to extend the significance to the Log2 values and not to the MANOVA. The lower are the values and the higher is the significance.

8) Figures 2 e 3, The caption says that the changes are reported for the two sampling times. But in the figure, the two results for the two sampling times I do not see them. I need clarification on how the two sampling times were treated.

In these figures the box plot of a given sample represents the whole data set of that samples, including intermediate time point (T1) and the endpoint (EP), and also including every replicate. From such data we have discounted the value of the baseline, in order to evidence the shifts.

Additionally, we have improved the original indications given on paragraph 2.7.2. *Quantification of main microbial VOCs*, as follows: …. 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 boxplots, where each boxplot is generated either from intermediate time points and endpoints values.

Highlights

- New salami formulations with replacement of NO_x by vegetal extracts are proposed
- Salami digestion and colonic fermentation were tested in an in vitro gut model
- The impact of salami on colon ecology was studied by microbiomics and metabolomics
- Any salami increased the proteolysis indicators of colon microbiota
- Salami without NO_x increased also some beneficial indicators of colon microbiota

Graphical Abstract

Click here to access/download;Graphical Abstract;graphical abstract FoodBioscience Jan 2023.jpg

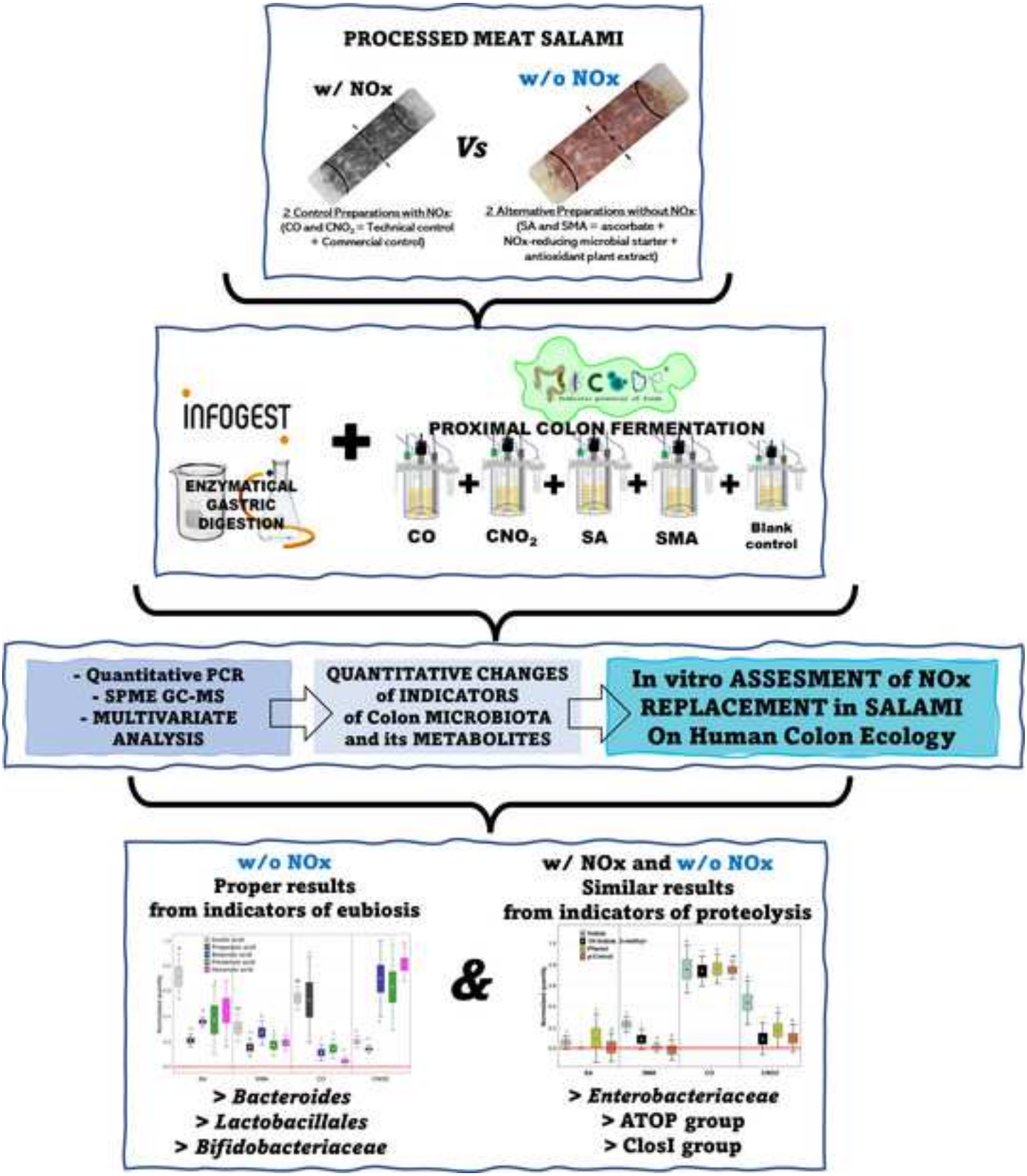


Figure 1

Click here to access/download;Figure;Figure 1 PCAs FoodBioscience Jan 2023.jpg

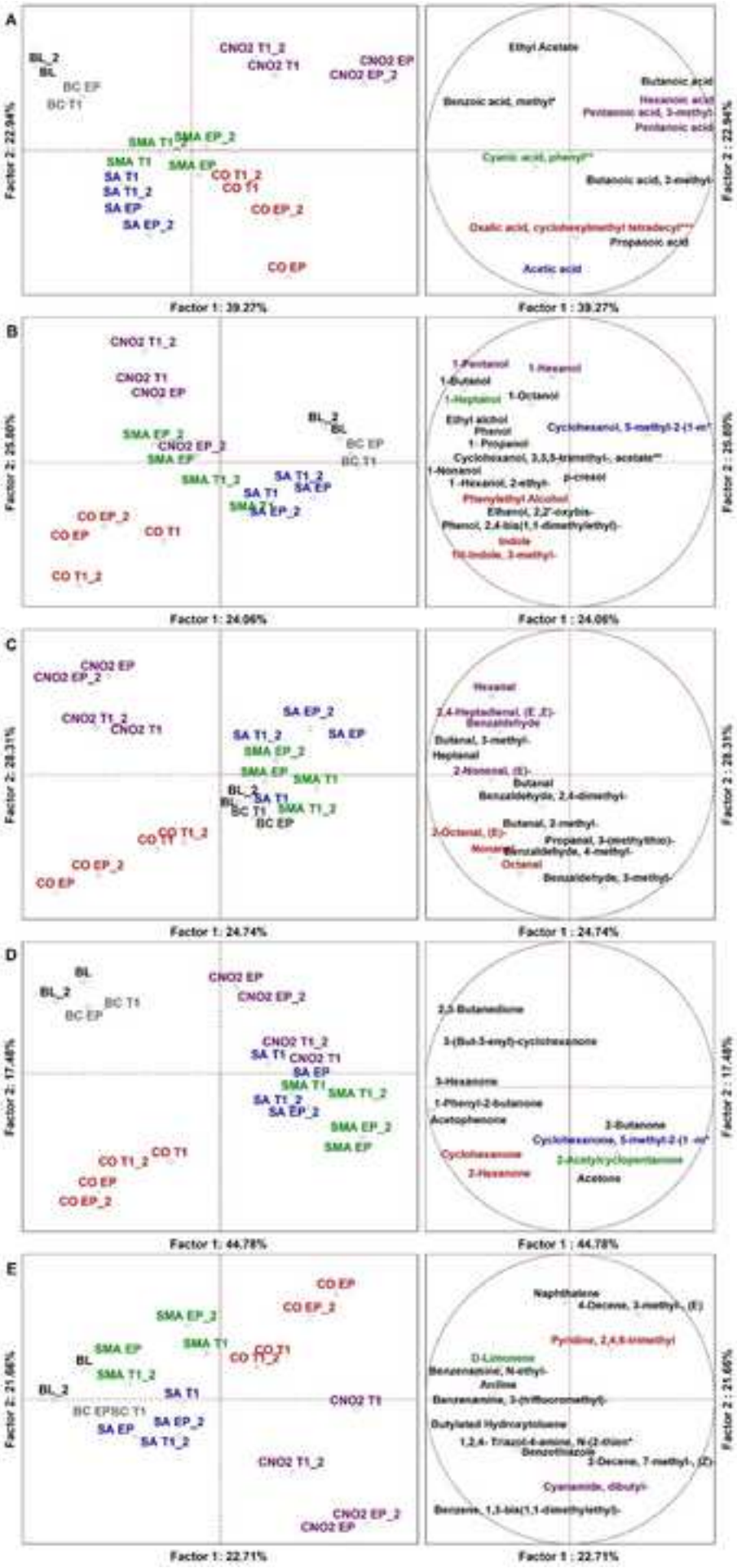


Figure 2

Click here to access/download;Figure;Figure 2 Beneficial VOCs FoodBioscience Jan 2023.jpg

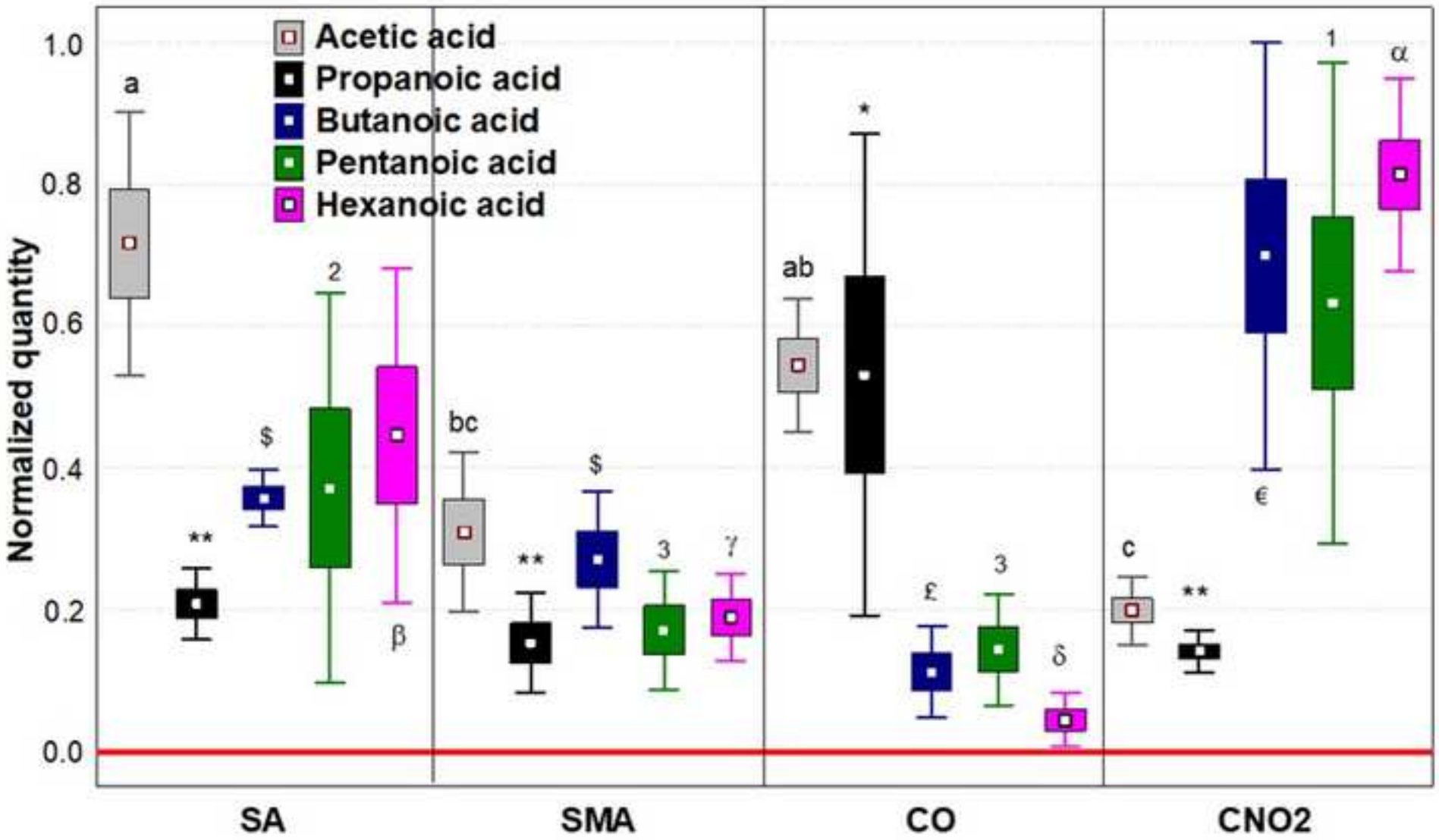
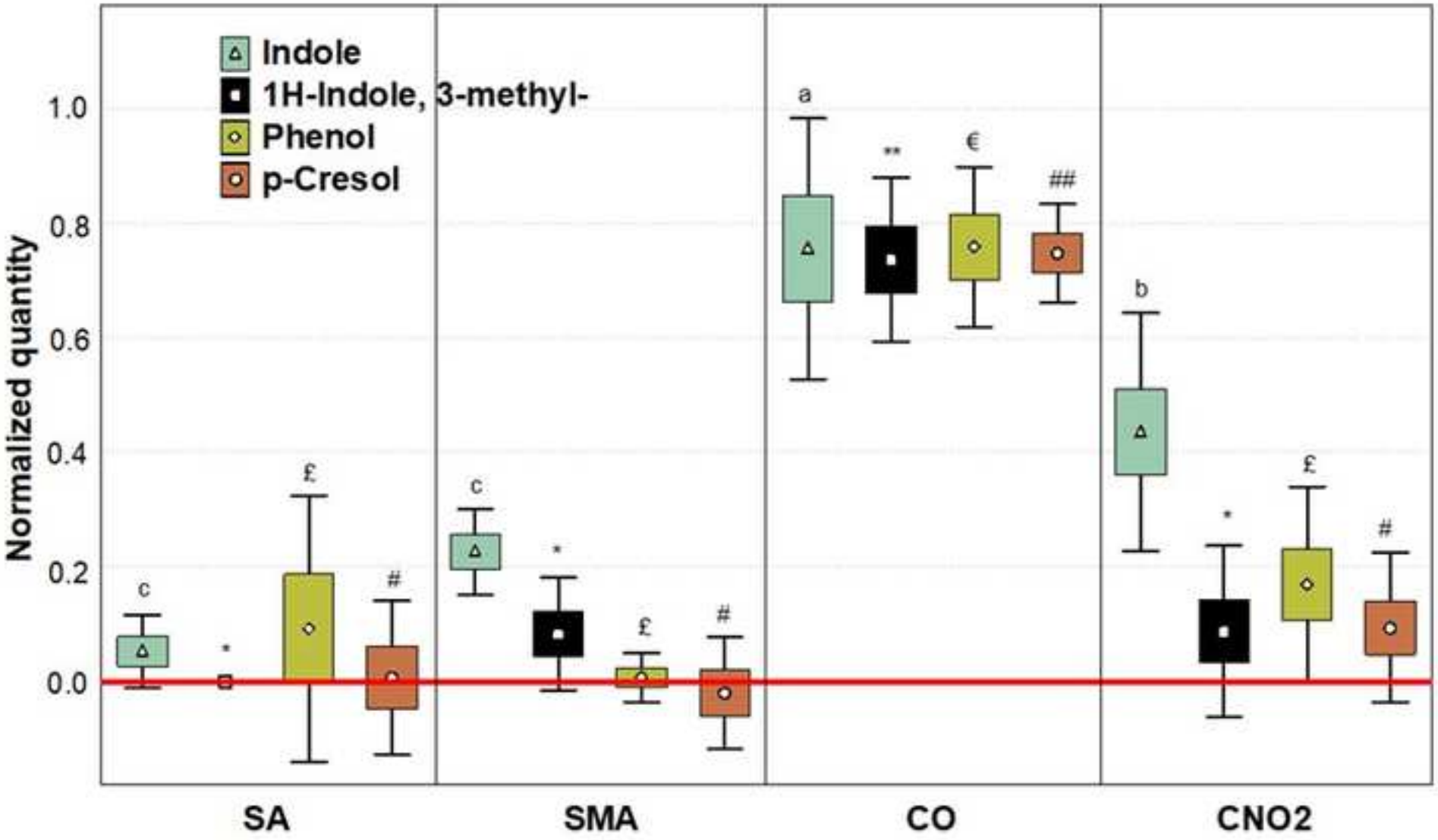


Figure 3

Click here to access/download;Figure;Figure 3 Detrimental VOCs Foodbioscience Jan 2023.jpg



Bologna, January 6th, 2023

Dear Editor,

For the article “Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota” authored by Lorenzo Nissen, Flavia Casciano, Mattia Di Nunzio, Gianni Galaverna, Alessandra Bordoni and Andrea Gianotti

No conflict of interest is linked to this research paper and their authors.

Sincerely yours,

Lorenzo Nissen,

Flavia Casciano,

Mattia Di Nunzio,

Gianni Galaverna,

Alessandra Bordoni,

Andrea Gianotti*.

***Andrea Gianotti, Ph.D.**
https://www.unibo.it/sitoweb/andrea.gianotti
Dipartimento di Scienze e Tecnologie Agro-Alimentari (DISTAL)
Department of Agricultural and Food Science
Alma Mater Studiorum, Università di Bologna,Via Fanin, 42 40127 Bologna, tel +39 051 2096577
Unità Organizzativa di Cesena, Piazza Goidanich 60, 47521 Cesena (FC), tel. : +39 0547 338134
Mob.: +39 338 7868519, e-mail: andrea.gianotti@unibo.it
https://orcid.org/0000-0002-8847-6251

Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota.

Lorenzo Nissen[1,2,3,*], Flavia Casciano[1,3], Mattia Di Nunzio[4], Gianni Galaverna[5], Alessandra Bordoni[1,2] and Andrea Gianotti[1,2,3*].

[1]DiSTAL - Department of Agricultural and Food Sciences, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[2]CIRI - Interdepartmental Centre of Agri-Food Industrial Research, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[3]CRBA, Center for Biomedical Applied Research, *Alma Mater Studiorum* – University of Bologna, Policlinico di Sant'Orsola, via Massarenti 9, 40138, Bologna, Italy.

[4]DeFENS, Department of Food, Environmental and Nutritional Sciences, University of Milan, via Celoria 2, 20133 Milan, Italy.

[5]Department of Food and Drugs, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy.

*Corresponding authors: andrea.gianotti@unibo.it +39 0547338134

and lorenzo.nissen@unibo.it +39 0547338146

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.

Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota.

Lorenzo Nissen[1,2,3,*], Flavia Casciano[1,3], Mattia Di Nunzio[4], Gianni Galaverna[5], Alessandra Bordoni[1,2] and Andrea Gianotti[1,2,3*].

[1]DiSTAL - Department of Agricultural and Food Sciences, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[2]CIRI - Interdepartmental Centre of Agri-Food Industrial Research, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[3]CRBA, Center for Biomedical Applied Research, *Alma Mater Studiorum* – University of Bologna, Policlinico di Sant'Orsola, via Massarenti 9, 40138, Bologna, ~~Italia~~ Italy.

[4]DeFENS, Department of Food, Environmental and Nutritional Sciences, University of Milan, via Celoria 2, 20133 Milan, Italy.

[5]Department of Food and Drugs, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy.

*Corresponding authors: andrea.gianotti@unibo.it +39 0547338134

and lorenzo.nissen@unibo.it +39 0547338146

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.

Keywords: processed meat; nitrates; gut microbiota; colon fermentation; proteolysis; clean-label.

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. ~~In general, excessive 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).~~ S~~Despite this, s~~alami is largely consumed around all the world (Blaiotta et al., 2018), but ~~excessive 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). A~~mong~~ others~~dditionally, 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 ~~those additives~~ 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 aminoacids. 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). ~~and for~~Due to ~~this~~ these reasons, there is a common interest by food technologists to~~for an~~ improve~~ment of their~~ the nutritional and health properties, and reduce the negative features. Several attempts were tried, for example, ~~e.g.,~~ 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. (~~2019~~2020), the authors added ~~a~~ 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., ~~2019~~2020).

~~Among others, 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 those additives 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 aminoacids. 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).~~ The request from consumers of ~~clean labeled~~ foods with "clean label" defined by~~,~~ minimal~~ly~~ process~~ed~~, ~~and with~~ 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; Shah et al., 2014; Shan et al., 2009; Pini et al., 2020). 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 ~~small intestine~~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 ~~intestinal~~ 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; Nissen et al., 2021a; Nissen et al., 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~~2~~2; 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 25-50 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 (Connoly et al, 2012; Nissen 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) (Wang et al, 2020; Nissen et al, 2022). Two biological experiments were performed within one week, employing fresh donations from the same donors.

2.4. In vitro ~~Gut~~ 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 hours in independent vessels using an *in vitro* ~~gut~~ colon model, MICODE (Nissen et al., 2021; Nissen et al., 2021a). The preparation of the experiments was made according to published procedures (Connoly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021a). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connoly et al., 2012; Nissen 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% ~~fecal~~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 et al. (2021a). Sampling was performed as reported in Nissen 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 ~~fecal~~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 17000 × 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 (Tanner et al., 2014; Westfall, Lomis & Prakash, 2018; Tsitko et al., 2019; Nissen et al., 2022a; Tamargo et al., 2022) following previous protocols (Modesto et al., 2011; Nissen et al., 2022; Nissen et al., 2022a). 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 (Nissen et al, 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; 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) (Di Cagno et al., 2011; Nissen et al., 2021; 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. ~~was done to compare sample productions of a single molecule.~~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~~analysed 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) ($p < 0.01$) (Table 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 et al., 2021a). 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 (Figure 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 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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. ~~Beneficial prebiotic~~ 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 hours of fermentation respectively), and the difference was measured and normalized (Figure 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 aminoacids (tyrosine, tryptophan, phenylaniline) fermentation. In particular, Phenol, Phenol, 2-methyl (a.k.a. p-cresol), Indole, and 1H-Indole, 3-methyl (a.k.a. skatole). Starting from the concentrations of these VOCs at the baseline (Table S2), the sample (Figure 3) that generated the ~~highest~~ 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

328 *Akkermansia muciniphila* to describe the commensal beneficial part of the core colon microbiota;
329 and *Enterobacteriaceae*, *Clostridium* group I, *Atopobium-Collinsella-Eggerthella* (ATOP) group,
330 *Escherichia coli* (total), *Escherichia coli* (toxigenic), *Desulfovibrio*. spp., to describe the commensal
331 opportunistic part of the core colon microbiota (Table S1).
332
333 3.3.1. Shift in taxa relative to the core microbiota.
334 Considering the total Eubacteria, with respect to the abundances at the BL and apart from the values
335 of the blank control (BC) (Table 1), the alternative formulations SA and SMA at EP significantly
336 fostered the growth of Eubacteria ($p < 0.05$), alike the commercial control CNO_2, and almost thrice
337 than the negative control CO. The quantifications of *Bacteroidetes* phylum (Table 1) have shown
338 changes principally at EP, when any samples, but SA, had significant differences in respect to BL
339 ($p < 0,05$). In particular, fermentations of CO and CNO_2 triggered a reduction, while that of salami
340 fostered growth. SMA was the best performer, able to significantly increase at the EP the loads of
341 this taxon around 1.7 and 4 times more than SA and CNO_2, respectively. Considering *Firmicutes*
342 (Table 1), significant increases were observed at EP for any sample, but SMA ($p > 0.05$). Although,
343 the surges after fermentation with both the controls were quite the double in respect to those of the
344 alternative formulations. For example, at the end point CNO_2 had level of this taxon at 6.56 E+09 ±
345 1.71 E+09 cells/mL, which was 1.90 times higher than SA. Based on the values of quantifications
346 relative to *Firmicutes* and *Bacteroidetes*, the ratios F/B (Table 1) was calculated to consider
347 changes in the condition of eubiosis defined at the BL. After colonic fermentation, SMA and SA
348 were able to keep the ratio similar to the BL ($p > 0.05$), but the controls were not ($p > 0.05$). In
349 particular, the ratio of CO was higher than 3, indicating a microbiota dysbiosis due to the
350 overrepresentation of *Firmicutes*. So far, the intensity of the capacity to maintain the microbiota
351 eubiosis among the salami tested was: SMA > SA > CNO_2 > CO.
352
353 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 of a healthier alcoholic fermentation, from the volatilome results, the control with no nitrite produced ~~toxic~~ skatole, which is highly toxic for the host intestinal mucosa (Wang et al., 2020)~~, while~~. Considering beneficial fermentation alcohols, their production was similar either by CNO_2 or SMA. Among these alcohols, a robust descriptor is ~~such as~~ 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)~~,~~ ~~were similarly produced either by CNO_2 or SMA.~~ 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.

405 The fermentation metabolite profile of the alternative formulations had a unique signature in the
406 production of ketones, such as Acetylcyclopentanone for SMA and Cyclohexanone, 5-methyl-2-(1 -
407 methylethyl)-, cis- for SA. More than oxidation products, these two VOCs seemed linked to
408 fermentation. These two VOC were also unique signature of fermentation (absent at its beginning)
409 and they were then produced homogeneously at the different time points of the process.
410 Interestingly, Acetylcyclopentanone is capable to protect cell cultures from oxidative stress-induced
411 toxicity and at a very low dosage of being able to prevent lethality in acetaminophen hepatotoxicity
412 mouse model (Zhang et al., 2013).
413 By multivariate analysis, alternative formulations were not discriminated by typical oxidative
414 aldehydes, which were instead specific descriptors of the controls. In particular, CNO_2 marked a
415 unique signature of 2,4-Heptadienal, (E,E)- and, along with CO was described by at least six other
416 oxidative aldehydes, such as Hexanal, Octanal, Nonanal, 2-Nonenal, (E)-, 2-Octenal, (E)-. All these
417 aldehydes are derived from lipid oxidation of food, and in a recent paper, the effect of the vegetal
418 extract was efficaciously tested to be a deterrent of their formation when added to roasted food in a
419 similar in vitro model (Hu et al., 2022). Analogously we could explain the effect of the vegetal
420 extract in SMA. The higher content of oxidative aldehydes in the controls reflects their higher
421 content in fatty acids, as previously reported (Di Nunzio et al., 2022). Also, the action of vegetal
422 extract brought a higher antioxidant potential to salami formulation than the controls, which could
423 have influenced the minor number of oxidative aldehydes.
424 D-Limonene increase after SMA fermentation was probably due to the changes of structure
425 occurring in the fermentation. Indeed, this VOC sourced from plant extracts is delivered in SMA
426 food matrix, increasing its bioaccessibility. The positive impact that this VOC could generate on the
427 host mucosa has been extensively studied, and its retainment and enrichment during colonic
428 fermentation within in vitro model have been similarly assessed in the past (Nissen, Casciano,
429 Valerii, Spisni, Gianotti, 2021). D-Limonene is a bioactive with a potent antioxidant activity
430 (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 aminoacids, 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.

~~As far as~~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 ~~*Bacteroides* spp.~~*E. coli* and *Clostridium* ~~spp~~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 (Wu et al., 2011; Arcidiacono et al., 2022).

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., ~~2019~~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 ~~e~~ 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., 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., ~~2019~~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 ~~has been~~was adopted for opportunistic ~~ones~~ microbes. T~~where t~~he effects of food formulations ~~were~~ are discussed ~~as their ability to~~in terms of ability to limit ~~limit~~ the growth of the selected ~~taxa~~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 sulfate 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 ~~gastro~~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.

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.

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/2018/632065.

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.

Conflicts of Interest: The authors declare no conflicts of interest.

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.

Supplementary Materials: Table S1: Primers pairs employed for PCR and qPCR reactions and quantifications; Table S2: Absolute quantifications of main microbial metabolites related to colonic microbiota at the baseline of colonic fermentation; Table S3: MANOVA categorical descriptors for the volatilome, categorized for the type of matrix; Table S4 MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation.

579 **References**

580 Agus, A., Planchais, J., & Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in
581 health and disease. *Cell Host Microbes, 23,* 716–724.

582 Arcidiacono, T., Macrina, L., Premaschi, S., Bologna, A., Magni, G., Foligno, N., et al. (2022).
583 Serum concentrations of free indoxyl and p-cresyl sulfate are associated with mineral
584 metabolism variables and cardiovascular risk in hemodialysis patients. *Journal of*
585 *Nephrology*, 1-9.

586 Arnal, M. E., Denis, S., Uriot, O., Lambert, C., Holowacz, S., Paul, F., et al. (2021). Impact of oral
587 galenic formulations of *Lactobacillus salivarius* on probiotic survival and interactions with
588 microbiota in human in vitro gut models. *Beneficial microbes*, *12*, 381-396.

589 Blaiotta, G., Murru, N., Cerbo, A. D., Romano, R., & Aponte, M. (2018). Production of probiotic
590 bovine salami using *Lactobacillus plantarum* 299v as adjunct. *Journal of the Science of*
591 *Food and Agriculture, 98(6),* 2285–2294.

592 Bonfrate L., Di Palo, D. M., Celano, G., Albert, A., Vitellio, P., De Angelis, M. et al. (2020).
593 Effects of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 in IBS
594 patients. *European Journal Clinical Investigations*, *50*, e13201.

595 Casciano, F., Nissen, L., & Gianotti, A. (2021). Effect of formulations and fermentation processes
596 on volatile organic compounds and prebiotic potential of gluten-free bread fortified by
597 spirulina (*Arthrospira platensis*). *Food & Function, 12(20),* 10226–10238.

598 Connolly, M. L., Tuohy, K. M., & Lovegrove, J. A. (2012). Wholegrain oat-based cereals have
599 prebiotic potential and low glycaemic index. *British Journal of Nutrition*, *108*, 2198-2206.

600 Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., et al.
601 (2011). Duodenal and faecal microbiota of celiac children: molecular, phenotype and
602 metabolome characterization. *BMC Microbiology*, *11(1)*, 1-21.

603 Di Nunzio, M., Loffi, C., Montalbano, S., Chiarello, E., Dellafiora, L., Picone, G., et al. (2022).
604 Cleaning the Label of Cured Meat; Effect of the Replacement of Nitrates/Nitrites on

Nutrients Bioaccessibility, Peptides Formation, and Cellular Toxicity of In Vitro Digested Salami. *International Journal of Molecular Sciences*, *23(20)*, 12555.

Dos Santos, J. M., Ignácio, E. O., Bis-Souza, C. V., & da Silva-Barretto, A. C. (2021). Performance of reduced fat-reduced salt fermented sausage with added microcrystalline cellulose, resistant starch and oat fiber using the simplex design. *Meat Science*, *175*, 108433.

Guerzoni, E., Vernocchi, P., Ndagijimana, M., Gianotti, A., & Lanciotti, R. (2007). Generation of aroma compounds in sourdough: Effects of stress exposure and lactobacilli-yeasts interactions. *Food Microbiology, 24*, 139-48.

Giello, M., La Storia, A., De Filippis, F., Ercolini, D., & Villani, F. (2018). Impact of *Lactobacillus curvatus* 54M16 on microbiota composition and growth of *Listeria monocytogenes* in fermented sausages. *Food Microbiology, 72*, 1-15.

Hammes, W. P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, *29(2)*, 151-156.

Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2015). Occurrence of volatile and non-volatile N-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, *48*, 163-169.

Hu, Y., Zhao, G., Yin, F., Liu, Z., Wang, J., Qin, L., et al. (2022). Effects of roasting temperature and time on aldehyde formation derived from lipid oxidation in scallop (*Patinopecten yessoensis*) and the deterrent effect by antioxidants of bamboo leaves. *Food Chemistry*, *369*, 130936.

Jiang, J., & Xiong, Y. L. (2016). Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. *Meat science, 120*, 107-117.

Johnson, I. T. (2017). The cancer risk related to meat and meat products. *British medical bulletin, 121(1)*.

629 Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., et
630 al. (2017). Association between body mass index and *Firmicutes/Bacteroidetes* ratio in an
631 adult Ukrainian population. *BMC Microbiology, 17,* 1-6.
632 Koutsos, A., Lima, M., Conterno, L., Gasperotti, M., Bianchi, M., Fava, F., et al. (2017). Effects of
633 commercial apple varieties on human gut microbiota composition and metabolic output
634 using an in vitro colonic model. *Nutrients, 9(6),* 533.
635 Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion
636 for RNA-seq data with DESeq2. *Genome Biology, 15,* 1-21.
637 Majou, D., & Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in
638 cured meats. *Meat Science, 145,* 273-284.
639 Martinez, J., Nieto, G., & Ros, G. (2014). Total antioxidant capacity of meat and meat products
640 consumed in a reference Spanish standard diet. *International Journal of Food Science and*
641 *Technology, 49(12),* 2610–2618.
642 Meurillon, M., & Engel, E. (2016). Mitigation strategies to reduce the impact of heterocyclic
643 aromatic amines in proteinaceous foods. *Trends in Food Science & Technology, 50,* 70-84.
644 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., et al.
645 (2014). A standardised static in vitro digestion method suitable for food—An international
646 consensus. *Food & Function, 5,* 1113–1124.
647 Modesto, M., Stefanini, I., D'Aimmo, M. R., Nissen, L., Tabanelli, D., Mazzoni, M., Bosi, P., et al.
648 (2011). Strategies to augment non-immune system based defence mechanisms against
649 gastrointestinal diseases in pigs. *NJAS - Wagening Journal of Life Sci*ence, *58,* 149-156.
650 Nissen, L., Aniballi, C., Casciano, F., Elmi, A., Ventrella, D., Zannoni, A., Gianotti, A., & Bacci,
651 M. L. (2022a). Maternal amoxicillin affects piglets colon microbiota: microbial ecology and
652 metabolomics in a gut model. *Applied Microbiology & Biotechnology, 106,* 7595–7614.

Nissen, L., Casciano, F., Chiarello, E., Di Nunzio, M., Bordoni, A., & Gianotti, A. (2021). Colonic in vitro model assessment of the prebiotic potential of bread fortified with polyphenols rich olive fiber. *Nutrients*, *13*, 787.

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B., & Cencic, A. (2009). Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: a functional study in the small intestinal cell model. *International Journal of Food Microbiology*, 135(3), 288-294.

Nissen, L., Rollini, M., Picozzi, C., Musatti, A., Foschino, R., & Gianotti, A. (2020). Yeast-Free Doughs by *Zymomonas mobilis*: Evaluation of Technological and Fermentation Performances by Using a Metabolomic Approach. *Microorganisms*, *8(6)*, 792.

Nissen, L., Cattivelli, A., Casciano, F., Gianotti, A., & Tagliazucchi, D. (2022). Roasting and frying modulate the phenolic profile of dark purple eggplant and differently change the colon microbiota and phenolic metabolites after *in vitro* digestion and fermentation in a gut model. *Food Research International*, *160*, 111702.

Nissen, L., Valerii, M. C., Spisni, E., Casciano, F., & Gianotti, A. (2021a). Multiunit in vitro colon model for the evaluation of prebiotic potential of a fiber plus D-limonene food supplement. *Foods*, *10*, 2371.

Oba, S., Sunagawa, T., Tanihiro, R., Awashima, K., Sugiyama, H., Odani, T., ... & Sasaki, K. (2020). Prebiotic effects of yeast mannan, which selectively promotes *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* in a human colonic microbiota model. *Scientific Reports*, *10*, 17351.

Perez-Burillo, S., Mehta, T., Pastoriza, S., Kramer, D. L., Paliy, O., & Rufi´an-Henares, J.´A. (2019). Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure. *Lebensmittel-Wissenschaft & Technologie*, *105*, 355–362.

678 Perez-Burillo, S., Pastoriza, S., Giron´es, A., Avellaneda, A., Pilar Francino, M., & Rufi´an-
679 Henares, J. A. (2020). Potential probiotic salami with dietary fiber modulates metabolism
680 and gut microbiota in a human intervention study. *Journal of Functional Foods, 66,* 103790.
681 Pini, F., Aquilani, C., Giovannetti, L., Viti, C., & Pugliese, C. (2020). Characterization of the
682 microbial community composition in Italian Cinta Senese sausages dry-fermented with
683 natural extracts as alternatives to sodium nitrite. *Food Microbiology*, *89,* 103417.
684 Saccani, G. Bergamaschi, M. Schivazappa, C. Cirlini, M. Galaverna, G. & Virgili, R. (2023).
685 Evaluation of the antioxidant effect of a phytocomplex addition in clean label pork salami
686 enriched in n-3 PUFA. *Food Chemistry, 399,* 133963
687 Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and
688 meat products. *Meat science, 98(1),* 21-33.
689 Shan, B., Cai, Y. Z., Brooks, J. D., & Corke, H. (2009). Antibacterial and antioxidant effects of five
690 spice and herb extracts as natural preservatives of raw pork. *Journal of the Science of Food*
691 *and Agriculture, 89(11),* 1879-1885.
692 Roager, H. M. & Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nature*
693 *Communications, 9,* 3294.
694 Rowan, F., Docherty, N. G., Murphy, M., Murphy, B., Coffey, J. C., & O'Connell, P. R. (2010).
695 *Desulfovibrio* bacterial species are increased in ulcerative colitis. *Diseases of the Colon &*
696 *Rectum, 53(11),* 1530-1536.
697 Tabanelli, G., Barbieri, F., Soglia, F., Magnani, R., Gardini, G., Petracci, M., Gardini, F., &
698 Montanari, C. (2022). Safety and technological issues of dry fermented sausages produced
699 without nitrate and nitrite. *Food Research International, 160,* 111685.
700 Tamargo, A., Cueva, C., Silva, M., Molinero, N., Miralles, B., Bartolomé, B., & Moreno-Arribas,
701 M. V. (2022). Gastrointestinal co-digestion of wine polyphenols with glucose/whey proteins
702 affects their bioaccessibility and impact on colonic microbiota. *Food Research*
703 *International, 111010.*

Taneyo-Saa, D. T., Turroni, S., Serrazanetti, D. I., Rampelli, S., Maccaferri, S., Candela, M., et al. (2014). Impact of Kamut® Khorasan on gut microbiota and metabolome in healthy volunteers. *Food Research International*, *63*, 227-232.

Tanner, S. A., Zihler Berner, A., Rigozzi, E., Grattepanche, F., Chassard, C., & Lacroix, C. (2014). In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for simultaneous testing on the same gut microbiota. *PloS One*, *9(4)*, e94123.

Tsitko, I., Wiik-Miettinen, F., Mattila, O., Rosa-Sibakov, N., Seppänen-Laakso, T., Maukonen, J., et al. (2019). A small in vitro fermentation model for screening the gut microbiota effects of different fiber preparations. *International Journal of Molecular Sciences*, *20*, 1925.

Valerii, M. C., Turroni, S., Ferreri, C., Zaro, M., Sansone, A., Dalpiaz, A., et al. (2021). Effect of a Fiber D-Limonene-Enriched Food Supplement on Intestinal Microbiota and Metabolic Parameters of Mice on a High-Fat Diet. *Pharmaceutics*, *13(11)*, 1753.

Vernocchi, P., Gili, T., Conte, F., Del Chierico, F., Conta, G., Miccheli, A., et al. (2020). Network analysis of gut microbiome and metabolome to discover microbiota-linked biomarkers in patients affected by non-small cell lung cancer. *International Journal of Molecular Sciences*, *21*(22), 8730.

Wang, X., Gibson, G. R., Sailer, M., Theis, S., & Rastall, R. A. (2020). Prebiotics inhibit proteolysis by gut bacteria in a host diet-dependent manner: A three-stage continuous in vitro gut model experiment. *Applied and Environmental Microbiology*, *86*, e02730-19.

Wang, M., Wichienchot, S., He, X., Fu, X., Huang, Q., & Zhang, B. (2019). In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. *Trends in Food Science & Technology*, *88*, 1-9.

Warren, Y. A., Citron, D. M., Merriam, C. V., & Goldstein, E. J. (2005). Biochemical differentiation and comparison of *Desulfovibrio* species and other phenotypically similar genera. *Journal of Clinical Microbiology*, *43(8)*, 4041-4045.

Wassenaar, T. M. (2018). *E. coli* and colorectal cancer: a complex relationship that deserves a critical mindset. *Critical Reviews in Microbiology, 44(5)*, 619-632.

Westfall, S., Lomis, N., & Prakash, S. (2018). A polyphenol-rich prebiotic in combination with a novel probiotic formulation alleviates markers of obesity and diabetes in *Drosophila*. *Journal of Functional Foods*, *48*, 374-386.

Wu, I. W., Hsu, K. H., Lee, C. C., Sun, C. Y., Hsu, H. J., Tsai, C. J., et al. (2011). p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease. *Nephrology Dialysis Transplantation, 26(3)*, 938-947.

Zhang, L., Gavin, T., Geohagen, B. C., Liu, Q., Downey, K. J., & LoPachin, R. M. (2013). Protective properties of 2-acetylcyclopentanone in a mouse model of acetaminophen hepatotoxicity. *Journal of Pharmacology and Experimental Therapeutics*, *346*(2), 259-269.

Zhou, S., Wang, Y., Jacoby, J. J., Jiang, Y., Zhang, Y., & Yu, L. L. (2017). Effects of medium-and long-chain triacylglycerols on lipid metabolism and gut microbiota composition in C57BL/6J mice. *Journal of Agricultural and Food Chemistry*, *65*, 6599-6607.

745 **Figure captions**

746 **Figure 1**. PCAs of the volatilome sorted by chemical classes of significant VOCs (ANOVA $p <$
747 0.05). The original dataset included the biological replicas of SA, SMA, CNO_2, CO, BC, and the
748 baseline (BL) and different time points (T1 = 18 h and EP = 24 h). A) Acids; B) Alcohols; C)
749 Aldehydes; D) Ketones; E) Other aromatic VOCs. Left side diagrams are for PCAs of cases; right
750 side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of
751 the respective group of cases. SA = Salami with ascorbate; SMA = Salami with ascorbate and plant
752 extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate. *Oxalic acid,
753 cyclohexylmethyl = Oxalic acid, cyclohexylmethyl tetradecyl ester; **Cyclohexanol, 5-methyl-2-
754 (1-m ac = Cyclohexanol, 5-methyl-2-(1-methylethyl) acetate; ***Cyanic acid phenyl = Cyanic acid
755 phenyl ester; * Benzoic acid, methyl = Benzoic acid, methyl ester; *Cyclohexanone, 5-methyl-2-(1-
756 m = Cyclohexanone, 5-methyl-2-(1 -methylethyl); *1,2,4- Triazol-4-amine, N-(2-thien = 1,2,4-
757 Triazol-4-amine, N-(2-thienethyl); *Benzene, 1,3-bis(1,1-dim = Benzene, 1,3-bis(1,1-
758 dimethylethyl).

759

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

Figure 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.

797

798 **Tables.**

799 **Table 1.** Quantification and changes of *Eubacteria*, *Bacteroidetes*, *Firmicutes* and the *Firmicutes* to

800 *Bacteroidetes* ratio of colonic fermentation of salami measured by qPCR.

qPCR Targets & Samples	**Quantification cells/mL**	**Changes log_2(F/C)**		**MANOVA (Time)**	
					$-log_{10}(p)$*
	Baseline	**T1 (18 h)**	**EP (24 h)**	*p*	
Eubacteria					
CO	2.77E+10 ± 8.47E+09	0.02	0.58^{AB}	0.810581	0.091203
SA	$2.77E+10 \pm 8.47E+09^{b}$	0.75^{ab}	1.63^{aA}	0.023662	1.625956
SMA	$2.77E+10 \pm 8.47E+09^{b}$	0.87^{ab}	1.38^{aA}	0.009666	2.014750
CNO_2	$2.77E+10 \pm 8.47E+09^{b}$	0.94^{a}	1.44^{aA}	0.019003	1.721188
BC	$2.77E+10 \pm 8.47E+09^{a}$	-0.15^{a}	-1.74^{bB}	0.015644	0.052740
MANOVA (Matrix) *p*		0.107124	0.000950		
Firmicutes					
CO	$2.46E+09 \pm 2.08E+08^{b}$	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	$2.46E+09 \pm 2.08E+08^{b}$	-0.39^{bB}	1.42^{aA}	0.000457	3.339918
BC	$2.46E+09 \pm 2.08E+08^{b}$	0.91^{abAB}	1.28^{aA}	0.000134	3.872407
MANOVA (Matrix) *p*		0.000012	0.002652		
Bacteroidetes					
CO	$4.80E+09 \pm 1.84E+09^{a}$	-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	$4.80E+09 \pm 1.84E+09^{b}$	0.11^{b}	1.38^{aA}	0.005644	2.248411
CNO_2	$4.80E+09 \pm 1.84E+09^{a}$	-0.11^{ab}	-0.62^{bB}	0.000008	5.071174
BC	$4.80E+09 \pm 1.84E+09^{a}$	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.

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
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 ~~*Different capital letters indicate significance difference within a column; 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~~-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.

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 ~~*This taxon was amplified by targeting cell division protein (FtsZ) rRNA; **This taxon was amplified by targeting cytolethal distending toxin (CDT IV) rRNA;~~ 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.

Figure 1.

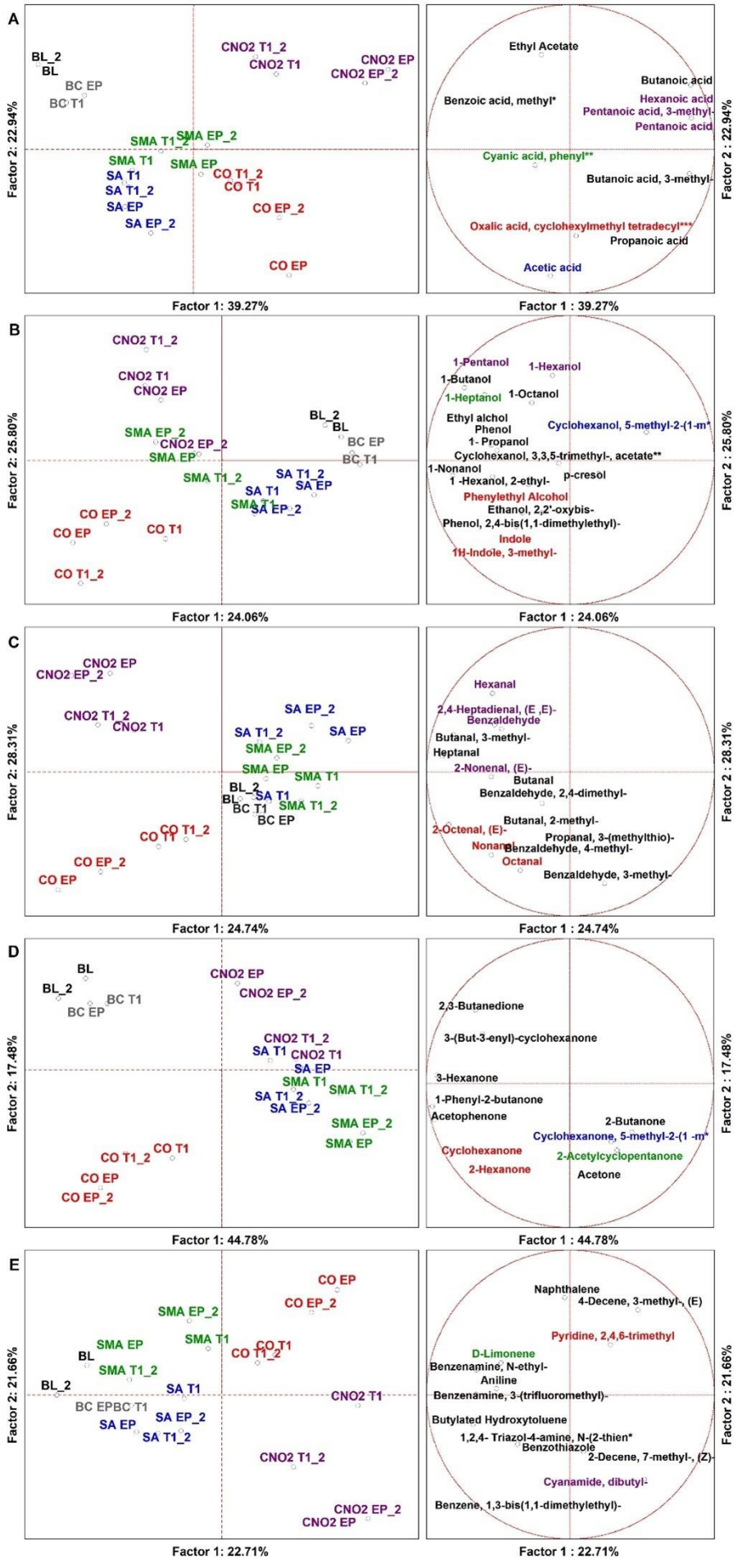
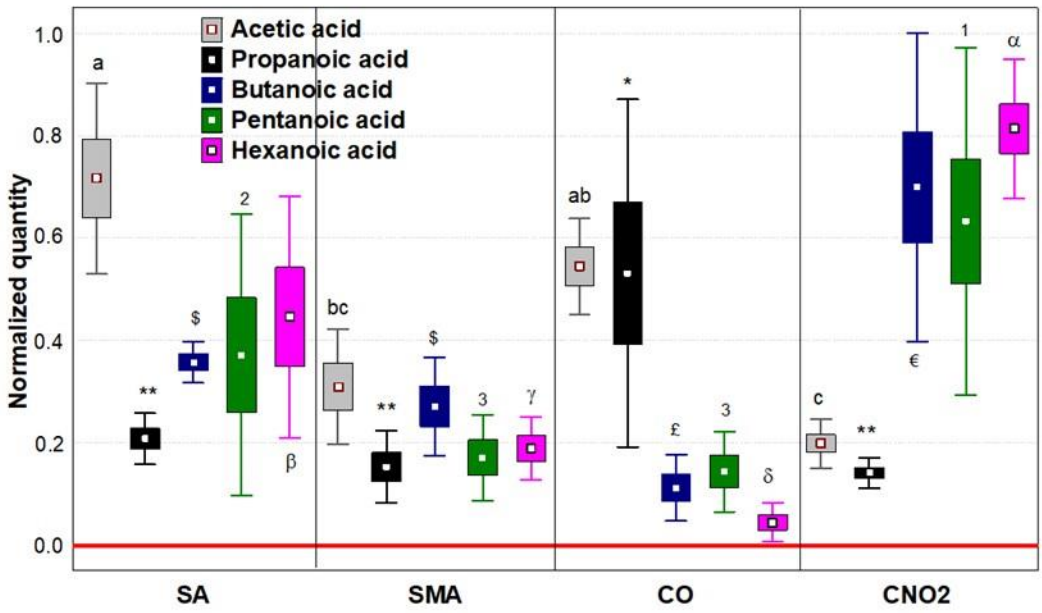
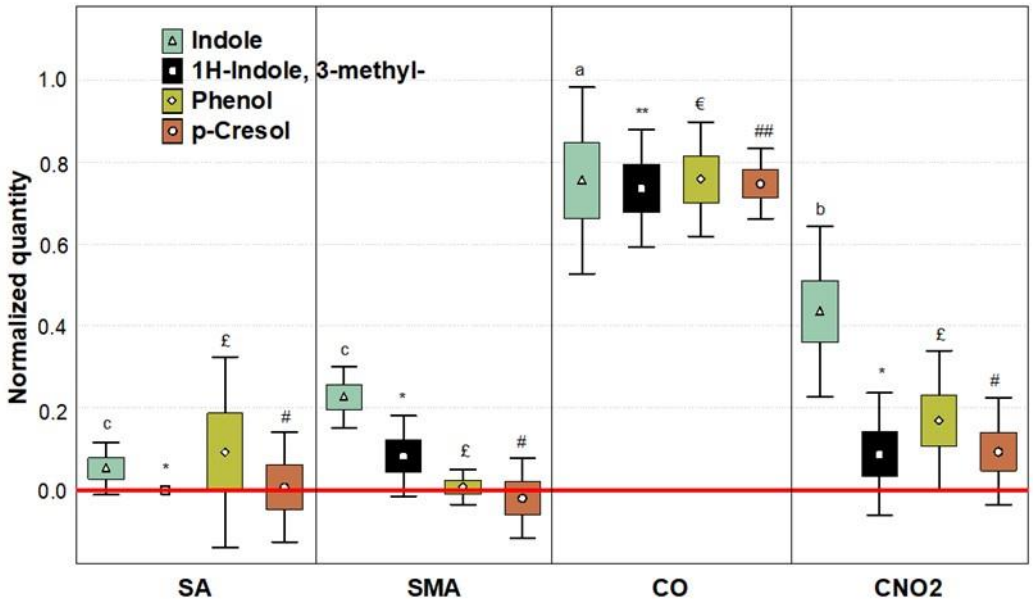

A
BL_2
BL
BC EP
BC T1
CNO2 T1_2
CNO2 T1
CNO2 EP
CNO2 EP_2
SMA T1_2
SMA EP_2
SMA T1
SMA EP
SA T1
SA T1_2
SA EP
SA EP_2
CO T1_2
CO T1
CO EP_2
CO EP
Factor 2: 22.94%
Factor 1: 39.27%
Ethyl Acetate
Butanoic acid
Benzoic acid, methyl*
Hexanoic acid
Pentanoic acid, 3-methyl-
Pentanoic acid
Cyanic acid, phenyl**
Butanoic acid, 3-methyl-
Oxalic acid, cyclohexylmethyl tetradecyl***
Propanoic acid
Acetic acid
Factor 2 : 22.94%
Factor 1 : 39.27%
B
CNO2 T1_2
CNO2 T1
CNO2 EP
BL_2
BL
BC EP
BC T1
SMA EP_2
CNO2 EP_2
SMA EP
SMA T1_2
SA T1_2
SA EP
SA T1
SMA T1
SA EP_2
CO EP_2
CO EP
CO T1
CO T1_2
Factor 2: 25.80%
Factor 1: 24.06%
1-Pentanol
1-Hexanol
1-Butanol
1-Heptanol
1-Octanol
Ethyl alchol
Phenol
Cyclohexanol, 5-methyl-2-(1-m*
1- Propanol
Cyclohexanol, 3,3,5-trimethyl-, acetate**
1-Nonanol
1 -Hexanol, 2-ethyl-
p-cresol
Phenylethyl Alcohol
Ethanol, 2,2'-oxybis-
Phenol, 2,4-bis(1,1-dimethylethyl)-
Indole
1H-Indole, 3-methyl-
Factor 2 : 25.80%
Factor 1 : 24.06%
C
CNO2 EP
CNO2 EP_2
CNO2 T1_2
CNO2 T1
SA EP_2
SA T1_2
SA EP
SMA EP_2
SMA EP
SMA T1
BL_2
BL
SA T1
BC T1
SMA T1_2
BC EP
CO T1_2
CO T1
CO EP_2
CO EP
Factor 2: 28.31%
Factor 1: 24.74%
Hexanal
2,4-Heptadienal, (E ,E)-
Benzaldehyde
Butanal, 3-methyl-
Heptanal
2-Nonenal, (E)-
Butanal
Benzaldehyde, 2,4-dimethyl-
Butanal, 2-methyl-
2-Octenal, (E)-
Propanal, 3-(methylthio)-
Nonanal
Benzaldehyde, 4-methyl-
Octanal
Benzaldehyde, 3-methyl-
Factor 2 : 28.31%
Factor 1 : 24.74%
D
BL
BL_2
BC T1
BC EP
CNO2 EP
CNO2 EP_2
CNO2 T1_2
SA T1
CNO2 T1
SA EP
SMA T1
SMA T1_2
SA T1_2
SA EP_2
SMA EP_2
SMA EP
CO T1_2
CO T1
CO EP
CO EP_2
Factor 2: 17.48%
Factor 1: 44.78%
2,3-Butanedione
3-(But-3-enyl)-cyclohexanone
3-Hexanone
1-Phenyl-2-butanone
Acetophenone
2-Butanone
Cyclohexanone, 5-methyl-2-(1 -m*
Cyclohexanone
2-Acetylcyclopentanone
2-Hexanone
Acetone
Factor 2 : 17.48%
Factor 1 : 44.78%
E
CO EP
CO EP_2
SMA EP_2
SMA EP
SMA T1
CO T1
CO T1_2
BL
SMA T1_2
BL_2
SA T1
CNO2 T1
BC EPBC T1
SA EP
SA EP_2
SA T1_2
CNO2 T1_2
CNO2 EP_2
CNO2 EP
Factor 2: 21.66%
Factor 1: 22.71%
Naphthalene
4-Decene, 3-methyl-, (E)
Pyridine, 2,4,6-trimethyl
D-Limonene
Benzenamine, N-ethyl-
Aniline
Benzenamine, 3-(trifluoromethyl)-
Butylated Hydroxytoluene
1,2,4- Triazol-4-amine, N-(2-thien*
Benzothiazole
2-Decene, 7-methyl-, (Z)-
Cyanamide, dibutyl-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Factor 2 : 21.66%
Factor 1 : 22.71%

Figure 2.


Acetic acid
Propanoic acid
Butanoic acid
Pentanoic acid
Hexanoic acid
Normalized quantity
1.0
0.8
0.6
0.4
0.2
0.0
a
**
$
2
β
bc
**
$
3
γ
ab
*
£
3
δ
c
**
€
1
α
SA
SMA
CO
CNO2

866 **Figure 3.**



867

Effects of the replacement of Nitrates/Nitrites in salami by plant extracts on colon microbiota.

Lorenzo Nissen[1,2,3,*], Flavia Casciano[1,3], Mattia Di Nunzio[4], Gianni Galaverna[5], Alessandra Bordoni[1,2] and Andrea Gianotti[1,2,3*].

[1]DiSTAL - Department of Agricultural and Food Sciences, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[2]CIRI - Interdepartmental Centre of Agri-Food Industrial Research, *Alma Mater Studiorum* – University of Bologna, P.za G. Goidanich, 60, 47521 Cesena, Italy.

[3]CRBA, Center for Biomedical Applied Research, *Alma Mater Studiorum* – University of Bologna, Policlinico di Sant'Orsola, via Massarenti 9, 40138, Bologna, Italy.

[4]DeFENS, Department of Food, Environmental and Nutritional Sciences, University of Milan, via Celoria 2, 20133 Milan, Italy.

[5]Department of Food and Drugs, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy.

*Corresponding authors: andrea.gianotti@unibo.it +39 0547338134

and lorenzo.nissen@unibo.it +39 0547338146

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.

Keywords: processed meat; nitrates; gut microbiota; colon fermentation; proteolysis; clean-label.

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 aminoacids. 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 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; Shah et al., 2014; Shan et al., 2009; Pini et al., 2020). 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; Nissen et al., 2021a; Nissen et al., 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 25-50 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 (Connoly et al, 2012; Nissen 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) (Wang et al, 2020; Nissen et al, 2022). 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 hours in independent vessels using an *in vitro* colon model, MICODE (Nissen et al., 2021; Nissen et al., 2021a). The preparation of the experiments was made according to published procedures (Connoly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021a). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connoly et al., 2012; Nissen 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 et al. (2021a). Sampling was performed as reported in Nissen 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 17000 × 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 (Tanner et al., 2014; Westfall, Lomis & Prakash, 2018; Tsitko et al., 2019; Nissen et al., 2022a; Tamargo et al., 2022) following previous protocols (Modesto et al., 2011; Nissen et al., 2022; Nissen et al., 2022a). 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 (Nissen et al, 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; 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) (Di Cagno et al., 2011; Nissen et al., 2021; 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 analysed 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) ($p < 0.01$) (Table 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 et al., 2021a). 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 (Figure 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 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 (Figure 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 (Figure 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).

252

253 3.1.4. Ketones.

254 A PCA of 11 statistically significant ketones distributed cases on the plot, separating the BL from
255 time points of fermentation of any substrates but not of the BC. Also, CNO_2 and CO were
256 discriminated from each other, but CNO_2 was partially separated from the alternative formulations.
257 Moreover, SA and SMA did not discriminate much one to each other but had their specific
258 descriptor (Figure 1D). CNO_2 did not have any specific descriptor. The ketones that described CO
259 were 2-Hexanone and Cyclohexanone (around 87% and 56% of contribution on total production) (p
260 < 0.05) (Table S3); the former was produced at the EP (100%) ($p > 0.05$), but the latter was also
261 ascribed to be already present at the BL (around 43%) ($p < 0.05$) (Table S4). The descriptors of the
262 alternative formulations were instead Acetylcyclopentanone for SMA (around 67%) ($p < 0.05$) and
263 Cyclohexanone, 5-methyl-2-(1 -methylethyl)-, cis- for SA (around 39%) (Table S3), that were both
264 absent at the BL and whose production was spread over the process either at T1 or EP ($p < 0.05$)
265 (Table S4).

266

267 3.1.5. Amines and alkenes.

268 A PCA of 13 statistically significant aromatic VOCs not previously sorted (accounting mainly for
269 amines and alkenes) distributed cases on the plot, separating the BL from time points of
270 fermentation of any substrates but not of the BC. Also, CNO_2 and CO were discriminated from each
271 other, but CNO_2 was partially separated from the alternative formulations. Lastly, SA and SMA did
272 not discriminate much one to each other, and only SMA had a specific descriptor (Figure 1E). The
273 VOCs that defined the controls were Cyanamide dibutyl for CNO_2 (around 48.9%) and Pyridine,
274 2,4,6-trimethyl for CO (around 94%) ($p < 0.05$) (Table S3). The former was absent at BL and was
275 produced for the most at T1 (around 74%) while the latter accounted to contribute for around 12%
276 at BL and around 84% at EP (Table S4). Interestingly, even considering chemical bias due to the
277 high volatility of D-Limonene, this bioactive was a unique descriptor of SMA fermentation. In this

278 case, D-Limonene enrichment was achieved during the whole process (around 33% and 49% at T1
279 and EP, respectively), starting from an initial amount (around 18%) ($p < 0.05$) (Table S4).
280
281 *3.2. Shift of microbial VOCs with functional properties.*
282 For their beneficial role, more specific attention was addressed to SCFAs (short chain fatty acids)
283 and MCFAs (medium chain fatty acids) (from fibrinolytic and saccharolytic metabolism). On the
284 other hand, some VOCs (mainly coming from proteolytic metabolism) were considered detrimental.
285 Indeed, they are in general related to proteolytic fermentation and are harmful to the mucosa and the
286 host and thereafter negatively correlated to prebiotic potential of foods.
287
288 3.2.1. VOCs related to beneficial and prebiotic effect.
289 Three short chain and two medium chain organic acids were considered: acetic, propanoic,
290 butanoic, pentanoic, and hexanoic. The absolute quantifications at the baseline (Table S2) were
291 compared to that at the two time points, T1 and EP (18 and 24 hours of fermentation respectively),
292 and the difference was measured and normalized (Figure 2). Considering the shift of fermentations
293 compared to the BL, the results of the recipient analyses demonstrated that any fermentation tested
294 produced low molecular organic acids. In particular, the control sample with nitrites (CNO_2)
295 generated the best fermentation outputs. Among the alternative formulation, SA fermentation
296 produced almost 7 times more acetic and 4 times more either pentanoic or hexanoic acids, than the
297 BL, while fermentation of SMA produced few amounts of the five VOCs. So far, the trend in
298 beneficial postbiotic VOCs production was CNO_2 > SA > SMA > CO.
299
300 3.2.2. Detrimental VOCs.
301 The fermentation of any salami has produced potentially detrimental VOCs derived from lipid
302 oxidation and aminoacids (tyrosine, tryptophan, phenylaniline) fermentation. In particular, Phenol,
303 Phenol, 2-methyl (a.k.a. p-cresol), Indole, and 1H-Indole, 3-methyl (a.k.a. skatole). Starting from

304 the concentrations of these VOCs at the baseline (Table S2), the sample (Figure 3) that generated
305 the top amount was the control with no nitrite (CO). In respect to this control, SA, SMA and CNO_2
306 produced similar overall amounts of any compounds ($p > 0.05$), except Indole ($p < 0.05$) and
307 skatole (not detected in SA), approximately 6 times less than CO ($p < 0.05$). The trend of
308 production of these VOCs was: CO > CNO_2 > SMA > SA.
309
310 *3.3. Microbiota analyses of colonic fermentations.*
311 qPCR absolute quantifications were targeted to 16 different bacterial taxa related to the core
312 microbiota of the human colon, including total Eubacteria, *Bacteroidetes* and *Firmicutes* to describe
313 the large picture; *Lactobacillales*, *Bifidobacteriaceae*, *Clostridium* group IV, *Bifidobacterium*
314 *longum*, *Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Faecalibacterium prausnitzii*, and
315 *Akkermansia muciniphila* to describe the commensal beneficial part of the core colon microbiota;
316 and *Enterobacteriaceae*, *Clostridium* group I, *Atopobium-Collinsella-Eggerthella* (ATOP) group,
317 *Escherichia coli* (total), *Escherichia coli* (toxigenic), *Desulfovibrio*. spp., to describe the commensal
318 opportunistic part of the core colon microbiota (Table S1).
319
320 3.3.1. Shift in taxa relative to the core microbiota.
321 Considering the total Eubacteria, with respect to the abundances at the BL and apart from the values
322 of the blank control (BC) (Table 1), the alternative formulations SA and SMA at EP significantly
323 fostered the growth of Eubacteria ($p < 0.05$), alike the commercial control CNO_2, and almost thrice
324 than the negative control CO. The quantifications of *Bacteroidetes* phylum (Table 1) have shown
325 changes principally at EP, when any samples, but SA, had significant differences in respect to BL
326 ($p < 0,05$). In particular, fermentations of CO and CNO_2 triggered a reduction, while that of salami
327 fostered growth. SMA was the best performer, able to significantly increase at the EP the loads of
328 this taxon around 1.7 and 4 times more than SA and CNO_2, respectively. Considering *Firmicutes*
329 (Table 1), significant increases were observed at EP for any sample, but SMA ($p > 0.05$). Although,

330 the surges after fermentation with both the controls were quite the double in respect to those of the
331 alternative formulations. For example, at the end point CNO_2 had level of this taxon at 6.56 E+09 ±
332 1.71 E+09 cells/mL, which was 1.90 times higher than SA. Based on the values of quantifications
333 relative to *Firmicutes* and *Bacteroidetes*, the ratios F/B (Table 1) was calculated to consider
334 changes in the condition of eubiosis defined at the BL. After colonic fermentation, SMA and SA
335 were able to keep the ratio similar to the BL ($p > 0.05$), but the controls were not ($p > 0.05$). In
336 particular, the ratio of CO was higher than 3, indicating a microbiota dysbiosis due to the
337 overrepresentation of *Firmicutes*. So far, the intensity of the capacity to maintain the microbiota
338 eubiosis among the salami tested was: SMA > SA > CNO_2 > CO.
339
340 3.3.2. Commensals Beneficial taxa.
341 Amongst the beneficial bacteria (Table 2) that were targeted, *Lactobacillales* and
342 *Bifidobacteriaceae* had different trend during fermentation. The former taxon increased after
343 fermentation with any substrate but significantly just for SA ($p < 0.05$), while the latter increased
344 significantly just for SA and SMA and decreased significantly for CNO_2 ($p < 0.05$). After SA
345 fermentation, the EP *Lactobacillales* loads was 2.10E+07 ± 3.92E+06 cells/mL, more than CNO_2.
346 After SMA fermentations, at the EP *Bifidobacteriaceae* accounted for 8.63E+08 ± 2.97E+08
347 cells/mL, 7.3 times more than CNO_2. Within this family, *B. longum* was particularly affected by the
348 controls recording dramatic losses after their fermentations but surged significantly ($p < 0.05$) and
349 similarly ($p > 0.05$) in abundances after SA and SMA fermentations (Table 2). Between
350 *Clostridiales*, the *Clostridium* group IV and the recipient *Faecalibacterium prausnitzii* were
351 underrepresented after any fermentation, but SMA, that anyhow scored no shift in respect to the BL
352 ($p > 0.05$). The group BPP (*Bacteroides-Prevotella-Porphyromonas)*, which mainly targets the
353 *Bacteroides* genus, increased significantly at the EP just for SMA ($p < 0.05$), as we previously have
354 observed at the phylum level. Lastly, *Akkermansia muciniphila* was not fostered by any substrates.
355

356 3.3.3. Commensals Opportunistic taxa.

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

373

374 **4. Discussion.**

375 *4.1. Volatilome.*

376 Considering the VOCs grouped as organic acids, the role of short and medium chain organic acids
377 is explained in section 4.2 by the shift observed later as absolute quantifications, but it is out of that
378 discussion the role of branched chain organic acids. From the results of the volatilome, the
379 alternative formulations with no nitrates/nitrites did not contribute to their production, but the
380 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 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 aminoacids, 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 (Wu et al., 2011; Arcidiacono et al., 2022).

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., 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.

485 4.3.2. Commensals opportunistic taxa.

486 Analogously to beneficial taxa, a similar approach was adopted for opportunistic microbes. The
487 effects of food formulations are discussed in terms of ability to limit the growth of the selected
488 opportunistic microbes. From our results, SA and SMA always limited more the growth of
489 opportunistic in comparison to the controls. SA was more potent than SMA because these taxa grew
490 less in four out of six cases. SMA was able to reduce the content of potentially toxigenic *E. coli*,
491 although the ANOVA model was not significant. Toxigenic *E. coli* harbors cyclomodulins, like
492 CDT (Cytholethal Distending Toxin), which take parts in the onset of colorectal cancer (Wassenaar,
493 2018). In contrast to our findings, in a recent paper done with similar methodologies but a different
494 in vitro model, the authors found that adding citrus fibers to salami also reduced the prevalence of
495 *Escherichia*/*Shigella* group (Perez-Burillo et al., 2020).
496 From our results, SA was also able to reduce the content of *Desulfovibrio*, that is a sulfurate-reducer
497 genus able to affect and shrink the mucin barrier and thereof the integral structure of the colon
498 mucosa by production of dimethyl sulfate and also inducer of colitis (Rowan et al., 2010), was
499 reduced by fermentation with SA. This feature gives evidence that the absence of nitrite in
500 formulation, which uses to be a substrate for this harmful taxon (Warren et al., 2005), results in its
501 containment and reduction due principally to the inhibitory and antioxidant action of ascorbate.
502 Lastly, the results of the ecological competition between *Enterobacteriaceae* and
503 *Bifidobacteriaceae* is an index of bifidogenic capacity of the substrates unveiling possible prebiotic
504 features. The *Bifidobacteriaceae* competition had shown to be higher when the alternative
505 formulations were fermented in respect to the controls. In this context, SMA was more potent than
506 SA; maybe because, even if ascorbate of SA can limit more *Enterobacteriaceae* than SMA, the
507 plant extract of SMA induced a higher growth of *Bifidobacteriaceae*, as it is reported that vegetal
508 fibers use to foster this health-related family (Wang et al., 2019).
509

510 **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.

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.

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/2018/632065.

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.

Conflicts of Interest: The authors declare no conflicts of interest.

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.

Supplementary Materials: Table S1: Primers pairs employed for PCR and qPCR reactions and quantifications; Table S2: Absolute quantifications of main microbial metabolites related to colonic microbiota at the baseline of colonic fermentation; Table S3: MANOVA categorical descriptors for

561 the volatilome, categorized for the type of matrix; Table S4 MANOVA categorical descriptors for
562 the volatilome, categorized for the time of fermentation.
563

References

Agus, A., Planchais, J., & Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbes, 23,* 716–724.

Arcidiacono, T., Macrina, L., Premaschi, S., Bologna, A., Magni, G., Foligno, N., et al. (2022). Serum concentrations of free indoxyl and p-cresyl sulfate are associated with mineral metabolism variables and cardiovascular risk in hemodialysis patients. *Journal of Nephrology*, 1-9.

Arnal, M. E., Denis, S., Uriot, O., Lambert, C., Holowacz, S., Paul, F., et al. (2021). Impact of oral galenic formulations of *Lactobacillus salivarius* on probiotic survival and interactions with microbiota in human in vitro gut models. *Beneficial microbes*, *12,* 381-396.

Blaiotta, G., Murru, N., Cerbo, A. D., Romano, R., & Aponte, M. (2018). Production of probiotic bovine salami using *Lactobacillus plantarum* 299v as adjunct. *Journal of the Science of Food and Agriculture, 98(6),* 2285–2294.

Bonfrate L., Di Palo, D. M., Celano, G., Albert, A., Vitellio, P., De Angelis, M. et al. (2020). Effects of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 in IBS patients. *European Journal Clinical Investigations*, *50,* e13201.

Casciano, F., Nissen, L., & Gianotti, A. (2021). Effect of formulations and fermentation processes on volatile organic compounds and prebiotic potential of gluten-free bread fortified by spirulina (*Arthrospira platensis*). *Food & Function, 12(20),* 10226–10238.

Connolly, M. L., Tuohy, K. M., & Lovegrove, J. A. (2012). Wholegrain oat-based cereals have prebiotic potential and low glycaemic index. *British Journal of Nutrition*, *108,* 2198-2206.

Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., et al. (2011). Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. *BMC Microbiology*, *11(1),* 1-21.

Di Nunzio, M., Loffi, C., Montalbano, S., Chiarello, E., Dellafiora, L., Picone, G., et al. (2022). Cleaning the Label of Cured Meat; Effect of the Replacement of Nitrates/Nitrites on

590 Nutrients Bioaccessibility, Peptides Formation, and Cellular Toxicity of In Vitro Digested
591 Salami. *International Journal of Molecular Sciences*, *23(20)*, 12555.
592 Dos Santos, J. M., Ignácio, E. O., Bis-Souza, C. V., & da Silva-Barretto, A. C. (2021). Performance
593 of reduced fat-reduced salt fermented sausage with added microcrystalline cellulose,
594 resistant starch and oat fiber using the simplex design. *Meat Science*, *175*, 108433.
595 Guerzoni, E., Vernocchi, P., Ndagijimana, M., Gianotti, A., & Lanciotti, R. (2007). Generation of
596 aroma compounds in sourdough: Effects of stress exposure and lactobacilli-yeasts
597 interactions. *Food Microbiology, 24*, 139-48.
598 Giello, M., La Storia, A., De Filippis, F., Ercolini, D., & Villani, F. (2018). Impact of *Lactobacillus*
599 *curvatus* 54M16 on microbiota composition and growth of *Listeria monocytogenes* in
600 fermented sausages. *Food Microbiology*, *72*, 1-15.
601 Hammes, W. P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a
602 specific group of fermented foods. *Food Microbiology*, *29(2)*, 151-156.
603 Herrmann, S. S., Duedahl-Olesen, L., & Granby, K. (2015). Occurrence of volatile and non-volatile
604 N-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, *48*,
605 163-169.
606 Hu, Y., Zhao, G., Yin, F., Liu, Z., Wang, J., Qin, L., et al. (2022). Effects of roasting temperature
607 and time on aldehyde formation derived from lipid oxidation in scallop (*Patinopecten*
608 *yessoensis*) and the deterrent effect by antioxidants of bamboo leaves. *Food Chemistry*, *369*,
609 130936.
610 Jiang, J., & Xiong, Y. L. (2016). Natural antioxidants as food and feed additives to promote health
611 benefits and quality of meat products: A review. *Meat science, 120*, 107-117.
612 Johnson, I. T. (2017). The cancer risk related to meat and meat products. *British medical*
613 *bulletin, 121(1)*.

614 Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., et
615 al. (2017). Association between body mass index and *Firmicutes/Bacteroidetes* ratio in an
616 adult Ukrainian population. *BMC Microbiology, 17,* 1-6.
617 Koutsos, A., Lima, M., Conterno, L., Gasperotti, M., Bianchi, M., Fava, F., et al. (2017). Effects of
618 commercial apple varieties on human gut microbiota composition and metabolic output
619 using an in vitro colonic model. *Nutrients, 9(6),* 533.
620 Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion
621 for RNA-seq data with DESeq2. *Genome Biology, 15,* 1-21.
622 Majou, D., & Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in
623 cured meats. *Meat Science, 145,* 273-284.
624 Martinez, J., Nieto, G., & Ros, G. (2014). Total antioxidant capacity of meat and meat products
625 consumed in a reference Spanish standard diet. *International Journal of Food Science and*
626 *Technology, 49(12),* 2610–2618.
627 Meurillon, M., & Engel, E. (2016). Mitigation strategies to reduce the impact of heterocyclic
628 aromatic amines in proteinaceous foods. *Trends in Food Science & Technology, 50,* 70-84.
629 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., et al.
630 (2014). A standardised static in vitro digestion method suitable for food—An international
631 consensus. *Food & Function, 5,* 1113–1124.
632 Modesto, M., Stefanini, I., D'Aimmo, M. R., Nissen, L., Tabanelli, D., Mazzoni, M., Bosi, P., et al.
633 (2011). Strategies to augment non-immune system based defence mechanisms against
634 gastrointestinal diseases in pigs. *NJAS - Wagening Journal of Life Sci*ence, *58,* 149-156.
635 Nissen, L., Aniballi, C., Casciano, F., Elmi, A., Ventrella, D., Zannoni, A., Gianotti, A., & Bacci,
636 M. L. (2022a). Maternal amoxicillin affects piglets colon microbiota: microbial ecology and
637 metabolomics in a gut model. *Applied Microbiology & Biotechnology, 106,* 7595–7614.

638 Nissen, L., Casciano, F., Chiarello, E., Di Nunzio, M., Bordoni, A., & Gianotti, A. (2021). Colonic
639 in vitro model assessment of the prebiotic potential of bread fortified with polyphenols rich
640 olive fiber. *Nutrients*, *13*, 787.
641 Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B., & Cencic, A. (2009). Gut health promoting
642 activity of new putative probiotic/protective *Lactobacillus* spp. strains: a functional study in
643 the small intestinal cell model. *International Journal of Food Microbiology*, 135(3), 288-
644 294.
645 Nissen, L., Rollini, M., Picozzi, C., Musatti, A., Foschino, R., & Gianotti, A. (2020). Yeast-Free
646 Doughs by *Zymomonas mobilis*: Evaluation of Technological and Fermentation
647 Performances by Using a Metabolomic Approach. *Microorganisms*, *8(6)*, 792.
648 Nissen, L., Cattivelli, A., Casciano, F., Gianotti, A., & Tagliazucchi, D. (2022). Roasting and frying
649 modulate the phenolic profile of dark purple eggplant and differently change the colon
650 microbiota and phenolic metabolites after *in vitro* digestion and fermentation in a gut
651 model. *Food Research International*, *160*, 111702.
652 Nissen, L., Valerii, M. C., Spisni, E., Casciano, F., & Gianotti, A. (2021a). Multiunit in vitro colon
653 model for the evaluation of prebiotic potential of a fiber plus D-limonene food supplement.
654 *Foods*, *10*, 2371.
655 Oba, S., Sunagawa, T., Tanihiro, R., Awashima, K., Sugiyama, H., Odani, T., ... & Sasaki, K.
656 (2020). Prebiotic effects of yeast mannan, which selectively promotes *Bacteroides*
657 *thetaiotaomicron* and *Bacteroides ovatus* in a human colonic microbiota model. *Scientific*
658 *Reports*, *10*, 17351.
659 Perez-Burillo, S., Mehta, T., Pastoriza, S., Kramer, D. L., Paliy, O., & Rufi´an-Henares, J.´A.
660 (2019). Potential probiotic salami with dietary fiber modulates antioxidant capacity, short
661 chain fatty acid production and gut microbiota community structure. *Lebensmittel-*
662 *Wissenschaft & Technologie*, *105*, 355–362.

663 Perez-Burillo, S., Pastoriza, S., Giron´es, A., Avellaneda, A., Pilar Francino, M., & Rufi´an-
664 Henares, J. A. (2020). Potential probiotic salami with dietary fiber modulates metabolism
665 and gut microbiota in a human intervention study. *Journal of Functional Foods, 66,* 103790.
666 Pini, F., Aquilani, C., Giovannetti, L., Viti, C., & Pugliese, C. (2020). Characterization of the
667 microbial community composition in Italian Cinta Senese sausages dry-fermented with
668 natural extracts as alternatives to sodium nitrite. *Food Microbiology*, *89,* 103417.
669 Saccani, G. Bergamaschi, M. Schivazappa, C. Cirlini, M. Galaverna, G. & Virgili, R. (2023).
670 Evaluation of the antioxidant effect of a phytocomplex addition in clean label pork salami
671 enriched in n-3 PUFA. *Food Chemistry, 399,* 133963
672 Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and
673 meat products. *Meat science, 98(1),* 21-33.
674 Shan, B., Cai, Y. Z., Brooks, J. D., & Corke, H. (2009). Antibacterial and antioxidant effects of five
675 spice and herb extracts as natural preservatives of raw pork. *Journal of the Science of Food*
676 *and Agriculture, 89(11),* 1879-1885.
677 Roager, H. M. & Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nature*
678 *Communications, 9,* 3294.
679 Rowan, F., Docherty, N. G., Murphy, M., Murphy, B., Coffey, J. C., & O'Connell, P. R. (2010).
680 *Desulfovibrio* bacterial species are increased in ulcerative colitis. *Diseases of the Colon &*
681 *Rectum, 53(11),* 1530-1536.
682 Tabanelli, G., Barbieri, F., Soglia, F., Magnani, R., Gardini, G., Petracci, M., Gardini, F., &
683 Montanari, C. (2022). Safety and technological issues of dry fermented sausages produced
684 without nitrate and nitrite. *Food Research International, 160,* 111685.
685 Tamargo, A., Cueva, C., Silva, M., Molinero, N., Miralles, B., Bartolomé, B., & Moreno-Arribas,
686 M. V. (2022). Gastrointestinal co-digestion of wine polyphenols with glucose/whey proteins
687 affects their bioaccessibility and impact on colonic microbiota. *Food Research*
688 *International, 111010.*

689 Taneyo-Saa, D. T., Turroni, S., Serrazanetti, D. I., Rampelli, S., Maccaferri, S., Candela, M., et al.
690 (2014). Impact of Kamut® Khorasan on gut microbiota and metabolome in healthy
691 volunteers. *Food Research International*, *63*, 227-232.
692 Tanner, S. A., Zihler Berner, A., Rigozzi, E., Grattepanche, F., Chassard, C., & Lacroix, C. (2014).
693 In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for
694 simultaneous testing on the same gut microbiota. *PloS One*, *9(4)*, e94123.
695 Tsitko, I., Wiik-Miettinen, F., Mattila, O., Rosa-Sibakov, N., Seppänen-Laakso, T., Maukonen, J.,
696 et al. (2019). A small in vitro fermentation model for screening the gut microbiota effects of
697 different fiber preparations. *International Journal of Molecular Sciences*, *20*, 1925.
698 Valerii, M. C., Turroni, S., Ferreri, C., Zaro, M., Sansone, A., Dalpiaz, A., et al. (2021). Effect of a
699 Fiber D-Limonene-Enriched Food Supplement on Intestinal Microbiota and Metabolic
700 Parameters of Mice on a High-Fat Diet. *Pharmaceutics*, *13(11)*, 1753.
701 Vernocchi, P., Gili, T., Conte, F., Del Chierico, F., Conta, G., Miccheli, A., et al. (2020). Network
702 analysis of gut microbiome and metabolome to discover microbiota-linked biomarkers in
703 patients affected by non-small cell lung cancer. *International Journal of Molecular*
704 *Sciences*, *21*(22), 8730.
705 Wang, X., Gibson, G. R., Sailer, M., Theis, S., & Rastall, R. A. (2020). Prebiotics inhibit
706 proteolysis by gut bacteria in a host diet-dependent manner: A three-stage continuous in
707 vitro gut model experiment. *Applied and Environmental Microbiology*, *86*, e02730-19.
708 Wang, M., Wichienchot, S., He, X., Fu, X., Huang, Q., & Zhang, B. (2019). In vitro colonic
709 fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and
710 changes in microbiota. *Trends in Food Science & Technology*, *88*, 1-9.
711 Warren, Y. A., Citron, D. M., Merriam, C. V., & Goldstein, E. J. (2005). Biochemical
712 differentiation and comparison of *Desulfovibrio* species and other phenotypically similar
713 genera. *Journal of Clinical Microbiology*, *43(8)*, 4041-4045.

714 Wassenaar, T. M. (2018). *E. coli* and colorectal cancer: a complex relationship that deserves a
715 critical mindset. *Critical Reviews in Microbiology*, *44(5)*, 619-632.
716 Westfall, S., Lomis, N., & Prakash, S. (2018). A polyphenol-rich prebiotic in combination with a
717 novel probiotic formulation alleviates markers of obesity and diabetes in *Drosophila*.
718 *Journal of Functional Foods*, *48*, 374-386.
719 Wu, I. W., Hsu, K. H., Lee, C. C., Sun, C. Y., Hsu, H. J., Tsai, C. J., et al. (2011). p-Cresyl sulphate
720 and indoxyl sulphate predict progression of chronic kidney disease. *Nephrology Dialysis*
721 *Transplantation*, *26(3)*, 938-947.
722 Zhang, L., Gavin, T., Geohagen, B. C., Liu, Q., Downey, K. J., & LoPachin, R. M. (2013).
723 Protective properties of 2-acetylcyclopentanone in a mouse model of acetaminophen
724 hepatotoxicity. *Journal of Pharmacology and Experimental Therapeutics*, *346*(2), 259-269.
725 Zhou, S., Wang, Y., Jacoby, J. J., Jiang, Y., Zhang, Y., & Yu, L. L. (2017). Effects of medium-and
726 long-chain triacylglycerols on lipid metabolism and gut microbiota composition in
727 C57BL/6J mice. *Journal of Agricultural and Food Chemistry*, *65*, 6599-6607.
728
729

730 **Figure captions**

731 **Figure 1**. PCAs of the volatilome sorted by chemical classes of significant VOCs (ANOVA $p <$
732 0.05). The original dataset included the biological replicas of SA, SMA, CNO_2, CO, BC, and the
733 baseline (BL) and different time points (T1 = 18 h and EP = 24 h). A) Acids; B) Alcohols; C)
734 Aldehydes; D) Ketones; E) Other aromatic VOCs. Left side diagrams are for PCAs of cases; right
735 side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of
736 the respective group of cases. SA = Salami with ascorbate; SMA = Salami with ascorbate and plant
737 extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate. *Oxalic acid,
738 cyclohexylmethyl = Oxalic acid, cyclohexylmethyl tetradecyl ester; **Cyclohexanol, 5-methyl-2-
739 (1-m ac = Cyclohexanol, 5-methyl-2-(1-methylethyl) acetate; ***Cyanic acid phenyl = Cyanic acid
740 phenyl ester; * Benzoic acid, methyl = Benzoic acid, methyl ester; *Cyclohexanone, 5-methyl-2-(1-
741 m = Cyclohexanone, 5-methyl-2-(1 -methylethyl); *1,2,4- Triazol-4-amine, N-(2-thien = 1,2,4-
742 Triazol-4-amine, N-(2-thienethyl); *Benzene, 1,3-bis(1,1-dim = Benzene, 1,3-bis(1,1-
743 dimethylethyl).
744
745 **Figure 2.** Changes in the abundance of beneficial microbial VOCs metabolites, expressed as
746 normalized scale from relative abundances with respect to the baseline (red line). The baseline
747 absolute quantifications in mg/kg are found in the Supplementary Material (Table S2). Changes
748 were recorded after 18, and 24 h (T1 and EP respectively) of *in vitro* fecal batch fermentations with
749 SA, SMA, and controls CO and CNO_2. Each plot is made with the raw data obtained from each
750 time point and replica. Samples were analyzed in duplicate from two independent experiments ($n =$
751 4). Marker = mean; box = mean ± S.D.; whiskers = Confidence Interval 0.95. Cases with different
752 letters or numbers or symbols among a single independent variable are significantly different
753 according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). SA = Salami with
754 ascorbate; SMA = Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 =
755 commercial control with nitrate.

Figure 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.

782

783 **Tables.**

784 **Table 1.** Quantification and changes of *Eubacteria*, *Bacteroidetes*, *Firmicutes* and the *Firmicutes* to

785 *Bacteroidetes* ratio of colonic fermentation of salami measured by qPCR.

qPCR Targets & Samples	**Quantification cells/mL**	**Changes log_2(F/C)**		**MANOVA (Time)**	
					$-log_{10}(p)$*
	Baseline	**T1 (18 h)**	**EP (24 h)**	*p*	
Eubacteria					
CO	2.77E+10 ± 8.47E+09	0.02	0.58^{AB}	0.810581	0.091203
SA	$2.77E+10 \pm 8.47E+09^{b}$	0.75^{ab}	1.63^{aA}	0.023662	1.625956
SMA	$2.77E+10 \pm 8.47E+09^{b}$	0.87^{ab}	1.38^{aA}	0.009666	2.014750
CNO_2	$2.77E+10 \pm 8.47E+09^{b}$	0.94^{a}	1.44^{aA}	0.019003	1.721188
BC	$2.77E+10 \pm 8.47E+09^{a}$	-0.15^{a}	-1.74^{bB}	0.015644	0.052740
MANOVA (Matrix) *p*		0.107124	0.000950		
Firmicutes					
CO	$2.46E+09 \pm 2.08E+08^{b}$	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	$2.46E+09 \pm 2.08E+08^{b}$	-0.39^{bB}	1.42^{aA}	0.000457	3.339918
BC	$2.46E+09 \pm 2.08E+08^{b}$	0.91^{abAB}	1.28^{aA}	0.000134	3.872407
MANOVA (Matrix) *p*		0.000012	0.002652		
Bacteroidetes					
CO	$4.80E+09 \pm 1.84E+09^{a}$	-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	$4.80E+09 \pm 1.84E+09^{b}$	0.11^{b}	1.38^{aA}	0.005644	2.248411
CNO_2	$4.80E+09 \pm 1.84E+09^{a}$	-0.11^{ab}	-0.62^{bB}	0.000008	5.071174
BC	$4.80E+09 \pm 1.84E+09^{a}$	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.

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
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.

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

820 log_2 (F/C). -**This taxon was amplified by targeting cell division protein (FtsZ) rRNA; *§This taxon was
821 amplified by targeting cytolethal distending toxin (CDT IV) rRNA; SA = Salami with ascorbate; SMA =
822 Salami with ascorbate and plant extract; CO = control with no nitrate; CNO_2 = commercial control with
823 nitrate; BC = Blank Control; T1 = 18 h of fermentation; EP = 24 h of fermentation. All values are obtained
824 as the mean of sextuplicate (3 technical and 2 biological replicates). Baseline values for each qPCR target are
825 obtained from sextuplicate and are equal for the fermentation of any sample, because indicate the
826 quantification after adaptation of the colon microbiota to in vitro colon ecology and prior in vitro colonic
827 fermentation.
828
829 **Figure 1.**

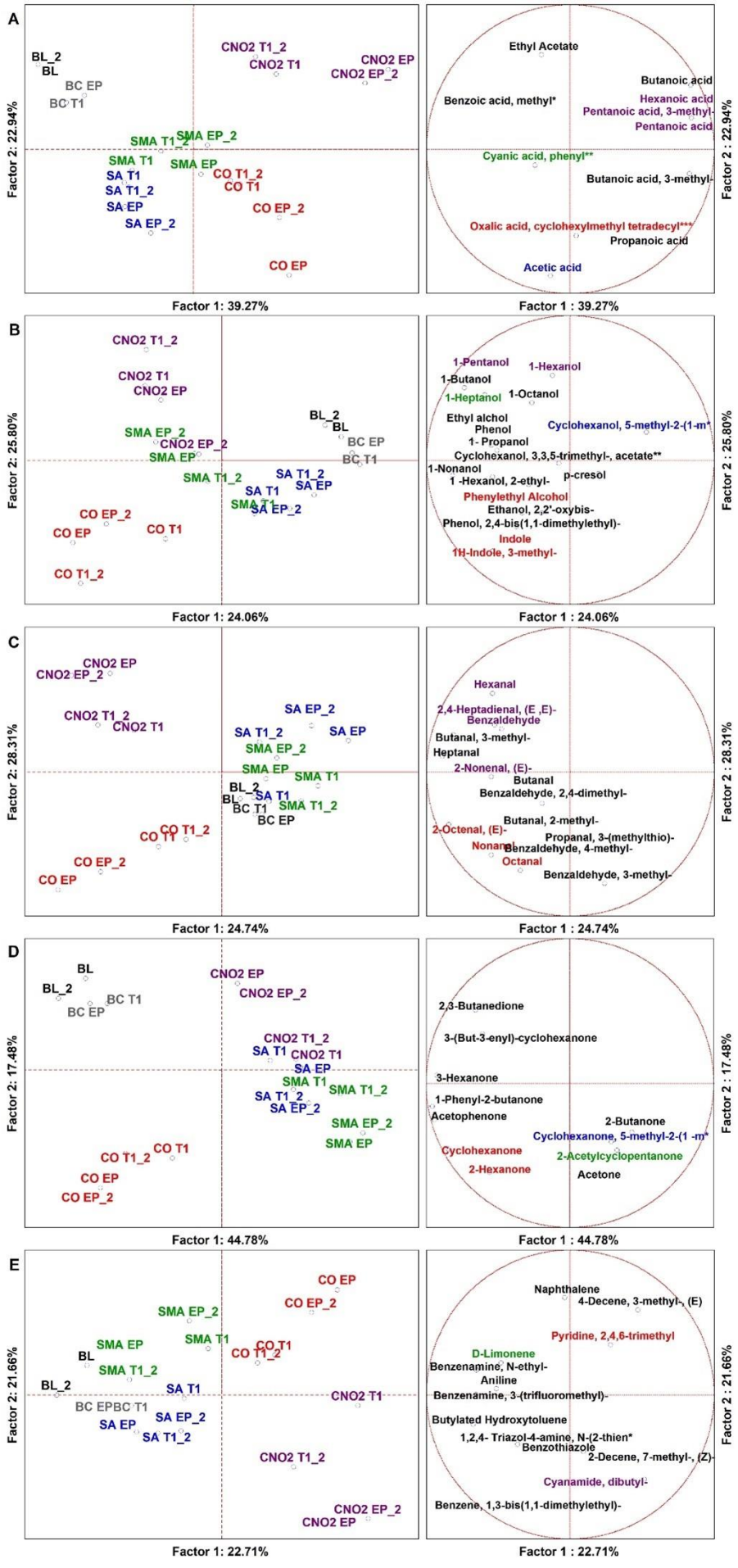

A
BL_2
BL
BC EP
BC T1
CNO2 T1_2
CNO2 T1
CNO2 EP
CNO2 EP_2
SMA T1_2
SMA EP_2
SMA T1
SMA EP
SA T1
SA T1_2
SA EP
SA EP_2
CO T1_2
CO T1
CO EP_2
CO EP
Factor 2: 22.94%
Factor 1: 39.27%
Ethyl Acetate
Butanoic acid
Benzoic acid, methyl*
Hexanoic acid
Pentanoic acid, 3-methyl-
Pentanoic acid
Cyanic acid, phenyl**
Butanoic acid, 3-methyl-
Oxalic acid, cyclohexylmethyl tetradecyl***
Propanoic acid
Acetic acid
Factor 2 : 22.94%
Factor 1 : 39.27%
B
CNO2 T1_2
CNO2 T1
CNO2 EP
BL_2
BL
BC EP
BC T1
SMA EP_2
CNO2 EP_2
SMA EP
SMA T1_2
SA T1_2
SA T1
SA EP
SMA T1
SA EP_2
CO EP_2
CO EP
CO T1
CO T1_2
Factor 2: 25.80%
Factor 1: 24.06%
1-Pentanol
1-Hexanol
1-Butanol
1-Heptanol
1-Octanol
Ethyl alchol
Phenol
Cyclohexanol, 5-methyl-2-(1-m*
1- Propanol
Cyclohexanol, 3,3,5-trimethyl-, acetate**
1-Nonanol
1 -Hexanol, 2-ethyl-
p-cresol
Phenylethyl Alcohol
Ethanol, 2,2'-oxybis-
Phenol, 2,4-bis(1,1-dimethylethyl)-
Indole
1H-Indole, 3-methyl-
Factor 2 : 25.80%
Factor 1 : 24.06%
C
CNO2 EP
CNO2 EP_2
CNO2 T1_2
CNO2 T1
SA EP_2
SA T1_2
SA EP
SMA EP_2
SMA EP
SMA T1
BL_2
BL
SA T1
BC T1
SMA T1_2
BC EP
CO T1_2
CO T1
CO EP_2
CO EP
Factor 2: 28.31%
Factor 1: 24.74%
Hexanal
2,4-Heptadienal, (E ,E)-
Benzaldehyde
Butanal, 3-methyl-
Heptanal
2-Nonenal, (E)-
Butanal
Benzaldehyde, 2,4-dimethyl-
Butanal, 2-methyl-
2-Octenal, (E)-
Propanal, 3-(methylthio)-
Nonanal
Benzaldehyde, 4-methyl-
Octanal
Benzaldehyde, 3-methyl-
Factor 2 : 28.31%
Factor 1 : 24.74%
D
BL
BL_2
BC T1
BC EP
CNO2 EP
CNO2 EP_2
CNO2 T1_2
SA T1
CNO2 T1
SA EP
SMA T1
SMA T1_2
SA T1_2
SA EP_2
SMA EP_2
SMA EP
CO T1_2
CO T1
CO EP
CO EP_2
Factor 2: 17.48%
Factor 1: 44.78%
2,3-Butanedione
3-(But-3-enyl)-cyclohexanone
3-Hexanone
1-Phenyl-2-butanone
Acetophenone
2-Butanone
Cyclohexanone, 5-methyl-2-(1 -m*
Cyclohexanone
2-Acetylcyclopentanone
2-Hexanone
Acetone
Factor 2 : 17.48%
Factor 1 : 44.78%
E
CO EP
CO EP_2
SMA EP_2
SMA EP
SMA T1
CO T1
CO T1_2
BL
SMA T1_2
BL_2
SA T1
CNO2 T1
BC EPBC T1
SA EP
SA EP_2
SA T1_2
CNO2 T1_2
CNO2 EP_2
CNO2 EP
Factor 2: 21.66%
Factor 1: 22.71%
Naphthalene
4-Decene, 3-methyl-, (E)
Pyridine, 2,4,6-trimethyl
D-Limonene
Benzenamine, N-ethyl-
Aniline
Benzenamine, 3-(trifluoromethyl)-
Butylated Hydroxytoluene
1,2,4- Triazol-4-amine, N-(2-thien*
Benzothiazole
2-Decene, 7-methyl-, (Z)-
Cyanamide, dibutyl-
Benzene, 1,3-bis(1,1-dimethylethyl)-
Factor 2 : 21.66%
Factor 1 : 22.71%

Figure 2.

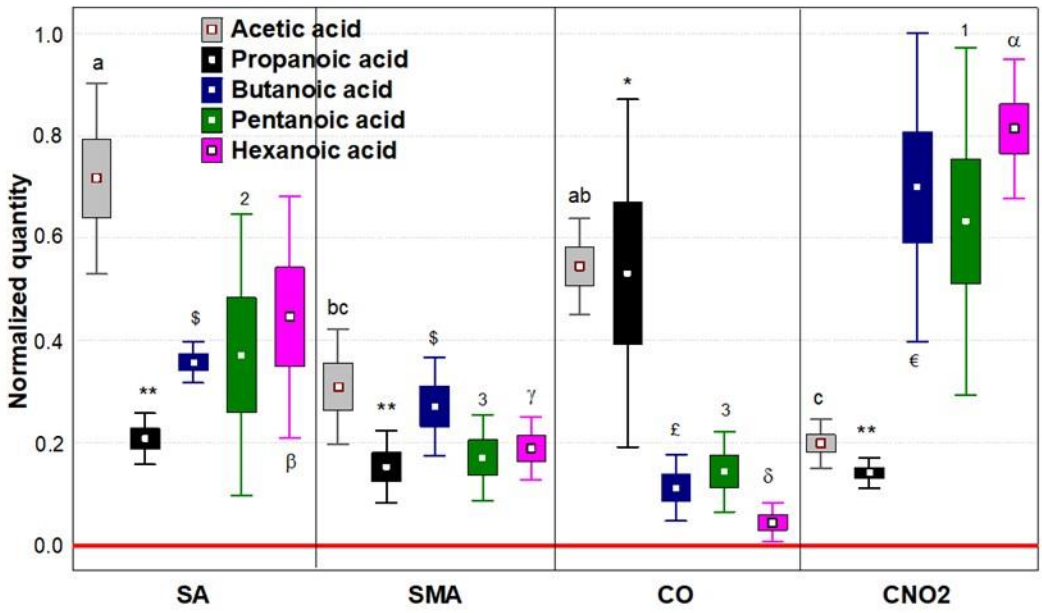
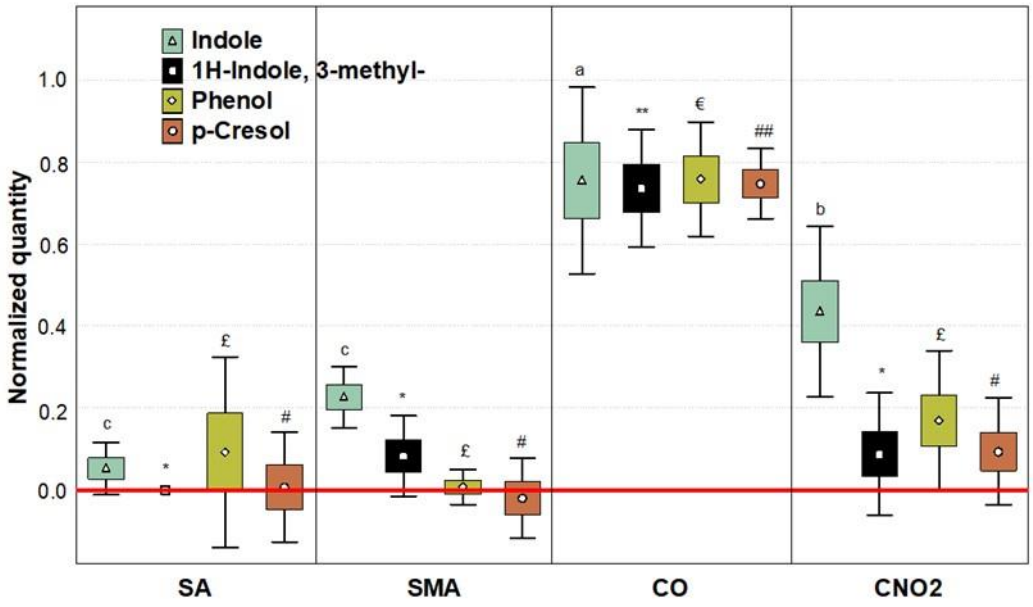

Acetic acid
Propanoic acid
Butanoic acid
Pentanoic acid
Hexanoic acid
Normalized quantity
1.0
0.8
0.6
0.4
0.2
0.0
a
**
$
2
β
bc
**
$
3
γ
ab
*
£
3
δ
c
**
€
1
α
SA
SMA
CO
CNO2

Figure 3.


Indole
1H-Indole, 3-methyl-
Phenol
p-Cresol
Normalized quantity
1.0
0.8
0.6
0.4
0.2
0.0
SA
SMA
CO
CNO2