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Potential probiotic salami with dietary fiber modulates metabolism and gut microbiota in a human intervention study

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ABSTRACT

A human intervention in 24 healthy volunteers was performed to test the potential health benefits of a fermented salami with a probiotic *Lactobacillus rhamnosus HN001* and added citrus fiber. Anthropometric measurements and blood biochemistry did not show any significant differences between pre- and post-intervention during 4 weeks with a daily intake of 30 g of salami, neither with regular salami (control group) nor with reformulated salami (intervention group). However, the inflammatory markers CRP and TNFα decreased significantly after intervention, suggesting a less inflammatory environment after reformulated salami consumption. Antioxidant plasmatic markers also improved within the intervention group. Butyrate production was significantly increased after reformulated salami consumption. Gut microbiota community structure, however, was not significantly shaped by neither regular nor reformulated salami. After the intervention with probiotic salami, *L. rhamnosus* was detected by quantitative polymerase chain reaction (qPCR) in all samples of the intervention group but not in the control group, showing probiotic effect.

1. Introduction

Salami is a dry-fermented sausage consisting of mixtures of lean meats and fatty tissues combined with salts, nitrate (curing agent), sugars and spices filled into casings to undergo a microbial fermentation, drying and maturation process (FAO, 2019). Salami is often considered as a food with unbalanced nutritional value due to the high fat and salt content and lack of bioactive molecules (Martínez, Nieto, & Ros, 2014). However, salami is highly consumed around the world, which could explain why in the last decade there has been a great focus of interest on improving meat products quality through (Blaiotta, Murru, Cerbo, Romano, & Aponte, 2018): (i) modifying meat products formulation by adding functional ingredients (Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013); (ii) modifying meat composition through cattle feeding (Gilmore et al., 2011); (iii) by innovations in processing/storage conditions (Cullere, Hoffman, & Dalle Zotte, 2013).

A major focus of interest was on the fat profile of meat products, which led to several attempts to improve it. Although there is much evidence that saturated fats are not to blame for cardiovascular diseases (CVD), changing them for unsaturated fats appears to be beneficial (Ekmekcioglu et al., 2018; de Souza et al., 2015). Accordingly, there were several research projects, involving human interventions, that were focused on achieving a healthier plasmatic lipid profile by increasing monounsaturated fatty acids and polyunsaturated fatty acids (MUFA and PUFA) content (Gilmore et al., 2011; Haug, Nyquist, Mosti,

Abbreviations: BMI, Body Mass Index; GL, glycaemia; TC, total cholesterol; cHDL, HDL cholesterol; cLDL, LDL cholesterol; TG, triglycerides; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyltransferase; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; TNF, tumor necrosis factor; IL, interleukin; CRP, C-Reactive protein; CAT, catalase; GPX, glutathione peroxidase; MDA, malondialdehyde; SCFA, short chain fatty acids; HPLC, high performance liquid chromatography; qPCR, quantitative polymerase chain reaction

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Andersen, & Høstmark, 2012) and adding plant sterols (Tikkanen et al., 2001). In these cases, human interventions showed an improved plasmatic lipid profile after consuming the reformulated meat product. Moreover, substitution of fats by dietary fiber has also been attempted (Verma & Banerjee, 2010). However, in such cases the focus of interest was usually on physico-chemical properties and organoleptic attributes of the product rather than in potential health benefits.

Fermented meat products have been also proposed as good carriers for probiotics (Vuyst, Falony, & Leroy, 2008), which are expected to play a role in host health. Recently, Thøgersen et al. (2018) studied the addition of inulin on Frankfurt sausages fed to rats and their effect on gut microbiota composition and short chain fatty acids (SCFAs) production (Thøgersen et al., 2018). They observed that rats fed with sausages enriched with inulin had an increased production of SCFA and higher abundances of *Bifidobacterium* spp. in their gut. However, related human studies are scarce and inconclusive. For instance, Jahreis et al. (2002) found that consumption of fermented sausages with a probiotic strain did not modify human lipid profile and the probiotic bacteria were only identified in some volunteers' feces (Jahreis et al., 2002).

Taking all this information into account, in this trial we aim to investigate the effects of the ingestion by healthy volunteers of 30 g/day (during 4 weeks) of salami supplemented with fiber and a probiotic starter on blood biochemistry, anthropometry, immunological markers, fecal microbiota composition, SCFAs production, and antioxidant capacity of plasma and feces.

2. Materials and methods

2.1. Subjects and trial design

Twenty-four healthy subjects were recruited in the University of Granada. Subjects were 20–30 years old and had a body mass index (BMI) within normal range (18.5–25). The trial consisted of a longitudinal nutritional intervention, double-blinded, with placebo and two parallel groups: control and intervention group (Fig. S1). Subjects were asked not to consume probiotics during a week before starting the trial. Participants were randomly divided into control and intervention groups (n = 12 for each group). The control group was given regular salami and instructed to intake 30 g/day (serving size) for 4 weeks. The intervention group was given the reformulated salami and instructed to intake 30 g/day for the same period of time. Both groups were given an isocaloric diet along the intervention period in order to assure the homogeneity of foods eaten. The day before starting the intervention, fecal and blood samples were taken and anthropometric measurements were also performed. At the end of the follow-up period, the same samples and measurements were obtained.

The trial complied with the principles of the declaration of Helsinki. The Ethics Committee of the University of Granada approved the trial protocol (SA/17/AYU/246) and informed consent was obtained from all participants.

2.2. Salami preparation

Salami samples were manufactured and provided by a local Spanish company (Elpozo Alimentación, S.A., Alhama de Murcia, Murcia). Each salami sample was formulated according to the following traditional recipe: a mixture of pork meat and fatty tissues was combined with salt, curing salt agent, black pepper, starch/fiber, and typical Mediterranean herbal extract.

For the control salami, 2% w/w of starch was added, and a standard starter composed of non-probiotic strains of lactic acid bacteria and catalase-positive streptococci was applied (*Lactobacillus fermentum* and *Staphylococcus xylosus*). Fiber-added salamis were supplemented with the probiotic *Lactobacillus rhamnosus HN001* as a starter culture, and citrus fiber (obtained from orange pulp dehydration and composed of 42% pectin, 25% cellulose and hemicellulose, and acquired from Fiberstars, USA). Fiber was added in a 1.5% w/w ratio. A mix of antioxidant herbal extract, composed of olive, coffee and tea extracts was also incorporated as ingredient in the improved salami (0.3% w/w), which is the usual proportion for seasoning in sausages and the one used by the company that provided the samples in their other commercial products (Table 1). Fiber and herbal extract were chosen after an *in vitro* test previously published (Pérez-Burillo et al., 2019). Salamis were put into casings to exclude oxygen and subjected to a ripening-drying process for 40 days. After curing, salamis were sliced and packaged as they are for commercial purposes.

Table 1
Composition of each salami formulation.

	Control salami	Fiber added-supplemented salami
Nutrient	*Content, in g/100 g*	*Content, in g/100 g*
Energy, kcal	333	326
Water, g	37.9	37.9
Protein, g	25.8	26.5
Lipids, g	22.9	22.1
Saturated, g	7.2	6.8
Monounsaturated, g	11.7	11.4
Polyunsaturated, g	3.9	3.8
Carbohydrates, g	3.2	3.3
Starch, g	2.0	0.0
Fiber, g	0.0	1.5
Herbal extract, g	0.0	0.3
Ash, g	4.2	4.4
Regular culture strains	5^9	5^{10}
L. rhamnosus HN001	0.0	2^{10}

Regular culture strains: *S. xylosus* and *L. fermentum*.

2.3. Anthropometric measurements

Guidelines of the International Society for the Advancement of Kinanthropometry (ISAK) were followed for the anthropometric analysis (Stewart, Marfell-Jones, Olds, & Ridder, 2011). All anthropometric measurements were carried out at the same place by an ISAK-certified level II anthropometry researcher. The following instruments were used: GPM Stadiometer (± 1 mm accuracy); Tefal scale (± 50 g accuracy); Holtain skinfold compass (± 1 mm accuracy); Holtain caliper (± 1 mm accuracy); Holtain flexible metallic metric belt (± 1 mm accuracy). The following measurements were taken: height, weight, skinfolds (triceps, biceps, subscapular, suprailiac, supraspinal, abdominal, thigh, and calf), perimeters (waist, hip, relaxed biceps, flexed and contracted biceps, anckle, forearm, chest, thigh and calf, and waist/hip index), and diameters (bicromial, biliocrestal, bicondylar humerus, bistiloid and bicondylar femur). The body mass index (BMI) was calculated from height and weight. Then, the percentages of fat mass, non-fat mass and water were calculated. Bone density, and visceral fat was also calculated along with the basal metabolism.

2.4. Blood biochemistry

Venous blood was used to determine health-related biochemical markers. The analysis was performed in the morning after a 12-hour fasting period. The samples were stored in the dark in containers with ice and processed within the hour after extraction. Plasma was separated by centrifugation at 1500 rpm for 20 min at 18–25 °C. The following parameters were measured with an automatized blood biochemistry system (DiaSys Diagnostic Systems GmbH, Germany): glycaemia (GL; mg/dL), total cholesterol (TC; mg/dL), HDL cholesterol (cHDL; mg/dL), LDL cholesterol (cLDL; mg/dL) and triglycerides (TG; mg/dL), urea (mg/dL), creatinin (mg/dL), uric acid (mg/dL), total proteins (g/dL), alanine aminotransferase (ALT) and aspartate transaminase (AST, U/L), GGT (gamma-glutamyltransferase, U/L), alkaline phosphatase (U/L), total billirrubine (mg/dL) and iron (μg/dL).

Oxidized LDL was measured through an ELISA kit (OxiSelect™ Human Oxidized LDL ELISA Kit, Cell Biolabs, Inc. USA).

Haematology was assessed through the following parameters: leukocytes (x10^3/μL), red blood cells (x10^6/μL), haemoglobin (g/dL), haematocrit (%), MCV (Mean Corpuscular Volume, fL), MCH (Mean Corpuscular Haemoglobin, pg) and MCHC (Mean Corpuscular Hemoglobin Concentration, g/dL). Leukocyte formula was also assessed: platelets ($\times 10^3$/μL), neutrophils, eosinophils, basophils, lymphocytes and monocytes (%).

2.5. Inflammatory and immunological markers

Venous blood was also used to analyze and quantify inflammatory and immunological markers. Tumor Necrosis Factor alpha (TNFα), Interleukin 6 (IL-6) and Interleukin 10 (IL-10) were measured through their corresponding ELISA kits from Thermo Fisher scientific (USA). C-Reactive Protein (CRP) was measured through the Human C-Reactive Protein ELISA Kit (CLIA) from Biomatik (USA). Phagocytic activity was measured as described in Gill and Rutherfurd (2001).

2.6. Fecal antioxidant capacity and plasma antioxidant status

The FRAP assay was used to measure the antioxidant capacity of feces, according to the procedure described in Benzie and Strain (1996). Results were expressed as mmol Trolox/g of sample.

Catalase (CAT) activity in plasma was determined following the method described by Pastoriza, Roncero-Ramos, Rufián-Henares, and Delgado-Andrade (2014). Results were expressed as μmol H_2O_2/min/mL.

Glutathione peroxidase (GPX) activity in plasma was determined as described in Pastoriza et al. (2014). Results were expressed as μmol GSH/min/mL.

Malondialdehyde (MDA) analysis in plasma was carried out as stated in Olusi (2002). Results were expressed as μmol MDA/L.

2.7. SCFAs analysis

The production of SCFAs was measured in feces and it was assessed by HPLC according to the procedure described in Pérez-Burillo et al. (2019). Results were expressed as mmol/kg of feces.

2.8. DNA extraction and sequencing

Fecal microbial ecology was assessed by analyzing the 16S rRNA gene. To do that, DNA extraction, sequencing and bioinformatics analyses were performed as described in Pérez-Burillo et al. (2019). DNA extraction was performed using a NucliSENS easyMAG platform (Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μl in 10 mM Tris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al. (2013). Primer sequences are: Forward 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA-G3′ and Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA-CHVGGGTATCTAATCC3′. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 ml of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

2.9. Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program (Schmieder & Edwards, 2011) applying the following parameters: a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3′-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite (Aronesty, 2011). Taxonomic affiliations were assigned using the RDP_classifier (Cole et al., 2009) from the Ribosomal Database Project (RDP). Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. Six taxonomic levels were assigned, which were kingdom, phylum, class, order, family and genus.

2.10. Lactobacillus rhamnosus HN001 qPCR

The HN001 primer sequences used were Forward 5′-CGCTTAGGACTCAGGATACA-3′ and Reverse 5′-GCTTGCGTCAGATTTTCAGTA-3′, according to published sequences (GenBank acc no. NZ_ABWJ00000000). PCR conditions used to amplify fecal DNA templates were pre-incubation at 95 °C for 10 min; followed by 45 cycles of denaturation at 95 °C for 10 s, annealing (69 °C) for 10 s and extension at 72 °C for 30 s; with a final cooling at 40 °C for 30 s. DNA amplification products were analysed with the QIAxcel System (QIAGEN, Hilden, Germany).

2.11. Statistical analysis

The homogeneity of variance was assessed using the Levene test and the normal distribution of the samples with the Shapiro-Wilk test. The Student's *t*-test was used to analyze parametric data. The significance level was set at 5% ($p < 0.05$) in all tests. SPSS 22.0 for Windows (IBM SPSS Inc., New York, USA) was used for data analyses.

3. Results and discussion

3.1. Anthropometric analysis

No significant differences in anthropometric parameters were detected in the control group after 4 weeks consuming the salami control (Supplemental Fig. S2A). In this case, results yield some interesting conclusions since regular salami consumption did not modify BMI index, weight, body fat percentage, or any skin-fold thickness measurement. Therefore, at least in relation with anthropometric measurements, daily salami intake does not translate in weight gain or increase of body fat percentage (Fig. S2A).

Similar results were observed for the intervention group, no significant differences were detected in any of the studied parameters after 4 weeks consuming the improved salami (Fig. S2B). In addition, no statistically significant differences were found between the control and intervention groups at the post-intervention time ($p > 0.05$) for any of the assessed parameters. From these results it can be concluded that the improved formulation (fiber, probiotic) does not have any effect on body composition, at least at the dose administered. Accordingly, our results showed that salami consumption with either of the two formulations could be part of the diet without affecting anthropometric parameters, contrary to the general perception about the unhealthy effects of salami (Martínez et al., 2014). This conclusion was reached after 4 weeks of salami intake, so the effect of a much longer intake could be different and should be analyzed in future studies.

3.2. Blood biochemistry

Salami consumption by the control group (Fig. S3A) did not change the levels of plasmatic lipids (total cholesterol, LDLc, HDLc or TG). These results suggest that regular salami can be part of a varied diet without influencing plasmatic lipids. However, since this trial was carried out with healthy volunteers and plasmatic lipids within the normal range, these results cannot be extrapolated to people with an unbalanced lipid profile and more experiments should be done in this population. Regarding the rest of the biochemical parameters (i.e. hematology and leukocyte formula), no significant differences were detected either. Therefore, it can be concluded here that regular salami consumption does not affect blood biochemistry, at least in healthy subjects during a 4 weeks period intake.

Regarding the intervention group, the results were in the same line as in the control group (Fig. S3B). Plasmatic lipids did not change significantly although a tendency to increase HDL levels ($p = 0.069$) and to decrease LDL concentration ($p = 0.058$) was detected. Jahreis et al. (2002) also found no modification is plasmatic lipid profile after consumption of sausages fermented with a probiotic strain (Jahreis et al., 2002). However, in our case the tendencies found could be derived from the presence of fiber, which is known to decrease blood LDL (Olmedilla-Alonso et al., 2013). Therefore, although the new ingredients added, at least at such dose, were not able to improve blood parameters, they didn't worsen them and tended to improve the lipid profile, indicating that future experiments with a larger population are needed.

3.3. Antioxidant capacity

To study the influence of salami intake, either control or intervention salami, on body antioxidant status we analyzed the fecal antioxidant capacity with the FRAP assay (Fig. 1A), and plasma antioxidant capacity through plasmatic GPX, catalase, Ox-LDL and MDA (Fig. 1B and C). Within the control group, no significant differences in any of the parameters measured were found, suggesting that incorporating salami to a varied diet neither increases nor decreases the redox status of the subject. The levels of MDA, catalase and GPX were in the same range as those reported for an adult population of the same body mass index (Olusi, 2002).

On the other hand, regarding the intervention group, the antioxidant capacity of feces did not show any changes before and after the intervention period, although there was a tendency ($p = 0.066$) to increase (Fig. 1A). However, plasmatic markers improved after the 4 weeks of intervention. In fact, GPX and catalase activity increased significantly ($p < 0.05$) whereas Ox-LDL and MDA decreased significantly ($p < 0.05$), suggesting a positive modulation of the redox status (Fig. 1B and C). In fact, MDA levels were below the usual range reported for individuals of the same body mass index (Olusi, 2002). This beneficial modulation could be due to the antioxidant extract incorporated to salami, since it has been demonstrated that complementing diet with antioxidants increases the expression of plasmatic antioxidant enzymes (Alshammari, Balakrishnan, & Al-Khalifa, 2017). Another possible explanation is a role of the added fiber. As our previous research showed *in vitro* salami added with this fiber showed a great potential as an antioxidant, probably due to fiber composition as well as to fiber metabolization by gut microbes (Pérez-Burillo et al., 2019). Thus, the antioxidant species released, either from the fiber matrix or from microbes' metabolic activity, could be absorbed at the large intestine and thus modulate the antioxidant status of plasma.

3.4. Inflammatory and immunological markers

Several markers involved in inflammation and immunological status were measured: CRP, IL-6, IL-10, TNFα and phagocytic activity. CRP is an unspecific inflammation marker that increases during inflammatory processes, IL-6 and TNFα behave as pro-inflammatory species while IL-10 is an anti-inflammatory interleukin. Inflammatory markers tested did not change significantly within the control group after the 4 weeks intake period (Fig. 2).

On the other hand, the intervention group showed an improvement in their inflammatory status after the intake period. A significant decrease ($p < 0.05$) was detected in the levels of CRP and TNFα, suggesting a less inflammatory environment (Fig. 2). The rest of the parameters did not change significantly. These results could be due to the improved formulation, including the probiotic bacteria and the fiber component. For example, an improvement of the inflammatory and immunological status has been demonstrated previously in elderly people supplemented with the immunostimulatory probiotic strain *L. rhamnosus* HN001 (Gill & Rutherfurd, 2001). However, it is known that modifications in phagocytic activity are only found in elderly population but not in adult population after exposure to a probiotic strain (Gill & Rutherfurd, 2001). In addition, as it will be explained below, butyrate levels were significantly ($p < 0.05$) increased in the intervention group after the intake period. This SCFA has been previously related to anti-inflammatory processes (Donohoe et al., 2014), being therefore another possible reason for the improved inflammatory status of volunteers, in addition to the probiotic strain used for the fermentation process.

3.5. Short chain fatty acids

Several health effects are attributed to SCFAs. Decreasing the luminal pH is the obvious effect. The three main SCFAs (acetate, propionate and butyrate) are all important for the maintenance of the gut barrier (Ríos-Covián et al., 2016). Moreover, while butyric acid is mostly metabolized by colonocytes as a source of energy and carbon, acetate and propionate are mainly absorbed and incorporated into different metabolic routes, which in turn could link the control of metabolic syndrome and diet-induced obesity (Lin et al., 2012). SCFAs also have an important role in colorectal cancer protection (Donohoe et al., 2014). In fact, it has been suggested that the protective effect of dietary fiber against colorectal cancer depends upon the production of butyrate and other SCFAs.

Regarding the control group no significant changes were detected after the intake period; in fact, SCFAs levels were almost the same before and after (Fig. 3). Acetate, propionate and butyrate production barely changed. On the other hand, within the intervention group, acetate production tended to increase ($p = 0.063$) after the intake period. In addition, there was a statistically significant increase ($p < 0.05$) on butyrate levels after intervention (Fig. 3). This result could explain, at least partially, why some inflammatory markers improved after consumption of the re-formulated salami. This higher butyrate production could be attributed to the fiber component of the new formulation. As it has been previously demonstrated (Flint, Duncan, Scott, & Louis, 2015), polysaccharide degradation leads to butyrate production, being *Faecalibacterium* and *Eubacterium* the most abundant butyrate producers in the human gut. As stated above, this new salami formulation is based on a previous *in vitro* study in which we investigated the potential of different fibers to produce SCFA (Pérez-Burillo et al., 2019). As citrus fiber was the most promising agent, we kept using it for the present formulation. As our previous research showed (Pérez-Burillo et al., 2019), results after intervention suggest that citrus fiber is also able to favor butyrate production. Higher butyrate production could be due to higher abundances for those genera known to be butyrate producers, with *Faecalibacterium, Eubacterium, Roseburia, Anaerostipes, Coprococcus, Butyricimonas, Clostridium XIVa, Intestinimonas* and *Butyricicoccus* as their major representatives (Flint et al., 2015; Ríos-Covián et al., 2016).

After analyzing gut microbial community structure by 16S rRNA, we checked these genera to study whether their abundance was higher after intervention, and to compare the control *Vs.* intervention group.

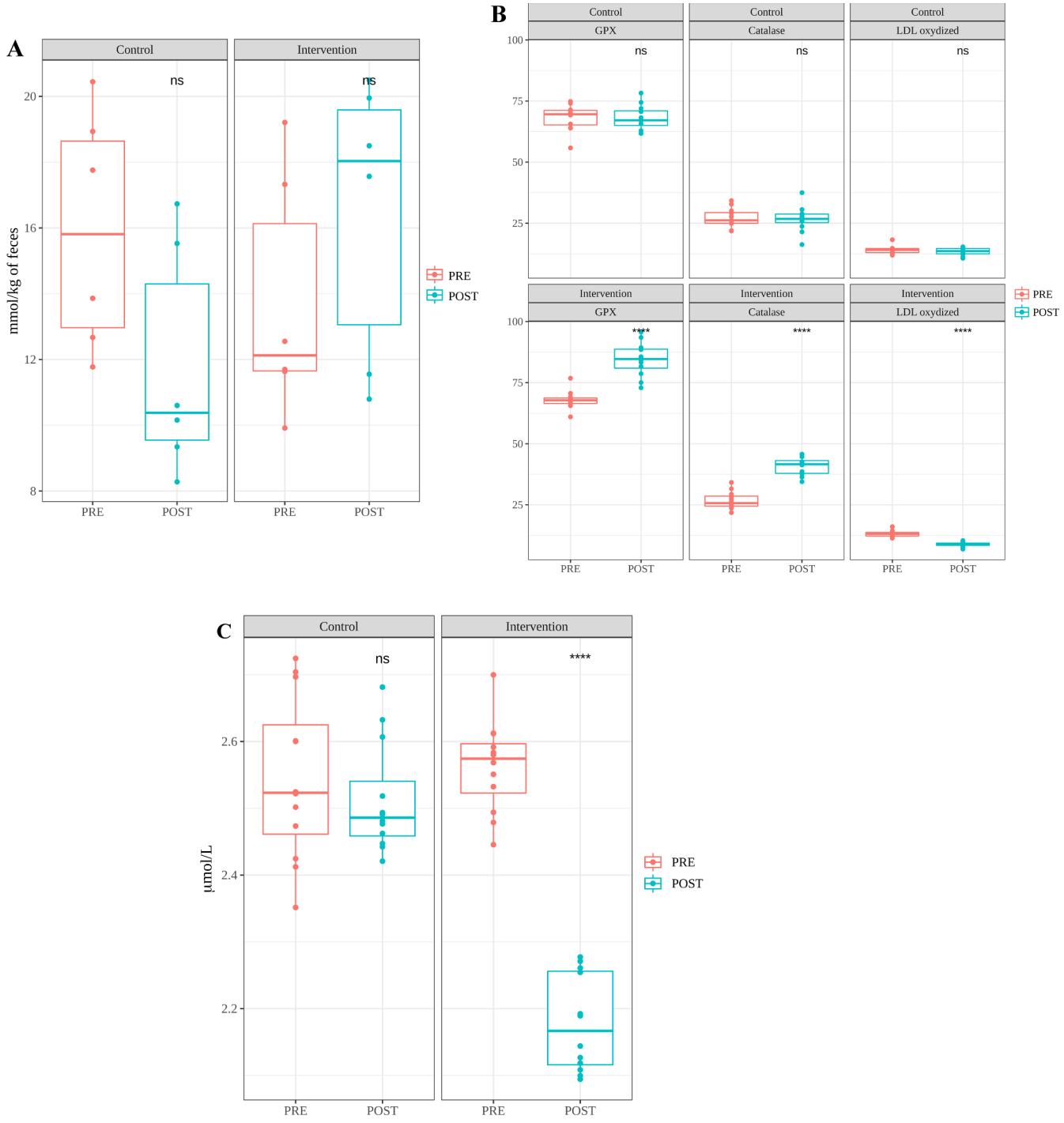

Fig. 1. Panel A shows antioxidant capacity of volunteers's feces measured by the FRAP method. Panel B shows plasmatic antioxidant markers GPx (µmol/min/ml), Catalase (µmol/min/ml), and oxydized LDL (mU/L). Panel C shows MDA plasmatic levels. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE as the reference group. Analyses were carried out in triplicate.

No significant differences were found before and after salami intake, neither in control nor in intervention group. However, in the intervention group some not statistically significant increases in the abundance of these genera were detected after salami intake: *Faecalibacterium, Eubacterium, Anaerostipes, Coprococcus, Butyricimonas, Clostridium XIVa,* and *Intestinimonas.* Even though these increases were not significant, their synergic action could led to a significantly ($p < 0.05$) higher butyrate production after salami consumption due to the sum of butyrate producers: *Faecalibacterium, Eubacterium, Roseburia, Anaerostipes, Coprococcus, Butyricimononas, Clostridium XVIa, Intestinimonas, Butyricicoccus* (Fig. S4). Moreover, after checking for Spearman correlations between butyrate concentrations and butyrate-producers abundance, we found a positive significant correlation between butyrate and *Clostridium XIVa* ($r^2 = 0.57$), *Faecalibacterium* ($r^2 = 0.46$) and total butyrate producers ($r^2 = 0.65$). These correlations were not found in the control group, reinforcing the idea that the improved salami slightly modifies the functionality of the gut microbiota so that it produces higher concentrations of healthy SCFAs.

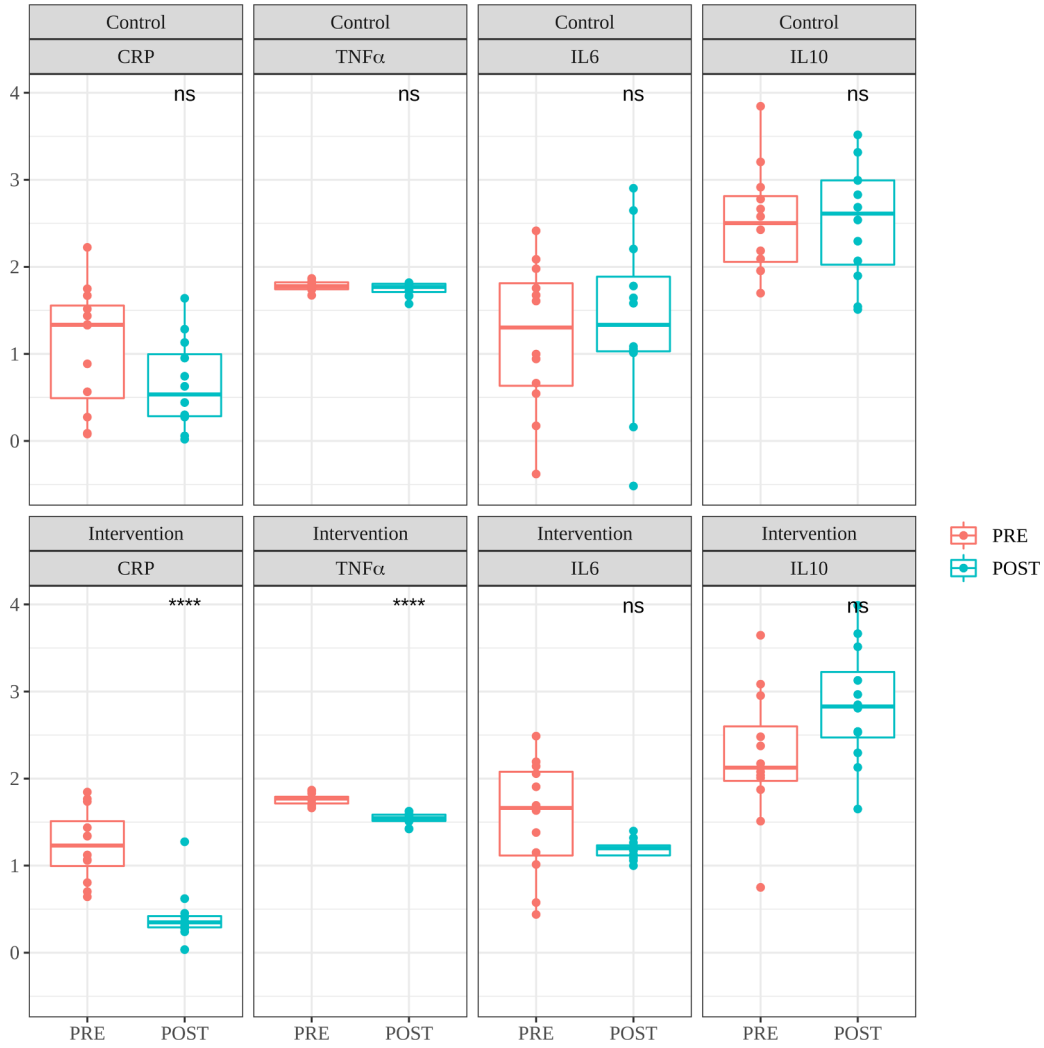

Fig. 2. Plasmatic levels of inflammatory markers CRP (mg/L), TNFα (pg/mL), IL-6 (pg/mL), and IL-10 (pg/mL). Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE as the reference group. Analyses were carried out in triplicate.

3.6. Gut microbial community structure

At phylum level, no significant differences between pre- and post-intervention were found, neither in the control nor in the intervention group (Fig. 4). After studying the community dissimilarity before and after salami intake through PCoA based on the Unifrac phylogenetic distance (Lozupone & Knight, 2005), no significant differences between both groups were found, showing no clear separation in the PCoA plot (Fig. 5A–B). At genus level, after applying OPLS-DA to investigate discriminant bacteria in each group before and after salami intake, we did not find any genus significantly associated to one or the other group. LefSE analysis was also applied (Segata et al., 2011), but again no significant differences were found. However, as stated before, some tendencies where discovered when investigating butyrate producers, and some of these bacteria were found to be higher (not significantly) after improved salami intake. These results are in line with those reported by other authors who found that the administration of different foods enriched in different *L. ramhnosus* strains did not modify the overall fecal microbiota, with slight modifications in the lactobacilli populations (Lahtinen et al., 2012).

Gut microbial community alpha diversity was also investigated through Shannon and Simpson indexes. As our previous analysis suggested, no significant differences were found, showing a very similar diversity before and after salami intake. Therefore, salami intake does not have any significant influence on gut microbiota community structure, at least in healthy people. However, though not significant, improved salami intake did result in a significantly higher butyrate production, probably due to some increase in certain butyrate producers.

3.7. qPCR Lactobacillus rhamnosus HN001

In order to classify a bacterial as a probiotic strain, one of the first conditions is to survive in the gastrointestinal tract in sufficient numbers to exert a positive health effect in the host (Saxelin et al., 2010). In general, $10^{8–10}$ bacterial cells/day has been recommended as the minimum number of a probiotic strain to provide a beneficial effect in humans (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011). In the case of the probiotic salami, about 4–5^{8} bacterial cells/g were obtained (Table 1), so that the consumption of 30 g/day potentially provides sufficient numbers of *L. rhamnosus* cells to obtain a health effect.

A probiotic strain not only should be viable in a food product but it also must survive the passage through the gastrointestinal tract (reach

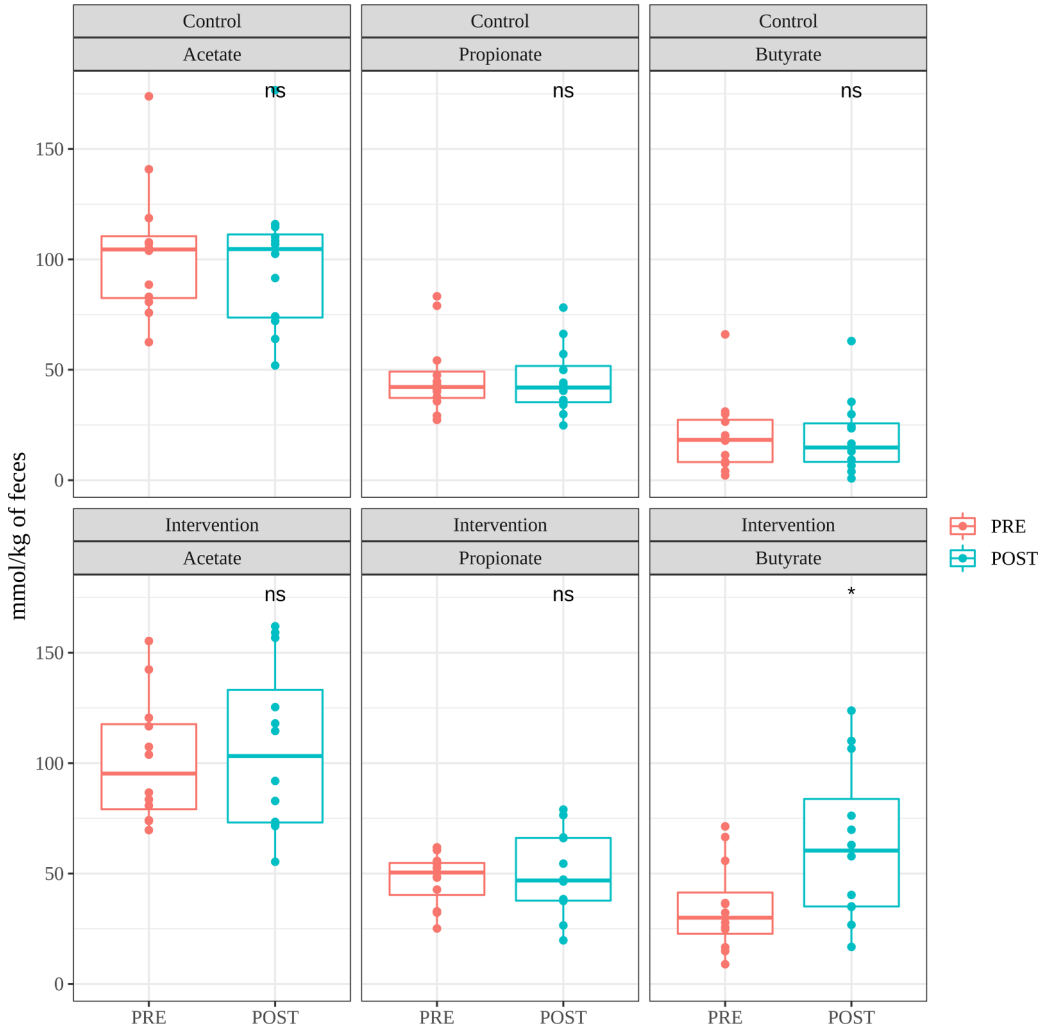

Fig. 3. Short chain fatty acids levels measured in volunteers's feces. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE as the reference group. Analyses were carried out in triplicate.

in a viable state the large and short intestine) in order to exert a positive effect on the host health (Dommels et al., 2009). Therefore, it is necessary to analyze the presence of the probiotic strain in human fecal samples. In this sense, *L. rhamnosus* was absent in all volunteers (both control and intervention groups) during the run-in phase. Then, after the intervention with probiotic salami, *L. rhamnosus* was detected in all samples of the intervention group (but not in the control group), with an average level of this species of log 6.78 cells/g. This level is similar to those reported by other authors for *L. rhamnosus* in probiotic fermented sausages (Rubio, Jofré, Aymerich, Guàrdia, & Garriga, 2014), and cheese (Lahtinen et al., 2012).

4. Conclusions

As conclusions, whereas no changes were observed in anthropometric measurements or blood biochemistry, reformulated salami improved some inflammatory and immunological markers (CRP and TNFα), antioxidant plasmatic markers and butyrate production. Moreover, *L. rhamnosus* was found by qPCR in all volunteers' feces from the intervention group, suggesting that this probiotic was able to resist passage through the gastrointestinal tract. As consequence, the probiotic bacteria along with the added fiber component could be responsible of the improved parameters observed. Accordingly, this reformulated salami could be used as a substitute of regular salami in common diets, or even included if not present, since it has no negative effects, but rather it does the above mentioned parameters. However, this trial has some limitations: i) it was carried out during four weeks, so it could not be enough time for more significant effects on gut microbial community structure or some blood markers to show up; ii) only 24 individuals were studied and maybe with a larger court more significant results would show; iii) volunteers court was composed of healthy individuals, which could make detecting changes more challenging; iv) trials with individuals suffering from bowel inflammation would be needed to assert the positive effects of enriched salami on inflammation.

5. Ethics statement

The study complied with the principles of the declaration of Helsinki. The Ethics Committee of the University of Granada approved the study protocol, and informed consent was obtained from all participants.

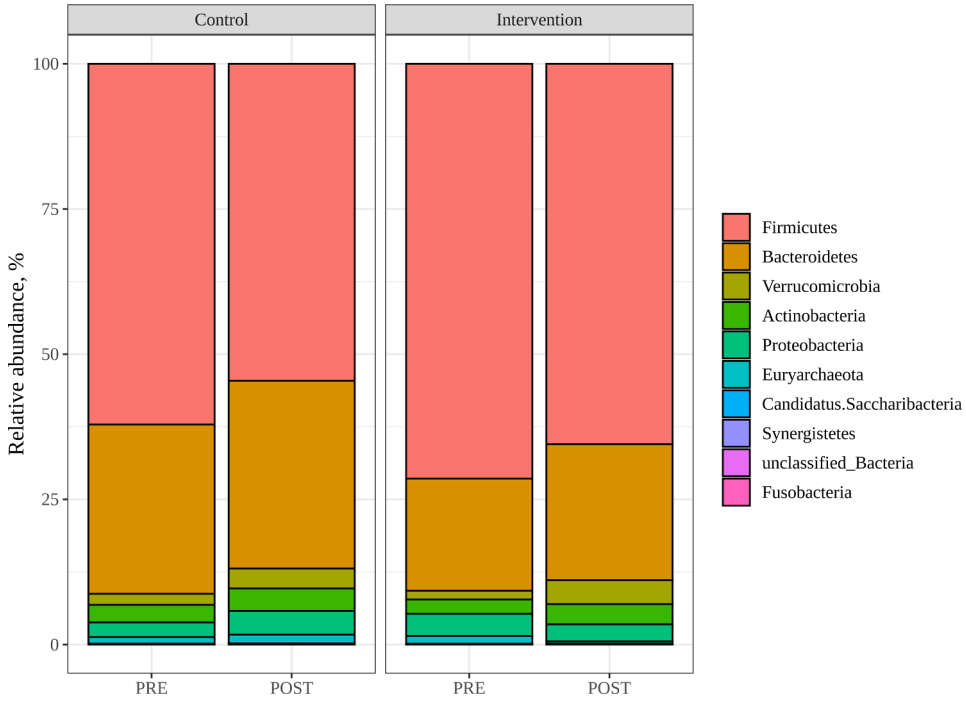

Fig. 4. Barplot of gut microbial community structure at *phylum* level. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE as the reference group.

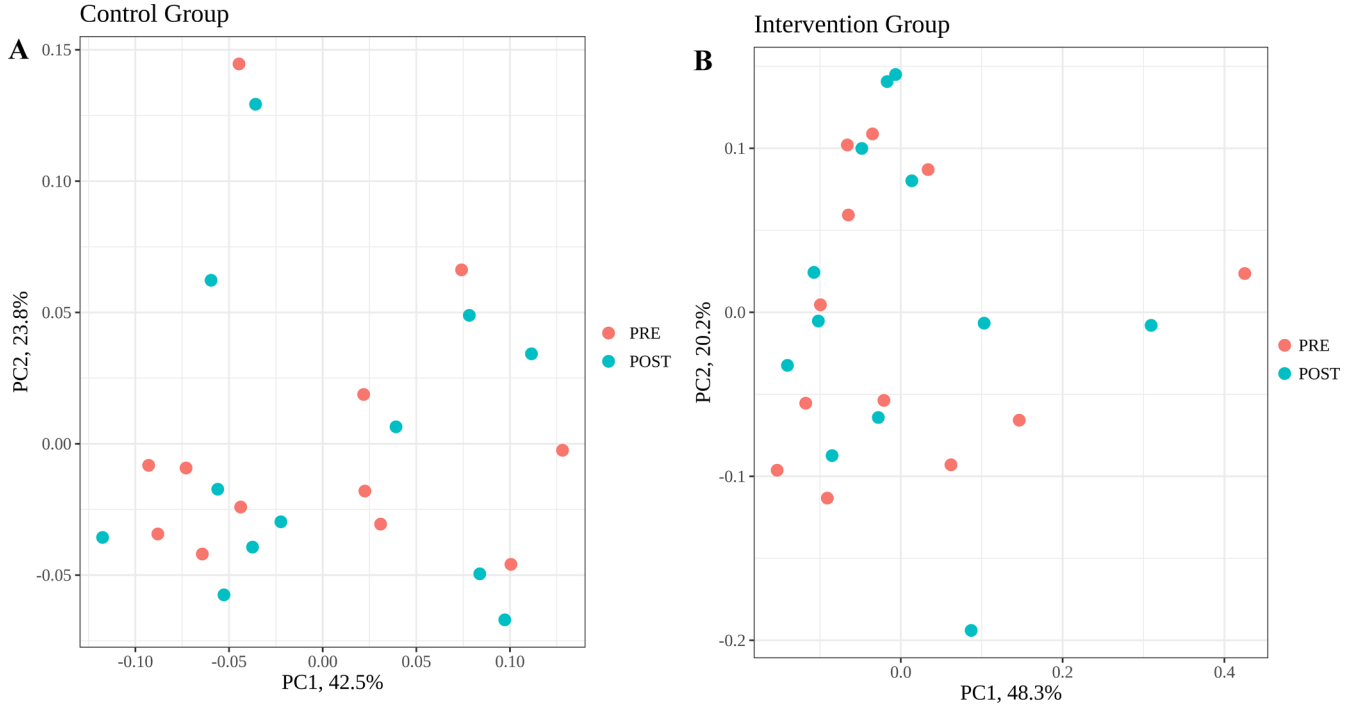

Fig. 5. Principal Coordinates Analysis with UniFrac Phylogenetic distance. Panel A shows control group, and Panel B shows intervention group. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE as the reference group.

CRediT authorship contribution statement

S. Pérez-Burillo: Methodology, Investigation, Writing - original draft. **S. Pastoriza:** Supervision, Writing - review & editing. **A. Gironés:** Formal analysis, Methodology, Investigation. **A. Avellaneda:** Conceptualization. **M. Pilar Francino:** Formal analysis, Conceptualization, Writing - review & editing. **J.A. Rufián-Henares:** Funding acquisition, Formal analysis, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103790.

References

Alshammari, G. M., Balakrishnan, A., & Al-Khalifa, A. (2017). Antioxidant effect of Arabian coffee (Coffea arabica L) blended with cloves or cardamom in high-fat diet-fed C57BL/6J mice. *Tropical Journal of Pharmaceutical Research, 16*(7), 1545–1552. https://doi.org/10.4314/tjpr.v16i7.12.

Aronesty, E. (2011). Command-line tools for processing biological sequencing data. Retrieved from https://github.com/ExpressionAnalysis/ea-utils.

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. https://doi.org/10.1002/jsfa.8717.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292.

Champagne, C. P., Ross, R. P., Saarela, M., Hansen, K. F., & Charalampopoulos, D. (2011). Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. *International Journal of Food Microbiology, 149*(3), 185–193. https://doi.org/10.1016/j.ijfoodmicro.2011.07.005.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., ... Tiedje, J. M. (2009). The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. *Nucleic Acids Research, 37*(Database issue), D141–D145. https://doi.org/10.1093/nar/gkn879.

Cullere, M., Hoffman, L. C., & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (Aspalathus linearis) in preventing lipid oxidation in meat products. *Meat Science, 95*(1), 72–77. https://doi.org/10.1016/j.meatsci.2013.04.018.

Dommels, Y. E. M., Kemperman, R. A., Zebregs, Y. E. M. P., Draaisma, R. B., Jol, A., Wolvers, D. A. W., ... Albers, R. (2009). Survival of Lactobacillus reuteri DSM 17938 and Lactobacillus rhamnosus GG in the human gastrointestinal tract with daily consumption of a low-fat probiotic spread. *Applied and Environmental Microbiology, 75*(19), 6198–6204. https://doi.org/10.1128/AEM.01054-09.

Donohoe, D. R., Holley, D., Collins, L. B., Montgomery, S. A., Whitmore, A. C., Hillhouse, A., ... Bultman, S. J. (2014). A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. *Cancer Discovery, 4*(12), 1387–1397. https://doi.org/10.1158/2159-8290.CD-14-0501.

Ekmekcioglu, C., Wallner, P., Kundi, M., Weisz, U., Haas, W., & Hutter, H.-P. (2018). Red meat, diseases, and healthy alternatives: A critical review. *Critical Reviews in Food Science and Nutrition, 58*(2), 247–261. https://doi.org/10.1080/10408398.2016.1158148.

FAO. Small-scale sausage production. From http://www.fao.org/3/x6556e/X6556E05.htm (retrieved September 17, 2019).

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society, 74*(01), 13–22. https://doi.org/10.1017/S0029665114001463.

Gill, H. S., & Rutherfurd, K. J. (2001). Probiotic supplementation to enhance natural immunity in the elderly: Effects of a newly characterized immunostimulatory strain Lactobacillus rhamnosus HN001 (DR20™) on leucocyte phagocytosis. *Nutrition Research, 21*(1), 183–189. https://doi.org/10.1016/S0271-5317(00)00294-3.

Gilmore, L. A., Walzem, R. L., Crouse, S. F., Smith, D. R., Adams, T. H., Vaidyanathan, V., ... Smith, S. B. (2011). Consumption of high-oleic acid ground beef increases HDL-cholesterol concentration but both high- and low-oleic acid ground beef decrease HDL particle diameter in normocholesterolemic men. *The Journal of Nutrition, 141*(6), 1188–1194. https://doi.org/10.3945/jn.110.136085.

Haug, A., Nyquist, N. F., Mosti, T. J., Andersen, M., & Høstmark, A. T. (2012). Increased EPA levels in serum phospholipids of humans after four weeks daily ingestion of one portion chicken fed linseed and rapeseed oil. *Lipids in Health and Disease, 11*(1), 104. https://doi.org/10.1186/1476-511X-11-104.

Jahreis, G., Vogelsang, H., Kiessling, G., Schubert, R., Bunte, C., & Hammes, W. P. (2002). Influence of probiotic sausage (Lactobacillus paracasei) on blood lipids and immunological parameters of healthy volunteers. *Food Research International, 35*(2), 133–138. https://doi.org/10.1016/S0963-9969(01)00174-0.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), e1. https://doi.org/10.1093/nar/gks808.

Lahtinen, S. J., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., ... Ouwehand, A. C. (2012). Probiotic cheese containing Lactobacillus rhamnosus HN001 and Lactobacillus acidophilus NCFM® modifies subpopulations of fecal lactobacilli and Clostridium difficile in the elderly. *Age, 34*(1), 133–143. https://doi.org/10.1007/s11357-011-9208-6.

Lin, H. V., Frassetto, A., Kowalik, E. J., Jr, Nawrocki, A. R., Lu, M. M., Kosinski, J. R., ... Marsh, D. J. (2012). Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS ONE, 7*(4), e35240. https://doi.org/10.1371/journal.pone.0035240.

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology, 71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005.

Martínez, J., Nieto, G., & Ros, G. (2014). Total antioxidant capacity of meat and meat products consumed in a reference 'Spanish standard diet'. *International Journal of Food Science & Technology, 49*(12), 2610–2618. https://doi.org/10.1111/ijfs.12577.

Olmedilla-Alonso, B., Jiménez-Colmenero, F., & Sánchez-Muniz, F. J. (2013). Development and assessment of healthy properties of meat and meat products designed as functional foods. *Meat Science, 95*(4), 919–930. https://doi.org/10.1016/j.meatsci.2013.03.030.

Olusi, S. (2002). Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans. *International Journal of Obesity, 26*(9), 1159–1164. https://doi.org/10.1038/sj.ijo.0802066.

Pastoriza, S., Roncero-Ramos, I., Rufián-Henares, J.Á., & Delgado-Andrade, C. (2014). Antioxidant balance after long-term consumption of standard diets including bread crust glycated compounds by adult rats. *Food Research International, 64*, 106–113. https://doi.org/10.1016/j.foodres.2014.06.004.

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

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C. G., & Salazar, N. (2016). Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00185.

Rubio, R., Jofré, A., Aymerich, T., Guàrdia, M. D., & Garriga, M. (2014). Nutritionally enhanced fermented sausages as a vehicle for potential probiotic lactobacilli delivery. *Meat Science, 96*(2), 937–942. https://doi.org/10.1016/j.meatsci.2013.09.008.

Saxelin, M., Lassig, A., Karjalainen, H., Tynkkynen, S., Surakka, A., Vapaatalo, H., ... Hatakka, K. (2010). Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *International Journal of Food Microbiology, 144*(2), 293–300. https://doi.org/10.1016/j.ijfoodmicro.2010.10.009.

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

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology, 12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60.

de Souza, R. J., Mente, A., Maroleanu, A., Cozma, A. I., Ha, V., Kishibe, T., ... Anand, S. S. (2015). Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: Systematic review and meta-analysis of observational studies. *BMJ, 351*, h3978. https://doi.org/10.1136/bmj.h3978.

Stewart, A., Marfell-Jones, M., Olds, T., & Ridder, J. (2011). *International standards for anthropometric assessment.* ISAK.

Thøgersen, R., Castro-Mejía, J. L., Sundekilde, U. K., Hansen, L. H., Hansen, A. K., Nielsen, D. S., & Bertram, H. C. (2018). Ingestion of an inulin-enriched pork sausage product positively modulates the gut microbiome and metabolome of healthy rats. *Molecular Nutrition & Food Research, 62*(19), 1800608. https://doi.org/10.1002/mnfr.201800608.

Tikkanen, M. J., Högström, P., Tuomilehto, J., Keinänen-Kiukaanniemi, S., Sundvall, J., & Karppanen, H. (2001). Effect of a diet based on low-fat foods enriched with non-esterified plant sterols and mineral nutrients on serum cholesterol. *The American Journal of Cardiology, 88*(10), 1157–1162. https://doi.org/10.1016/S0002-9149(01)02053-7.

Verma, A. K., & Banerjee, R. (2010). Dietary fibre as functional ingredient in meat products: A novel approach for healthy living — a review. *Journal of Food Science and Technology, 47*(3), 247–257. https://doi.org/10.1007/s13197-010-0039-8.

Vuyst, L. D., Falony, G., & Leroy, F. (2008). Probiotics in fermented sausages. *Meat Science, 80*(1), 75–78. https://doi.org/10.1016/j.meatsci.2008.05.038.