Official Title: Evaluation of the Beneficial Health Effects of an Additive-free Meat Product in Healthy Subjects (HIPOCARNE**)**

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1.1. Trial Design

This study consisted of a double-blind, randomised, controlled, unicentric trial with two parallel arms depending on the cooked meat product consumed (control and reformulated), with a duration of 5 weeks. The study was approved, according to the Declaration of Helsinki, by the Bioethics Committee of the University of Murcia (UMU) with code 550/2023. The research took place at the Department of Nutrition and Bromatology of the Faculty of Veterinary Medicine of the University of Murcia. Investigators provided a detailed explanation of the protocol to participants, and those choosing to participate gave their informed consent. Each participant received a unique code based on their arrival order. Following this, an external researcher then assigned the participants to the different groups with the aim of having two similar groups based on sex, age, and IMC. The specific group assignments were not known to either the researchers or the participants.

Three weeks before the start of the study, a recruitment phase was carried out, in which potential participants were recruited and informed about the details of the study. From a total of 63 individuals initially recruited, fifty-eight volunteers completed the trial. Participants who abandoned the trial were for personal reasons unrelated to the study. Two visits were made during the experimental phase of the intervention: the initial and the final visit. At both visits, blood, faeces, and urine samples were obtained from participants, and anthropometric measurements were also taken. Two deliveries of 15 blister packs of 100g control or reformulated cooked meat product were made, at baseline and after 17 days. Individuals consumed one blister pack per day for 6 days per week. Throughout the trial, subjects recorded their complete diet in a collection notebook, which was given to the researchers at the end of the study.

1.2. Participants

The study comprised a total of 58 subjects divided into two groups of 29 individuals with similar characteristics (Table 2). To participate in the study, subjects were required to be between 18 and 65 years of age and body mass index (BMI) between 18.5 and 30 kg/m2. Participants also had to avoid all of the exclusion criteria: use of antibiotics in the last 3 months; use of medication for hypertension or for the treatment of hyperlipaemia; consumption of pre- or probiotic supplements; consumption of omega-3 or omega-6 supplements; eating , intestinal or similar problems; diabetes mellitus, chronic kidney disease, liver disease, infectious diseases or any type of cancer; having donated blood in the last month; having undergone surgery within the last three months; abusive alcohol consumption; being vegetarian; being pregnant; being a smoker; and participation in other clinical trials in the last 3 months.

Table 1. Distribution of volunteer demographics

	Control	Intervention	p-value[1]
Age (years)	26.6 ± 11.5	26.7 ± 11.7	0.97
Men	15	14	1.00
Women	14	15	
BMI (kg/m2)	23.7 ± 2.7	23.6 ± 2.8	0.98
Weight (kg)	66.6 ± 11.8	68.4 ± 12.7	0.58
Fat mass (%)	26.0 ± 9.0	26.9 ± 9.0	0.71
Total colesterol (mg/dl)	175.8 ± 34.6	178.0 ± 30.9	0.80
Oxidized LDL (ng/mL)	238.6 ± 52.1	233.6 ± 52.4	0.71

[1] p-values between control and intervention groups were examined using a one-way analysis of variance (Tukey's test). The results are shown as mean ± SD (n=29).

1.3. Study Analyses

1.3.1. Anthropometric measurements

The anthropometric analysis of the participants followed the protocol of the International Society for the Advancement of Kinanthropometry (ISAK). The following measurements were taken with the corresponding equipment: height (213 measuring rod, Seca deutschland, Germany), weight (body fat monitor with scale BF400, OMRON Healthcare, Japan), body mass index (BMI) and percentage of fat mass (body fat monitor BF306, OMRON Healthcare, Japan). Abdomen circumference was also measured (201 antrophometric tape, Seca deutschland, Germany). In addition, hip and waist circumferences were measured to calculate the waist:hip ratio (WHR) (cm waist:cm hip), which is used to evaluate the development cardiovascular disease.

1.3.2. Blood samples

Subjects fasted for 12 hours prior to blood collection and were not allowed to perform moderate-intensity exercise in the previous 24 hours. Blood samples were collected from the antecubital vein in gel and clot activator tubes (Vacutest Kima Srl, Arzergrande, Italy). These samples were centrifuged at 3500 rpm for 5 min at 4 °C to separate the serum from the cell fraction. After collection, the serum portions were stored at -80 °C until further analysis.

Biochemical markers such as basal glucose, GOT, GPT, hs-CRP and lipid markers (triglycerides (TG), LDL-C, HDL-C, and total cholesterol (TC)) were measured using a clinical chemistry analyzer (BA400 Biosystems).

Interleukin (IL) 1β, IL-6, IL-10, and tumour necrosis factor-alpha (TNFa) were analysed using commercially available kits (Milliplex Human High Sensitivity T Cell; Life Science, Darmstadt, Germany) according to manufacturer's indications. Catalase activity was assessed by an automatic method previously described by Slaughter et al. FRAP measurement was based on the method described by Benzie and Strain, with some modifications. Glutathione peroxidase (GPx) was determined using a commercially available method (Ransel, Randox Laboratories Limited, Crumlin, United Kingdom). Catalase, FRAP and GPx were measured in an automated chemistry analyzer (Olympus AU400, Olympus Diagnostica Europe GmbH, Ennis, Ireland). The TBARS assay was determined following the method by Buege and Aust. Oxidized Low Density Lipoprotein (ox-LDL) was determined using commercially available ELISA Kit (Human ox-LDL(Oxidized Low Density Lipoprotein) ELISA Kit, Texas, USA). TBARS and ox-LDL were measured using a microplate reader (Powerwave XS, Biotek instruments).

1.3.3. Stool samples

Faecal samples were collected by participants at home, using a sterile kit provided to them previously. Once the sample was collected, participants stored it under refrigeration (for no more than 24-48h) until it was delivered to the researchers. Once received, it was stored at -20°C until the corresponding analyses were performed.

1.3.3.1. Sample preparation for FRAP and short chain fatty acids analysis.

The faecal sample was previously defrosted at 4°C for 24h. Then, samples were diluted 1:10 with 0.1M phosphate buffer (2.33 g PO_4HNa_2 and 14.03 g PO_4H2Na in 2L of water, pH 7.4) and sterilised. The mixture was centrifuged for 20 min at 5000 rpm in order to collect the supernatant and centrifuged again for 10 min. The supernatant was collected again and separated into 2 fractions for SCFA and FRAP assay analysis. The sample for AGCC was stored at -80°C until use. The FRAP assay was performed on the same day of sample preparation.

1.3.3.2. FRAP assay.

The antioxidant capacity of faeces was measured using the FRAP assay, according to the procedure described by Benzie and Strain. For this purpose, the supernatant portion was previously diluted with water at 1:10 dilution.

1.3.3.3. Short chain fatty acids analysis

The production of SCFAs was assessed according to the procedure described in Panzella et al. with some modifications. The supernatant portion was filtered through 0.20 µm PTFE filters and analysed by reversed-phase HPLC with diode array detector (1260 Infinity II LC System, Agilent, Santa Clara,

CA, USA). The results were obtained in ppm and converted to mmol/kg faeces, expressing the result as the increase of SCFAs in 24 hours.

1.3.3.4. DNA extraction and metagenomic sequencing

Bacterial genomic DNA was isolated from each fermentation pellet sample using NZY Soil gDNA Isolation kit (Nzytech, Lisboa, Portugal). Sequencing of the V3 and V4 regions of the 16S rRNA gene was performed on the MiSeq (Illumina, Essex, UK) instrument using 2 x 300 bp reads. The oligonucleotide primers targeting the 16S rRNA gene V3 and V4 regions were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3', respectively. The 16S-V4 sequencing library was first reviewed with FastQC for overall quality assessment, and the libraries were processed in R package DADA2 (v.1.8.0.). Reads were quality trimmed with the "filterAndTrim" function with "maxEE (2,5)" and reads below 165 bp were discarded. Forward and reverse reads are merged below to generate a table of sequences, and the resulting Amplicon Sequence Variants (ASVs) were subjected to de novo chimera detection, using DADA2 and any artifacts were removed.

1.3.3.5. Bioinformatic analysis

For bacteria taxonomic assignment, ASVs were queried against the Silva database v.132 [The SILVA ribosomal RNA gene database project: improved data processing and web-based tools] using IDTAXA implemented in the R package DECIPHER. Sequences identified as non-bacterial were discarded.

The abundance matrix, the taxonomy assignment and the metadata obtained from each sample were merged and imported with the phyloseq v3.12 package. Alpha diversity was calculated in R using the phyloseq package, and several alpha indices were generated, such as ASV numbers, Chao1, ACE, Shannon, Simpson, InvSimpson, and Fisher and plotted using the function "plot richness". Beta diversity was calculated using weighted and unweighted Unifrac distances. To test for significant differences in community composition among different seasons, permutational multivariate analysis of variance using distance matrices (PERMANOVA) was conducted using the Adonis function in the R package, and the results were visualized by Principal Coordinates Analysis (PCoA).

1.3.4. Urine samples

Participants collected the first urine sample of the morning in a sterile container given to them previously. Once the sample was collected, participants stored it under refrigeration (for no more than 24-48h) until it was delivered to the researchers. Samples were stored at -20°C until use.

1.3.4.1 Nitrates and nitrites analysis

Urine samples were used to analyse nitrate and nitrite content. The samples were diluted 1:10, then 1 ml of Carrez I ($K_4[Fe(CN_6)\cdot 3H_20$) and 1 ml of Carrez II ($ZnSO_4$ x $7H_2O$) were added and stirred for 30 minutes at room temperature. After this time, the mixture was filtered through a 150 mm Ø filter paper. Finally, the samples were analysed in a SmartChem.

1.4. Statistical analysis

Data analysed were expressed as mean ± standard deviation (SD). Student's t-test was used to analyse significant differences over time (baseline and final). To analyse the differences between groups in the evolution of the different variables, an analysis of variance (ANOVA) was performed for repeated measures, with time (baseline and final) as the within-subject factor, and the product consumed (Control and Intervention) as the between-subject factor. Tukey´s test was carried out for post hoc analysis. Cross-tabulation and chi-square tests were conducted to determine the perception of respondents on food additives based on demographic data.

Data processing and statistical analysis were performed using SPSS version 28.0 (IMB Corp., Armonk, NY, USA) with a significant level of $p \leq 0.05$.

INFORMED CONSENT STATEMENT

Ms. /Mr. ..... of ..... years of age and with ID card No. ..... declares that she has been informed about the benefits that my participation could bring to cover the objectives of the research project entitled "RESEARCH AND DEVELOPMENT OF MINIMALLY PROCESSED AND HYPOALLERGENIC MEAT FOODS (HIPOCARNE)", directed by Gaspar Ros Berruezo, with the aim of determining the effects of meat products without synthetic additives at blood, urine and feces level, with certificate of the Ethical Committee of the UM, contact telephone: +34 968 364794 and e-mail: gros@um.es.

I have been informed of the possible prejudices that participation in this project may have on my well-being and health by having read the participant information sheet about the study in question.

I have been informed that my personal data will be subject to processing for scientific research purposes by the University of Murcia. The period of conservation of the data will be the minimum necessary to ensure the realization of the study or project. However, my identification data, to ensure optimal privacy conditions and when the study procedure allows it, could be subjected to anonymization or pseudo-anonymization, in any case, the identifying information that could be collected will be deleted when it is not necessary.

I have been informed that any inquiry regarding the processing of personal data in this study or to request access, rectification, deletion, limitation or opposition may be directed to the address protecciondedatos@um.es likewise, I have been informed of my right to file a complaint with the Spanish Data Protection Agency.

I have been given an information sheet and a copy of this informed consent, dated and signed.

Taking this into consideration, I hereby give my consent for this data collection and sample to take place and to be used for the purposes specified in the project.

Full name Participant Signature of Participant Date

Signature of Researcher: