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Effect of the Consumption of Lean Red Meat from Beef (Pirenaica Breed) Versus Lean White Meat (Chicken) on the Gut Microbiota: A Randomized Cross-Over Study in Healthy Young Adults

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ABSTRACT

Limited evidence exists regarding the impact of meat consumption on the human gut microbiota, with factors such as animal source, production system, and cooking methods often overlooked. This study evaluates the effect of Pirenaica breed beef or conventional chicken-based diets on the gut microbiota of healthy adults. A randomized cross-over controlled trial with two 8-week periods, separated by a 5-week washout, is carried out. Participants consume either Pirenaica breed beef or chicken three times per week with their diet. Stool samples are collected at the beginning and end of each period. Gut microbiota is analyzed via amplification and sequencing of V3–V4 regions of 16S rRNA. Alpha diversity and relative abundances at phylum and genus levels are calculated. Sixteen participants are included (mean age 20.12 ± 2.36 years). Both diets induce modest changes in microbial composition, with no significant differences between groups. At the phylum level, *Bacillota* increased, while *Synergistota*, *Chloroflexota*, and *Pseudomonadota* decreased. Alpha diversity parameters declined significantly after the chicken-based diet, although overall reduction in microbial diversity was observed across both interventions. The consumption of lean red meat or lean white meat as part of habitual diet produces similar effects on the gut microbiota.

1 | Introduction

The gut microbiota has emerged as a key factor in human health, as evidence indicates that intestinal bacteria play a role in disease etiology [1]. Similarly, diet is recognized as a potent modulator of microbiome composition, diversity, and metabolic activity [2]. Understanding how diet influences microbial communities could provide insights into the mechanisms underlying the association between food consumption and health outcomes [3]. However, it is critical to note that the quality of food and its nutrients rather than quantity, should be considered the most important factors [4].

Meat consumption has been in the spotlight in recent years as it has been criticized from ethical, environmental, and health perspectives [5]. Both unprocessed and processed red meat consumption have been reported to be risk factors for cardiovascular diseases and type 2 diabetes mellitus [6]. In this context, it is crucial to understand the biological function of the nutrients included in a variety of types of meat, which have different effects on health [7]. There is no consistent evidence of the association between meat consumption and gut microbiota-related outcomes in humans. A systematic review of meat consumption and the gut microbiota revealed that bacterial composition may be affected differently at lower taxonomic levels, such as genus or species level, or according to overall alpha diversity [8]. Moreover, most of these studies did not consider factors such as animal sources, feeding, processing, or cooking methods, which could be crucial for determining the nutritional profile of meat products [9–11].

In recent years, consumption patterns for animal-derived products have evolved considerably. In addition to their flavor, shape, and tenderness, consumers are particularly interested in their origin, potential health effects, and sustainable production methods [12]. Rural areas contribute to the promotion of local agriculture and livestock farming systems, providing an optimal environment for sustainable meat production [12]. The Pirenaica breed is an autochthonous beef breed closely linked to the Pyrenees Mountains. It follows an extensive husbandry system model and is raised on local feedstuffs and collective grazing lands. Livestock farming systems in some specific areas are based on sustainable and local practices. It is crucial to provide safe and high-quality meat, which will have positive effects on consumers' health.

The present study aimed to evaluate the effect of the consumption of lean red meat from beef (Pirenaica breed) in healthy young adults on their gut microbiota composition, compared with lean white meat (chicken).

2 | Experimental Section

2.1 | Study Design

This was a sub-study in the context of the DIETAPYR2 study (*Innovaciones aplicadas a la cadena productiva pirenaica de vacuno para valorizar una carne identificable por el consumidor*). This was a randomized crossover controlled trial (NCT04832217, clinicaltrials.gov) that included two experimental periods lasting 8 weeks. Participants were randomly assigned either a Pirenaica breed beef or a conventional chicken-based diet. They were instructed to consume the allocated products three times per week within their usual diet. A 5-week washout period was implemented between the two intervention phases to minimize residual effects from the previous diet, and to allow participant's baseline status to be restored before staring the second phase.

Figure 1 shows the study design. Two weeks before the beginning of the study, participants were contacted and informed consent was obtained. At the first visit, medical history, sociodemographic, and lifestyle behavior questionnaires were completed. Additionally, anthropometric, blood pressure (systolic and diastolic blood pressure) and heart rate measurements were taken. Following an overnight 12-h fast, blood was drawn. As a voluntary test of participation, stool samples were collected.

At the initial visit, participants were randomly assigned to a beef (Pirenaica breed) (Intervention Group) or chicken-based diet (Control Group). After the first experimental period, participants attended the second visit in the afternoon and the following morning, during which blood extraction and stool samples were collected under the same conditions. Following the crossover design, participants were switched to an alternative diet, either a chicken or Pirenaica breed-based diet, for the second 8-week period. Detailed experimental explanations were previously published [13].

2.2 | Participants

The participants were healthy young adults over the age of 18 years with no chronic, metabolic, endocrine, or nutrition-related diseases. Volunteers who completed stool sample collection at all four time points were included in this sub-study. Three university accommodation halls, two of them in Huesca (Colegio Mayor Universitario Ramón Acín and Residencia Misioneras del Pilar) and one in Zaragoza (Residencia Baltasar Gracián) (Spain), were the recruitment settings. The study protocol was approved by the Research Ethics Committee of the Government of Aragón (Spain) (N° 17/2018, 11 October 2018). All procedures were performed following the ethical guidelines of the Edinburgh revision of the 1964 Declaration of Helsinki (2024) [14].

2.3 | Dietary Intervention

Participants were urged to follow their usual diet and randomly assigned to consume allocated food products three times per week. The nutritional value of both diets, including protein and fats sources, was similar in both groups. Participants consumed 150 g of boneless chicken or Pirenaica breed beef (200 g with bones). The Pirenaica breed beef was obtained from entire young bulls that were raised through an extensive husbandry farming system for approximately 6 months. They were fed using cereal-based concentrates and cereal straw until they reached approximately 13.5 months of age. The nutritional composition of the Pirenaica breed beef has been previously analyzed [15]. The meat from Pirenaica breed young bulls is lean and is a

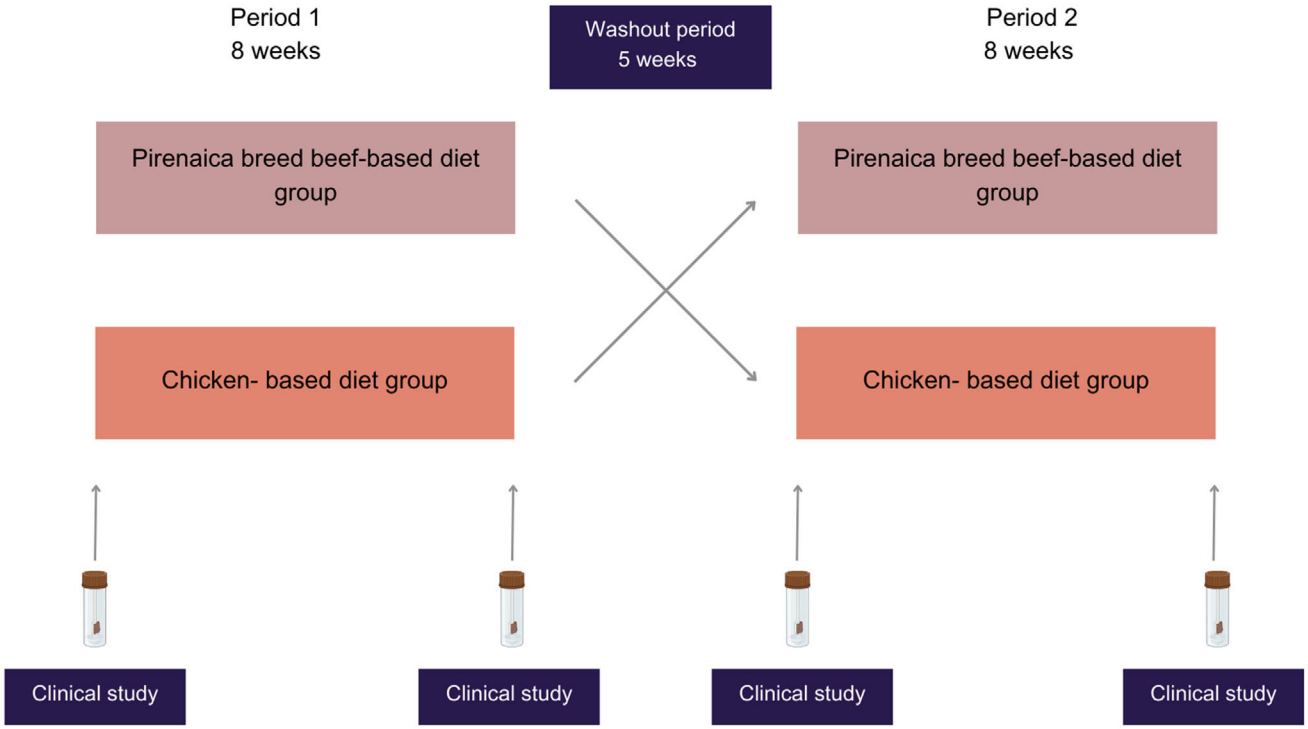

FIGURE 1 | Study design. A total of 16 participants were randomly assigned to start with either the Pirenaica breed beef or chicken-based diet for 8 weeks, followed by a 5-week washout period, after which they changed to the alternative diet for another 8 weeks. Stool samples were collected at the beginning and end of each period.

source of potassium and phosphorus, high in protein, zinc, and vitamins B3, B6, and B12, and low in sodium [15]. The chicken meat was sourced from conventional poultry farms. Meat cuts included loin, silverside and brisket for beef and thigh, drumstick, and breast. There were no analysis of macronutrient and micronutrient intakes between accommodation halls since the menus were similar. To ensure the same conditions, the study products were served during lunchtime and each chef in the three student accommodation halls was provided with cooking methods instructions. The cooking methods included breading, stewing, and grilling for beef and breading, stewing and roasting for chicken [13]. Dietary assessment was conducted for each participant using a food frequency questionnaire, at the beginning and end of each 8-week period. The questionnaire was previously validated [16, 17]. To evaluate overall dietary habits beyond the assigned products and account for potential variability in total intake, the Diet Quality Index (DQI) [18] was also calculated for each participant. This composite index is widely used to assess overall quality of the diet and includes three components: dietary quality, dietary diversity, and dietary equilibrium. A detailed description of the index is available in the previously published manuscript based on the same cohort [19].

2.4 | Stool Sample Collection

Stool samples were collected by participants at the beginning and end of each intervention period. The samples were frozen at −20 °C immediately after collection and stored at −80 °C until analysis.

2.5 | Extraction of DNA

Bacterial DNA was extracted from frozen samples using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions. Fecal samples were mixed with 1 mL InhibitEX buffer in SK38 tubes and processed by using the Precellys 24 homogenizer for 2 × 30 s at 6500 rpm and 10 s delay between cycles. Lysis was completed at 95 °C for 5 min. Finally, DNA was eluted in 40 µL elution ATE buffer. Once DNA was extracted, DNA concentrations were measured with Qubit 4.0 fluorometer (Invitrogen) and dsDNA HS (high sensitivity) Assay Kit (Invitrogen). DNA purity was assessed by measuring the A260/A280 with NanoDrop ND-1000 Spectrophotometer V3.0.1 (Thermo Scientific Waltham, MA, USA) [20].

2.6 | Sequence Analysis and Bioinformatics

The extracted DNA was amplified by PCR using specific primers for 16S, targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene [21], as well as a primer for dimer cleanup. Using the Illumina Novaseq 6000 platform and 250 PE, the libraries were sequenced.

The primer sequences for 341F and 806R are commonly used to amplify the V3-V4 region of the bacterial 16S rRNA gene. The 341F forward primer sequence is 5′-CCTACGGGAGGCAGCAG-3′ and the 806R reverse primer sequence is 5′-GGACTACHVGGGTWTCTAAT-3′. To confirm that there was no contamination, a negative control containing water was obtained. Using Illumina bcl2fastq2 Conversion

Software v2.20, the raw sequences were demultiplexed, and raw data was imported into QIIME 2 2023.7 open-source software [22] using the q2-tools-import script which uses Paired-end with Quality input format. The denoising process was performed with DADA2 [23], which uses a quality-aware model of Illumina amplicon errors to obtain a distribution of sequence variances, each differing by one nucleotide. To truncate and trim the forward reads at positions 240 and 7, the q2-dada2-denoise script was used following the retrieval of the quality scores. After truncating the reverse reads at position 240, we trimmed them at position 7. We removed chimeras using the consensus filter, which detects chimeras in samples individually and removes those found in a sufficient number of samples. In addition, forward and reverse reads are merged during this step. We constructed phylogenies using FASTTREE2 (via q2-phylogeny) [24], using all amplicon sequence variants (ASVs) aligned with MAFFT [25] via q2-alignment. With a similarity threshold of 99%, a naive Bayes taxonomy classifier was employed (via q2-feature-classifier) [26] against the SILVA 16S V3-V4 v138_99 [27]. The data filtering process excluded samples with fewer than 10,000 reads. The diversity of the samples was examined using the vegan library of R programming language version 4.2.2 (The R Foundation for Statistical computing, Vienna, Austria) [28]. In the present study, alpha diversity indices such as Fisher [29], Shannon [30], Simpson, inverse Simpson [31], and species richness indices, as well as Pielou's evenness index were examined [32].

2.7 | Functional Profiles

To explore the functional profiles of the sequenced samples, the PICRUSt2 tool was employed [33]. This method infers metagenomic content by placing identified phylotypes into a reference phylogenetic tree built from approximately 20,000 full-length 16S rRNA gene sequences obtained from the Integrated Microbial Genomes (IMG) database. Functional characteristics of theses genomes were performed using Clusters of Orthologous Groups of Proteins (COG) and the Enzyme Commission numbers (EC) databases. To reconstruct metabolic pathways, EC numbers were mapped to MetaCyc reactions, allowing the inference of MetaCyc pathway abundances. These were calculated as the harmonic mean of the contributing key reactions within each sample. Final pathway predictions were adjusted for 16S rRNA gene copy number and scaled to the relative abundance of each phylotype, providing estimated abundances for each predicted gene family [20].

2.8 | Statistical Analysis

The sample size for the global study was calculated assuming a two-tailed alpha error of 0.05, with a statistical power of 90% and a 20% loss to follow-up; the required sample size was 60 participants. Due to the exploratory nature of the results of gut microbiota data, combined with the lack of previous research and limitations in reproducibility across studies, it proved challenging to perform an a priori power calculation for this sub-study. The power calculation was performed for the estimated differences between the intervention and the control groups, along with the variance calculation of the crossover study design. Sociodemographic data are reported as mean ± standard deviation (SD) for continuous variables or as the number of cases and percentages for categorical variables. Baseline differences were calculated with the Student's t-test for continuous variables or the Chi^2 test for categorical variables. All quantitative data were tested for normality assumptions with the Shapiro-Wilk test. The gut microbiota composition was analyzed for the relative abundance at the phylum and genus levels. The updated taxonomic designations for *Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria*, and *Synergistetes* are referred to as *Actinomycetota*, *Bacteroidota, Chloroflexota, Bacillota, Pseudomonadota*, and *Synergistota*, respectively [34]. To assess differences between the beginning and the end of the experimental period in the relative abundance of bacteria (phylum and genus levels), as well as for the alpha diversity indices, a paired Wilcoxon signed rank test was applied. A linear mixed-effects model for repeated measures was applied to evaluate intervention effects. The model included the intervention/control condition, period, sequence, and DQI values as fixed effects, with participants as random effects. The dependent variable was defined as the difference between the post- and pre-intervention values for each individual. This was to account for within-subject correlations and repeated measures over time. This approach is particularly suitable for crossover studies with small sample sizes, as it improves statistical power and controls for individual variability [35]. We conducted a robust linear mixed model analysis using the nlme package as it employs modern, efficient linear algebra methods, avoiding the constraints of homogeneity of variances, sphericity, and other distributional assumptions. To better illustrate the control and intervention effects, the intercept was set to zero, and the random forest displayed the beta coefficients along with their 95% confidence intervals. Statistical analyses were conducted in R Studio (Integrated Development Environment for R. Posit Software, PBC, Boston, MA) with the nlme and forest libraries and the Statistical Package for the Social Sciences (SPSS Version 25; SPSS, Chicago, IL, USA), with visualizations generated in GraphPad Prism (Version 9). Based on a Rivera-Pinto analysis, it is possible to identify microbial signatures, that is, groups of microbes that can predict particular phenotypes of interest. Based on an individual's unique microbiota, these microbial signatures may be used to diagnose, prognosticate, or predict therapeutic responses. To identify microbial signatures, we modelled the response variable as well as selected the taxa that yield the highest level of classification or prediction accuracy. As part of the Rivera-Pinto method and Selbal algorithm, we evaluated specific signatures at the phylum and genus levels to select a sparse model that adequately explains the response variable. The microbial signatures are calculated using geometric means based on data collected from two groups of taxa. The name implies that these groups are those with relative abundances, or balances, that are related to the response variable of interest [36].

3 | Results

Among the 52 participants who initially agreed to participate, 47 completed the study with acceptable follow-up compliance. Data from 16 participants who collected the stool samples at all four study points were ultimately analyzed for this sub-study. The mean age of participants was 20.16 years (± 2.43), 68.8% females. The average body mass index (BMI) was

23.75 kg/m^2 (± 2.68). No significant differences in sociodemographic characteristics, including age, BMI, or parental education, were observed between participants who began the study with the Pirenaica breed beef-based diet and those who started with the chicken-based diet. The DQI was assessed at the beginning and end of each intervention period. Baseline DQI values were comparable between groups. However, a significant reduction was observed after chicken-based diet (p = 0.009). The overall variation between both groups was also statistically significant (p = 0.0025) (see Table S1). Additionally, the baseline gut microbiota profile for all participants is provided in Table S2.

Table 1 presents the phylum-based abundances and alpha diversity indices, including the median with interquartile range before and after each intervention period. Variation and mean differences between the experimental periods were also considered. After 8 weeks of intervention, the Pirenaica breed beef-based diet group exhibited a modest decrease in the relative abundance of the phyla *Bacteroidota, Chloroflexota, Pseudomonadota*, and *Synergistota*, along with an increase in the relative abundance of *Actinomycetota*. The dominant phylum, *Bacillota*, remained relatively stable. However, these changes did not reach statistical significance ($p < 0.05$), with the exception of *Chloroflexota* (p = 0.043), suggesting stability across in response to the dietary interventions. Additionally, no significant changes were observed in alpha diversity measures, including species richness, Fisher's alpha, Shannon, Pielou's evenness, Simpson, and the Inverse Simpson indices (Figure 2). A decrease in the relative abundance of the phyla *Actinomycetota*, *Bacteroidota*, *Chloroflexota*, *Pseudomonadota*, and *Synergistota* was observed after the chicken-based diet, with statistically reductions in *Chloroflexota* (p = 0.003) and *Synergistota* (p = 0.002). Remarkably, the chicken-based diet group exhibited a significant decrease in richness (p = 0.005), Fisher (p = 0.005) and Shannon indices (p = 0.034). When comparing the variations observed before and after each intervention in both dietary groups, no statistically significant differences were observed at the phylum level or in the alpha diversity indices. Figure 3 shows the relative abundances at the phylum level of stool samples provided by all participants at the beginning and end of the two periods of the study.

At the genus level (see Table S3), a significant increase in the relative abundance of *Blautia* (p = 0.044) was observed after the Pirenaica breed beef-based diet, while *Coprococcus* (p = 0.044), *Eubacterium hallii* group (p = 0.049), and *Roseburia* (p = 0.013) showed a significant decrease. The chicken-based diet group exhibited significant decreases in the relative abundance of *Lachnopiraceae NK4A136* group (p = 0.010), *Lachnospira* (p = 0.049), *Eubaterium eligens* group (p = 0.009), *Adlercreutzia* (p = 0.006), *GCA-900066575* (p = 0.039), *Saccharofermentans* (p = 0.047), *Thermovirga* (p = 0.003), and *Bacteroides* (p = 0.048). Conversely, significant increases were observed in *Sphingomonas* (p = 0.005), *Family XIII AD3011* group (p = 0.004), and *Ruminococcaceae UCG-009* (p = 0.034). Interestingly, *Blautia* also showed a significant increase in this groups (p = 0.034).

Table 2 summarizes the findings from the linear mixed model analysis, which assessed the effects of dietary interventions (Pirenaica breed-beef or chicken-based diets), period, sequence, and DQI values at the phylum-level abundances and alpha diversity indices. After adjusting for DQI, both dietary interventions continued significantly influenced specific phyla, with consistent directional trends. Both diets were associated with an increase in *Bacillota*, and reductions in *Chloroflexota* and *Synergistota*, with all changes remaining statistically significant ($p < 0.005$). Regarding alpha diversity, both interventions led to significant reductions in richness, Fisher's index, and the Shannon index; a significant reduction in the Inverse Simpson index was observed only following the chicken-based diet. Significant period-related effects were observed for several variables. During the second period, the relative abundance of *Bacillota* decreased (p = 0.005) and significant increases in *Synergistota* ($p < 0.001$) and *Chloroflexota* ($p < 0.001$). Alpha diversity indices showed notable variability between periods, with significant increases recorded in richness (p = 0.007), Fisher's index (p = 0.007), Shannon (p = 0.030) and the Inverse Simpson index (p = 0.015) during the second period compared to the first. A sequence effect was observed only for the Shannon index (p = 0.015) showed significant reductions associated with the sequence in which diets were administered.

3.1 | Bacteria Metabolic Pathways

The predicted metabolic profiles of the sequenced samples were assessed, focusing on pathways related to amino acid and polyamine metabolism, lipid metabolism, energy production, and fermentation. Overall, the chicken-based diet was associated with a broader reduction in microbial functional capacity, while the Pirenaica breed beef-based diet induced more limited changes. Following the Pirenaica breed beef-based diet, microbial functional activity remained relatively stable, except to L-methione biosynthesis pathways (p = 0.026) and L-methione biosynthesis II (p = 0.017). The chicken-based diet induced significant reductions in pathways related to aromatic amino acid biosynthesis (p = 0.026), fatty acid biosynthesis initiation (p = 0.010), and gluconeogenesis (p = 0.007). Moreover, reduced activity was observed in L-isoleucine degradation (p = 0.015) and fatty acid biosynthesis (p = 0.004) (see Table S3).

3.2 | Rivera-Pinto Analysis

Based on the geometric mean of data derived from two groups of taxa whose relative abundances correlate with the response variable, the Rivera-Pinto method [36] is used to determine a microbial signature. In the chicken-based diet group, *Coprococcus*, *Eubacterium eligens* group, and *Eubacterium hallii* group genera were most closely associated with the baseline (Figure 4A). A higher balance score was associated with a significant relative abundance of the *Coprococcus*, *Eubacterium eligens* group, and *Eubacterium hallii* group genera. This was in comparison to the *Sphingomonas*, *Streptococcus*, *Collinsella*, and *Ruminoclostridium* 5 genera. The *Coprococcus* 2 and *Agathobacter* genera, as well as the *Bacteroidota* phylum, were most closely associated with baseline in the Pirenaica breed beef-based diet group (Figure 4B). Significant relative abundances of *Coprococcus* 2, *Bacteroidota*, and *Agathobacter* were associated with a higher balance score. Comparatively to the *Phascolarctobacterium* and *Lachnospira* genera and the family *Ruminococcaceae*. In the comparison between the chicken-based diet group and Pirenaica breed

TABLE 1 | Relative abundances at phylum level and alpha diversity indices in the intervention and control groups.

	Pirenaica breed beef-based diet group, $n = 16$				Chicken-based diet group, $n = 16$				Mean differences in the variations between both groups		
	Before	After	Δ	p^a	Before	After	Δ	P^a	Mean difference	CI 95%	P^b
Sequences	213,560 (203,841–217,903)	212,831 (209,374–216,142)	592.5	0.959	206,240 (203,103–209,348)	209,374 (201,483–214,603)	2447.6	0.301	1855.125	−19,360.693; 23,070.9429	0.642
Phylum											
Actinomycetota	1.86 (1.26–2.56)	2.11 (1.43–2.57)	−0.02	0.938	3.05 (2.63–3.57)	2.54 (1.81–3.53)	−0.36	0.501	−0.35	−1.93; 1.24	0.642
Bacteroidota	0.04 (0.02–0.08)	0.01 (0.00–0.11)	−0.02	0.222	0.03 (0.01–0.12)	0.02 (0.00–0.05)	−0.03	0.109	−0.01	−0.05; 0.03	0.836
Chloroflexota	0.00 (0.00–0.59)	0.00 (0.00– 0.00)	−0.36	0.043	1.25 (0.16–1.53)	0.00 (0.00–0.16)	−0.82	0.003	−0.46	−1.15; 0.22	0.155
Bacillota	97.45 (94.87–98.07)	97.49 (96.84–98.35)	1.21	0.605	92.08 (90.76–94.71)	96.44 (93.60–97.40)	1.98	0.134	0.77	−3.02; 4.56	0.877
Pseudomonadota	0.10 (0.02–0.75)	0.07 (0.00–0.11)	−0.25	0.112	0.77 (0.15–1.07)	0.29 (0.10–0.68)	0.48	0.733	0.73	−0.57; 2.02	0.352
Synergistota	0.00 (0.00–0.62)	0.00 (0.00–0.00)	−0.41	0.068	1.51 (0.32–2.14)	0.00 (0.00–0.00)	−1.18	0.002	−0.77	−1.65; 0.11	0.079
Alpha diversity indices											
Richness	71.00 (66.00–79.25)	70.00 (66.25–75.00)	−5.13	0.737	98.00 (79.75–101.00)	71.00 (62.25–91.75)	−16.75	0.005	−11.36	−25.06; 1.80	0.079
Fisher's index	11.26 (10.33–12.84)	11.08 (10.38–12.02)	−1.01	0.717	16.56 (12.95–17.17)	11.26 (9.64–15.33)	−3.24	0.005	−2.23	−4.87; 0.40	0.098
Shannon index	3.40 (3.30–3.55)	3.32 (3.11–3.50)	−0.12	0.098	3.53 (3.40–3.68)	3.41 (3.05–3.65)	−0.18	0.034	−0.07	−0.22; 0.09	0.326
Pielou's evenness	0.79 (0.78–0.81)	0.78 (0.76–0.82)	−0.02	0.109	0.77 (0.76–0.80)	0.79 (0.73–0.80)	−0.01	0.877	0.01	−0.001; 0.04	0.255
Simpson index	0.93 (0.92–0.95)	0.92 (0.90–0.94)	−0.02	0.148	0.93 (0.92–0.95)	0.93 (0.88–0.95)	−0.02	0.148	−0.002	−0.02; 0.02	0.877
Inverse Simpson	14.82 (12.30–19.36)	12.53 (10.09–16.90)	−1.85	0.134	14.81 (11.80–20.51)	15.00 (8.63–18.87)	−1.83	0.098	0.01	−2.74; 2.77	0.717

Abbreviation: SD, standard deviation.

Note: Data are expressed as median and interquartile range; Δ: mean change (final-beginning) of each diet group.

[a]Multivariate contrast between the beginning and end of each corresponding food product (Pirenaica breed beef, $n = 16$ or chicken, $n = 16$).

[b]Multivariate contrasts between the variations (final-beginning) of both diet groups (Pirenaica breed beef or chicken). Significant differences ($p < 0.05$).

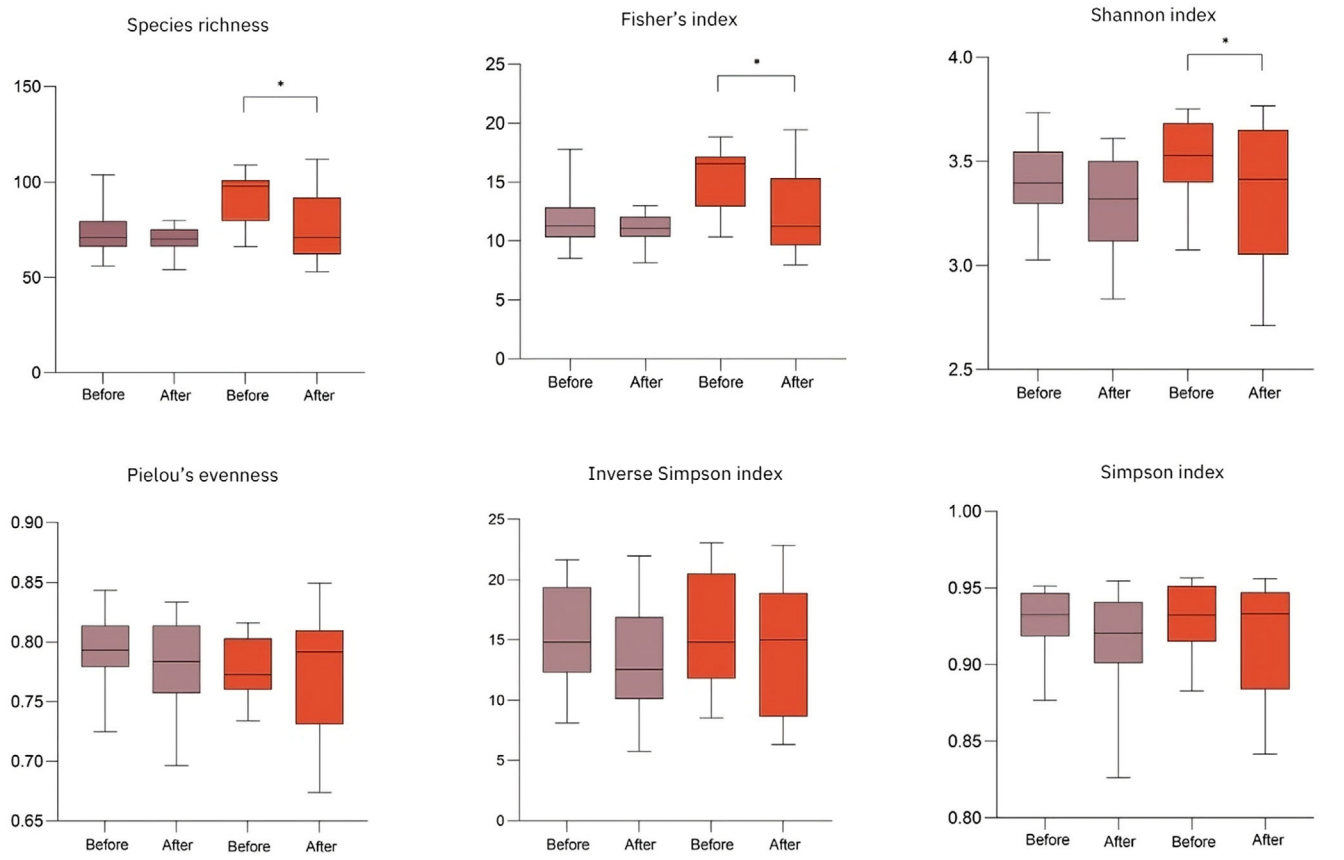

FIGURE 2 | Alpha diversity indices of the intervention and control groups. The data are expressed as the median and interquartile range according to the Wilcoxon test. Richness and Fisher's index, which quantify the relationship between the number and abundance of species, were calculated. For microbial diversity and evenness, Shannon, Pielou, Simpson, and inverse Simpson's indices were performed. Pirenaica breed beef-based diet group ($n = 16$); chicken-based diet group ($n = 16$). Box plots represent the interquartile range (25th–75th percentile); the line inside within each box indicates the median, and the whiskers extend to minimum and maximum values. * Indicates statistically significant differences ($p < 0.05$).

beef-based diet group at baseline, *Gracilibacter*, *Negativibacillus*, *Subdoligranulum*, and *Eubacterium hallii* group genera and the *Actinomycetota* phylum were most closely associated with the chicken-based diet group (Figure 4C). A higher balance score was associated with a significant relative abundance of *Gracilibacter*, *Negativibacillus*, *Subdoligranulum*, and *Eubacterium halli* group genera and the *Actinomycetota* phylum. This was compared to the *Coprococcus* 2, *Lachnospiraceae* ND3007 group, and CAG56 genera. At the end of the intervention, the *Pseudomonadota* phylum and *Ruminococcaceae* UCG013 genus were most closely associated with the chicken meat group (Figure 4D). Significant relative abundances of *Pseudomonadota* phylum and *Ruminococcaceae* UCG013 genus were associated with a higher balance score. Comparatively to the *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* uncultured, *Butyricicoccus*, *Negativibacillus*, and *Collinsella* genera.

4 | Discussion

In this study, the effects of an intensive intervention involving the inclusion of lean red meat from beef (Pirenaica breed) or lean white meat on gut microbiota composition and structure were examined. The gut microbiota influences critical processes such as digestion, nutrient absorption, and immune modulation; dysbiosis has been associated to various metabolic, autoimmune, and gastrointestinal diseases. While meat consumption has long been regarded as a risk factor for cardiometabolic diseases, its relationship with the microbial ecosystem is increasingly recognized [37]. Most studies do not focus on meat intake but rather, more broadly on dietary patterns with or without meat and their impact on gut microbiota. The positive effects of certain food groups on the gut microbiota are well-documented. For instance, dairy products, among animal-origin foods, have been demonstrated to promote gut health by increasing the abundance of probiotic bacteria as *Bifidobacterium* and *Lactobacillus* [38]. Similarly, dietary fiber from fruits, vegetables and whole grains may influence the gut microbiota composition and function through the increase of specific genera within the phyla *Bacillota* and *Actinomycetota* [39, 40]. In contrast, the effect of meat consumption on gut microbiota has typically been examined in the context of a Western dietary pattern, characterized by increased animal fats, refined sugars, and animal proteins [41]. This dietary pattern leads to an increase in *Bacillota* and *Pseudomonadota*, while decreasing *Bacteroidota*, as well as the genera *Prevotella* and *Bifidobacterium* [42].

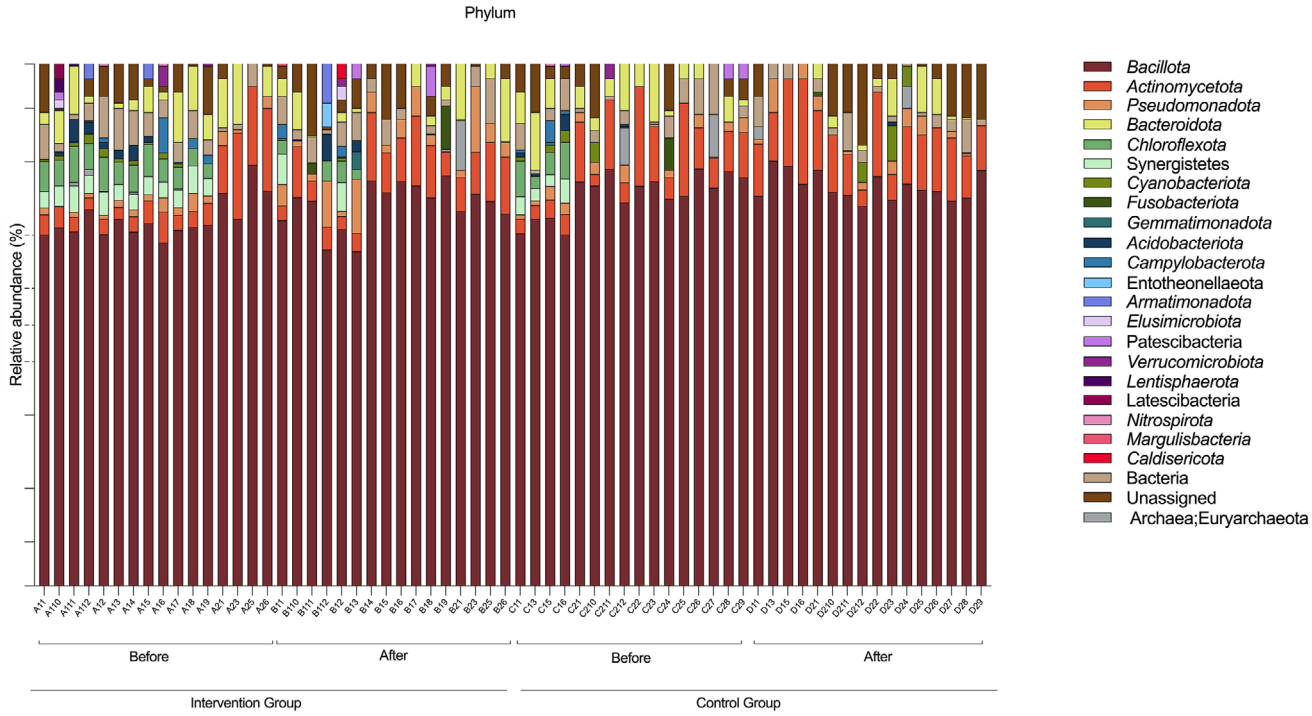

FIGURE 3 | Relative abundances at the phylum level by all participants at the beginning and end of the two periods of the study. Pirenaica breed beef-based diet group ($n = 16$); chicken-based diet group ($n = 16$). The stacked bar plots represent the composition of microbial communities for each sample.

TABLE 2 | Effects of the type of intervention, period, sequence, and Diet Quality Index (DQI) on gut microbiota composition and alpha diversity between both groups.

	Pirenaica breed beef-based diet		Chicken-based diet		Period		Sequence		Diet Quality Index	
	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*
Phylum										
Actinomycetota	−4.01	0.098	−3.82	0.110	1.23	0.131	−0.31	0.692	0.07	0.155
Bacteroidota	0.04	0.632	0.04	0.632	0.01	0.687	−0.03	0.328	0.00	0.441
Chloroflexota	−1.22	0.049	−1.05	0.083	1.26	0.000	−0.17	0.392	0.00	0.928
Bacillota	12.99	0.013	11.39	0.025	−5.12	0.005	0.90	0.571	−0.18	0.074
Pseudomonadota	−3.68	0.119	−2.87	0.214	0.34	0.644	−0.41	0.597	0.08	0.134
Synergistota	−1.81	0.027	−1.78	0.028	1.61	0.000	−0.02	0.927	0.00	0.777
Alpha diversity indices										
Richness	−40.09	0.023	−41.44	0.019	21.61	0.007	−11.49	0.051	0.47	0.161
Fisher's index	−7.82	0.024	−8.04	0.020	4.23	0.007	−2.15	0.060	0.09	0.166
Shannon index	−0.66	0.013	−0.65	0.014	0.18	0.030	−0.22	0.015	0.01	0.049
Pielou's evenness	−0.06	0.211	−0.06	0.264	−0.01	0.665	−0.02	0.235	0.00	0.233
Simpson index	−0.08	0.056	−0.07	0.077	0.02	0.083	−0.02	0.094	0.00	0.144
Inverse Simpson	−5.94	0.195	−4.23	0.348	3.49	0.015	−2.73	0.096	0.05	0.606

Note: Data are expressed as coefficients and *p* values; the coefficients represent the estimated effect of dietary intervention (Pirenaica breed beef or chicken), period, sequence, and DQI on the relative abundances at the phylum level and alpha diversity indices.

Abbreviation: DQI, Diet Quality Index.

[a]Linear mixed model for repeated measures ($n = 16$ per group) within individual to evaluate the effect of the type of intervention, period, sequence, and DQI. Significant differences ($p < 0.05$).

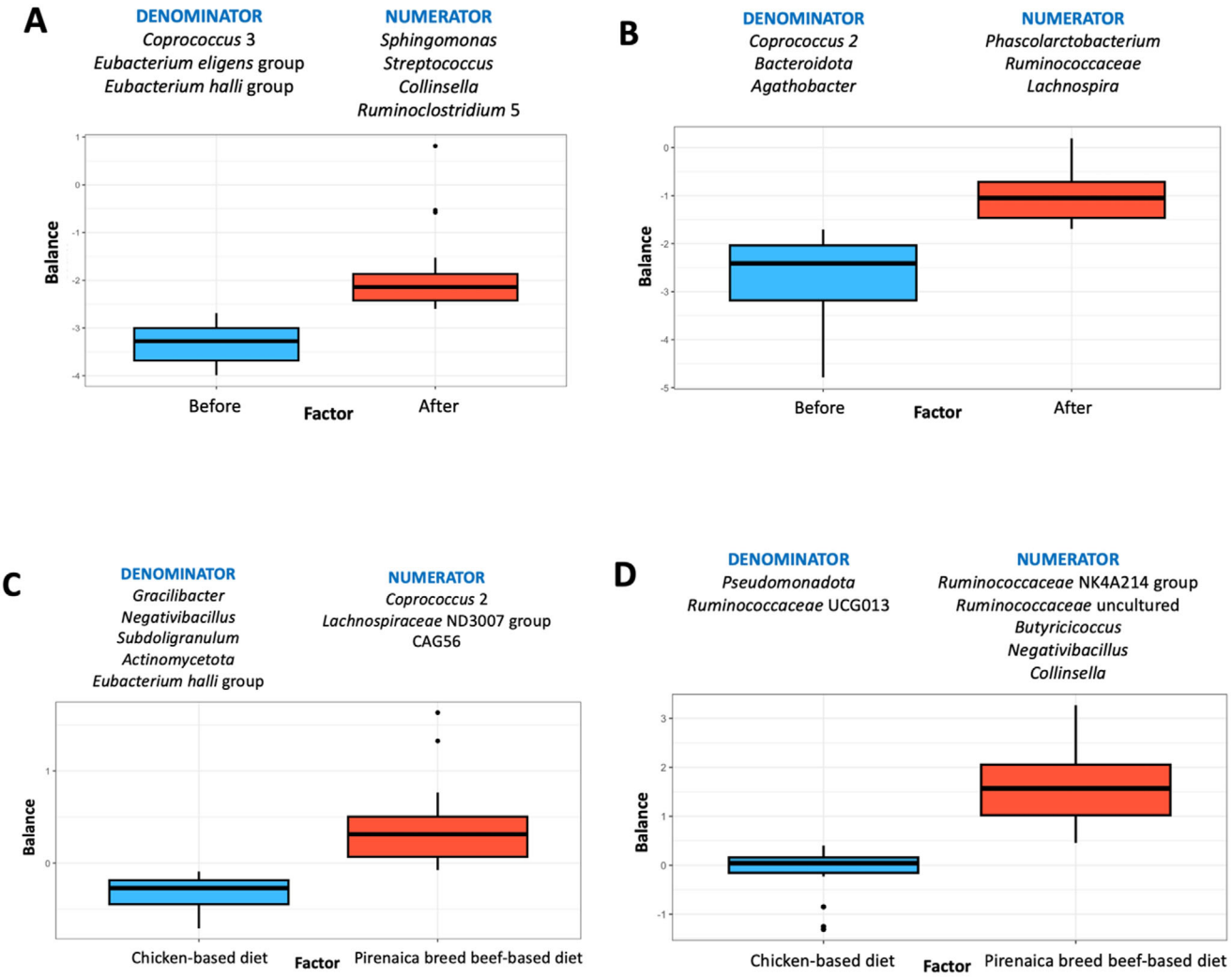

FIGURE 4 | Group balances are presented in an overview. Box plots illustrate the distribution of balance scores for before and after intervention in the chicken-based diet group (A), before and after intervention in the Pirenaica breed beef-based diet group (B), at the beginning of both diets (C), and after both diets (D). Pirenaica breed beef-based diet group ($n = 16$); chicken-based diet group ($n = 16$).

4.1 | Meat Consumption and Relative Abundance at the Phylum and Genus Levels

Our results revealed modest changes in microbial composition at the phylum level following both dietary interventions, with no statistically significant differences observed between the Pirenaica breed beef and chicken-based diet groups. Both diets were associated with a slight increase in the relative abundance of *Bacillota*, the dominant phylum, and a reduction in *Bacteroidota*. Notably, *Pseudomonadota* decreased in both groups, although its relative abundance remained consistently higher in participants who received the chicken-based diet. These trends were further supported by the linear mixed model analysis, accounting for type of dietary intervention, sequence, period, and DQI effects. After adjustments, both diet continued to influence microbial composition, with increases in *Bacillota* and reductions in *Synergistota* and *Chloroflexota* observed across interventions. These findings suggest that both meat diets exerted comparable, directional effects on the gut microbiota at the phylum level, regardless of diet quality (Figure 5).

At the genus level, *Blautia* increased in response to both interventions. This genus has previously associated with higher meat consumption [43, 44], and in some studies, with less favorable metabolic profile, although its remains unclear [8]. *Coprococcus*, *Roseburia*, and *Lachnospira* decreased after the interventions. These genera are well-established producers of short-chain fatty acids (SCFAs), particularly butyrate, and their abundance has been reported to decline in meat rich diets [45]. Although some genera changed differently depending on the meat consumed, no significant differences were found between groups, suggesting a similar overall impact on microbial composition.

Few studies have directly explored the effect of the type of meat consumed on the gut microbiota composition. In Chinese volunteers, bacterial taxa responses varied from our findings [46]. The relative abundances at both phylum and genus levels did not significantly change with meat-based diet replacement. After 2 weeks of a beef and chicken based diets, *Pseudomonadota* showed the most significant change during the meat replacement, increasing after a beef-based diet and decreasing

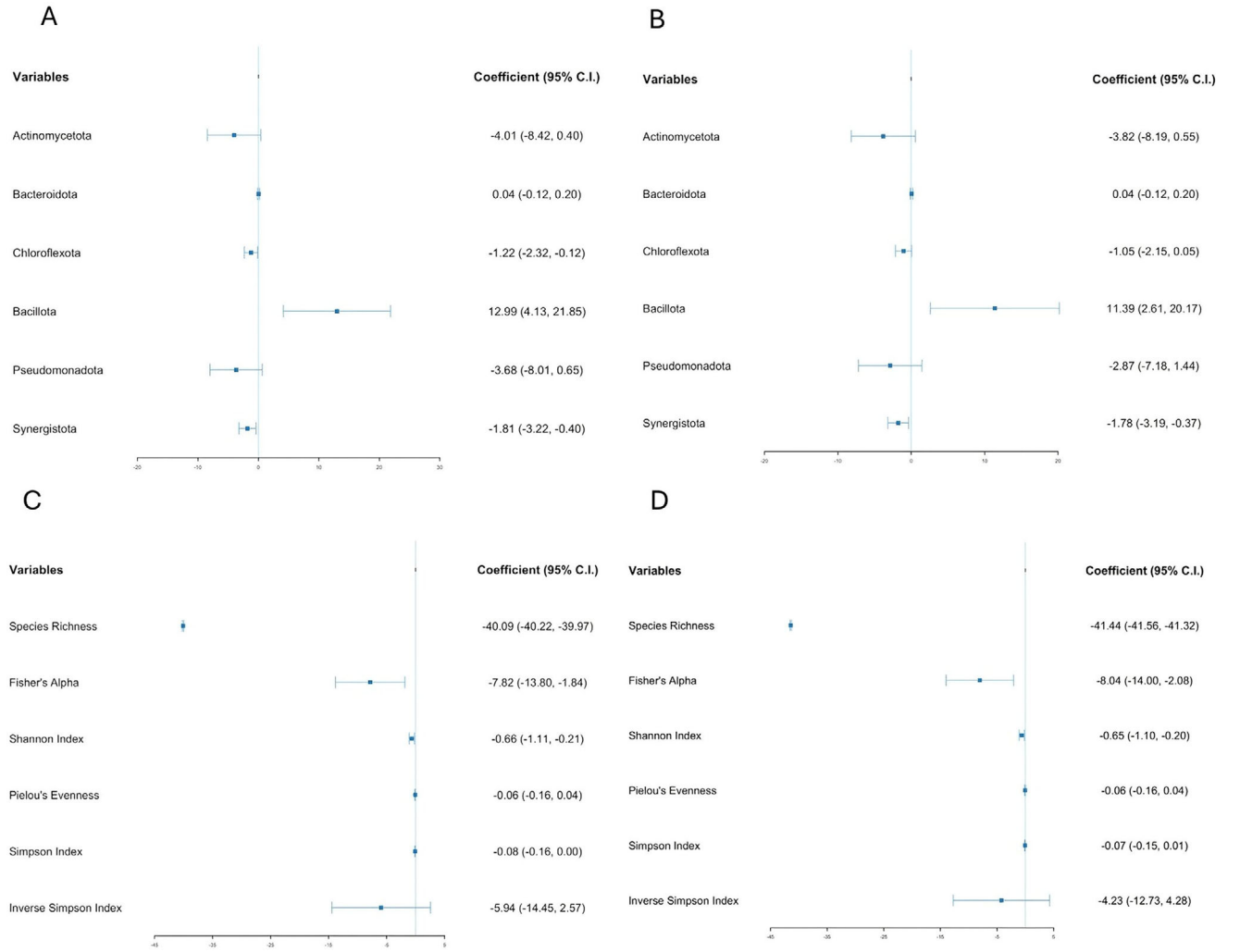

FIGURE 5 | Forest plots showing the effects of the intervention type at the phylum level abundances and alpha diversity indices. Relative abundances at the phylum level for the Pirenaica breed beef-based diet (A), relative abundance at the phylum level for the chicken-based diet (B), alpha diversity indices for the Pirenaica breed beef-based diet (C), and alpha diversity indices for the chicken-based diet (D). Data are presented as estimated coefficients and 95% confidence intervals obtained from linear mixed-effects models. Analysis was conducted using repeated measures ($n =$ 16 per group). A negative coefficient indicates a reduction in the corresponding variable following the intervention, while a positive coefficient indicates an increase.

after a chicken-based diet [46]. An increase in *Pseudomonadota* abundance has been associated with dysbiosis, characterized by reduced mucous production in the gut barrier and low-grade inflammation [47]. At the genus level, our findings differ from this study, with reported increases in *Lachnospira*, *Lachnospiraceae* group NK4A136, and *Ruminococcus* 2 detected after consuming the beef-based diet [46]. A study compared the effect of consuming pork versus chicken within a healthy dietary pattern for 10 days in adults; a reduction in the abundance of *Bacteroidota* and the family *Bacteriodaceae* but an increase in the family *Christensenellaceae*, independent of pork or poultry intakes was observed [48]. However, no other types of meat, seafood, or whole eggs were allowed [48], and the short periods limits comparison with our findings. In an observational study, meat preferences caused significant variations in the gut microbiota composition between pork eaters and chicken eaters. Pork eaters showed a greater relative abundance of *Bacillota* and a lower relative abundance of *Bacteroidota* than chicken eaters [49]; these observations may not be attributed to red meat intake in the absence of controlled full-feeding diets. Similarly, after 5 days of consuming red meat or mushrooms as part of an uncontrolled diet, the relative abundances of *Bacteroidota* and *Bacillota* decreased and increased, respectively [45]. However, it may be challenging to compare our results with those of previous studies due to methodological differences, including shorter interventions periods and the absence of washout periods between interventions.

4.2 | Meat Consumption and Alpha Diversity

After consuming both diets, there was a decrease in microbial diversity and richness, assessed by the Fisher, Shannon, and Simpson indices. However, statistically significant decreases were only observed following the chicken-based diet group, particularly in species richness, Fisher and the Shannon indices. This pattern suggests that the Pirenaica breed beef-based diet may exert a comparatively milder impact on overall microbial diversity

than the chicken-based diet. The analysis of intervention type, period, sequence, and DQI effects highlighted complex interactions modulating the gut microbiota. Notably, DQI significantly declined following the chicken-based diet, which may partially explain the observed microbial shifts. However, after adjusting for DQI, it did not emerge as significant predictor of alpha diversity parameters dietary habits during this phase may not have influenced responses. Interestingly, diversity indices increased during the second intervention period, pointing toward potential temporal or adaptive host-related responses. No consistent sequence effect was observed, supporting the adequacy of the crossover design and the effectiveness of the washout period. A previous study showed that the addition of unprocessed or processed lean red meat to a healthy lacto-ovo vegetarian diet for 3 weeks did not modify the overall microbiota structure, as assessed by metric values such as the Chao1, ACE, Shannon, and inverse Simpson indices [50]. The Chao and Shannon indices showed lower richness and diversity in pork eaters than in the chicken-eaters [49]. Furthermore, a randomized controlled trial revealed that fried meat (including chicken and fish) consumption decreased microbial community richness in overweight adults. At the phylum level, the ratio of *Bacillota*/*Bacteroidota* decreased in both the fried meat and control groups, with a significantly lower decrease in the control group, along with differences at the genus level [51]. High-heat cooking methods, such as frying and grilling, produce higher amounts of advanced glycation end-products [52]. Therefore, it is possible that the type of meat and the cooking method could modify the gut microbiota composition and structure.

4.3 | Functional Profiles

Regarding microbial metabolic pathways, a greater reduction was observed following the chicken-based diet, particularly in aromatic amino acid biosynthesis and gluconeogenesis pathways. However, the overall pattern of functional response was similar between both dietary interventions. These findings highlight the value of integrating microbial functional analysis in dietary intervention studies. Previous intervention comparing meta-rich diets to vegetarian diets have not reported difference sin microbial functionally after 4 weeks of intervention [43].

Our study assessed the effects of different types of meat consumption on gut microbiota, taking into consideration an extensive meat production system. Young bulls from Pirenaica beef, raised under extensive husbandry conditions, is naturally lean (1.75–2.28 g fat/100 g), high in high-quality protein (23–24 g/100 g), and rich in micronutrients such as potassium, phosphorus, selenium, zinc, and B vitamins (B3, B6, B12), while being low in sodium [15]. These attributes support its inclusion in balanced into dietary patterns and justify its selection as a model of lean red meat for gut microbiota research. The results from this intervention have already been published, showing similar body composition parameters, lipid profiles, and fatty acid values after both intervention diets [13]. These findings suggest that the integration of lean red meat from beef (Pirenaica breed) into a healthy dietary pattern could positively influence not only the gut microbiota but also some health indicators. Overall, 8 weeks of intensive nutritional intervention provided sufficient time for changes in the gut microbiota to occur. Indeed, studies have reported significant changes in microbiota composition after 3 weeks of intervention [53]. In this study, a 5-week washout period was established to reduce the influence of the previous intervention. The absence of a sequence effect across phylum-level abundances and alpha diversity parameters indicates that this period was adequate to re-establish baseline microbial conditions. However, the presence of period effect may reflect temporal adaptation, or that the diet consumed during this interval could have interacted with the outcomes.

4.4 | Strengths and Limitations

The study design (crossover clinical trial) is a notable strength as it provides a high level of evidence for comparisons of the outcomes between diets. The inclusion of an institutionalized young adult population minimized lifestyle variability, enhancing the result's validity. Moreover, the control of cooking methods across university accommodation halls is another strength to be considered. Furthermore, other interventions do not take into account the nutritional composition of the meat studied. In our study, the Pirenaica breed beef sourced from extensive husbandry practices yields a unique nutritional profile influenced by breed, feeding practices, sex, and age at slaughter [15].

The study also presented several limitations. It is important to note that this sub-study required voluntary participation, which may have limited the final sample size. To address this limitation, a linear mixed-effects model was applied for repeated measurements. This approach accounted for individual-level variability and enhanced the reliability of the statistical analysis, thereby improving the statistical power to detect effects. Although dietary intake was assessed using a validated food frequency questionnaire and overall dietary quality was considered using the DQI, the potential influence of unmeasured or residual dietary factors on gut microbiota parameters cannot be entirely excluded. Additionally, carry-over, period, or sequence effects could influence the effect of the intervention. The reported results are relevant to a particular type of meat, the Pirenaica breed beef, although this meat could be similar to others from similar husbandry conditions in extensive mountain production systems. However, when comparing our results with other studies, there is a lack of specificity in defining the term "meat". The varied uses of these methods add to research evidence that is inconsistent and controversy [8].

5 | Conclusion

Overall, our results suggest that lean red meat from beef (Pirenaica breed) responds comparable to lean white meat from chickens in terms of gut microbiota composition and structure in healthy young adults. A more comprehensive analysis of the gut microbial community and its correlation with health parameters should be further considered.

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Consent

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

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available in article supplementary material.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information file 1: mnfr70189-sup-0001-SuppMat.docx