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# Chia (*Salvia hispanica* L.) flour modulates the intestinal microbiota in *Wistar* rats fed a high-fat and high-fructose diet

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**Declarations of interest**

none

[1] **Abbreviations**

[1] **SCFA**: short-chain fatty acids; **IgA**: Immunoglobulin A; **HFHF**: high-fat high-fructose; **HFHF + CF**: high-fat high-fructose plus chia flour; **AIN-93M**: Murino standard diet; **BMI**: Body mass index; **ALA**: Alpha-linolenic acid; **ω-6**: linoleic acid; **NCBI**: National center for biotechnology information; **OTUs**: Operational Taxonomy Units; **PCoA**: Principal coordinate analysis; **PICRUSt2**: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; **PERMANOVA**: Permutational multivariate analysis of variance; **HPLC**: high performance liquid chromatography. **KEGG**: Kyoto Encyclopedia of Genes and Genomes, **LEfSe:** Linear discriminant analysis Effect Size.



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**Chia (*Salvia hispanica* L.) flour modulates the intestinal microbiota in *Wistar* rats fed a high-fat and high-fructose diet**

***Abstract*:** A diet rich in sugar and fat can promote metabolic disorders development, especially in the intestine. Chia flour (*Salvia hispanica*. L) is a source of dietary fiber, alpha-linolenic fatty acid (ALA), bioactive peptides, and phenolics, promoting health benefits. This study aimed to analyze chia flour's effect on gut microbiota modulation and intestinal health in adult male *Wistar* rats fed a high-fat and high-fructose diet (HFHF). Male *Wistar* rats (n = 10/group) were fed the diets standard (AIN-93M) or HFHF (31% saturated fat and 20% fructose) in the first phase to induce metabolic disorders. In the second phase, the rats were fed AIN-93M, HFHF, or HFHF plus 14.7% c chia flour (HFHF + CF) for 10 weeks. The consumption of chia flour increased the ALA (3.24 ± 0.24) intake and significantly improved immunoglobulin A (IgA) levels (1126.00 ± 145.90), goblet cells number (24.57 ± 2.76), crypt thickness (34.37 ± 5.86), crypt depth (215.30 ± 23.19), the longitudinal muscle layer (48.11 ± 5.04), cecum weight (4.39 ± 0.71), Shannon index ($p < 0.05$), and significantly increased the production of acetic (20.56 ± 4.10) and butyric acids (5.96 ± 1.50), *Monoglobus sp., Lachnospiraceae sp.,* and *Prevotellaceae sp.* abundance. Furthermore, chia significantly reduced the cecal pH content (7.54 ± 1.17), body mass index (0.62 ± 0.03) and weight (411.00 ± 28.58), and Simpson index ($p < 0.05$). Therefore, chia intake improved intestinal health parameters and functionality in rats with metabolic disorders, which demonstrates to be an effective strategy for gut microbiota modulation.

***Keywords:*** Chia flour; Bioactive compounds; Short-chain fatty acids; Intestinal morphology; Immunoglobulin A; Cecal pH content; Diversity analysis; Functionality analysis.

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## 1. Introduction

The Western diet is based on ultra-processed foods rich in fat, fructose, salt, and sugar, which contribute to hyperphagia, obesity, intestinal dysbiosis, inflammatory bowel diseases, and other noncommunicable diseases, such as diabetes, and cardiovascular diseases (Woodie et al., 2020; Attal et al., 2021; Maurya et al., 2023; Newsome et al., 2023). The intestinal dysbiosis caused by ultra-processed food intake impairs the intestinal membrane through microbial community modification, leads to an increase of pathogenic bacteria proliferation, and a reduction of probiotic bacteria (Kang et al., 2023; Lee et al., 2023; Saranya & Viswanathan, 2023). Besides, dysbiosis can modify the enterocyte morphology of villus and crypts and reduce mucus synthesis, which may increase the passage of pathogens and liposaccharides and decrease secretory IgA, which forms part of the first line of defense on the intestinal surface against pathogens (Ou et al., 2022; Lu et al., 2023).

The consumption of bioactive compounds promotes the restoration and maintenance of healthy intestinal homeostasis, assisting the short-chain fatty acids (SCFAs) synthesis, the beneficial bacteria strains proliferation, and the improvement of intestinal bacterial morphology (Liang et al., 2021; Mishima et al., 2022a). The chia flour (*Salvia hispanica* L.) is the source of bioactive compounds, dietary fiber (37.9% total dietary fiber = 34.5% insoluble dietary fiber, 3.4% soluble dietary fiber), unsaturated fatty acids (27.5% lipids = 82.2% polyunsaturated fatty acids and 7.5% monounsaturated fatty acids), proteins (20.7%), bioactive peptides and phenolics compounds (rosmarinic, ferulic and caffeic acids) (Da Silva et al., 2017; Enes et al., 2020; Amaya Cano et al., 2021; Grancieri et al., 2022; Moreira et al., 2022).

Chia flour soluble extracts (0.5%) by intra-amniotic administration improve intestinal morphology since it increases the villus surface area, villus length, villus width, and the goblet cells diameter and number and promotes the growth of *Bifidobacterium sp*. and *Lactobacillus sp.* genera and reduces *E. coli sp.* and *Clostridium sp.* (Da Silva et al., 2019). The consumption

of chia flour with a standard diet by adult female ovariectomized *Wistar* rats increased acetic and butyric acids concentrations longitudinal and circular muscle layers, and crypt thickness, decreased the cecal pH content, and enriched *Bacteroides sp.* genus and Muribaculaceae and Lachnospiraceae families (Mishima et al. 2022a). Moreover, chia flour added to a standard diet increased SCFAs production, faecal moisture, and circular muscle layer width in young male rats without metabolic disorders (Mishima et al. 2022b).

In this present study, adult male *Wistar* rats were submitted to metabolic disorders (liver steatosis - grade 2, insulin resistance, and high triglyceride and uric acid levels) (Enes et al., 2020; Moreira et al., 2022) by a high-fat and high-fructose (HFHF) diet to investigate chia flour effects on the functionality, morphology, and gut microbiota modulation. Our hypothesis is that chia flour intake increases SCFAs production, decreases cecal pH content, increases the beneficial bacterial strain proliferation, improves intestinal morphology and IgA levels in rats with metabolic disorders caused by HFHF diet consumption.

## 2. Material and Methods

### 2.1. Samples

Chia seeds were obtained in Rio Grande do Sul, Brazil. After harvesting, the seeds were stored in a freezer, at -18 ºC in vacuum packaging. The chia seeds were ground in a blender (PHILIPS WALITA® model RI2035 500W) to obtain the flour and prepare the experimental diet. The flour was stored at -18°C in a black polypropylene bag to prevent lipid oxidation.

### 2.2. Animals and Experimental Diets

Thirty adult male *Wistar* rats (*Rattus norvegicus*), aged 45 to 50 days, were obtained from the Central Animal House of the Center for Biological Sciences and Health, Federal University of Viçosa, Minas Gerais, Brazil. The sample calculation equation was determined by Marineli et al., (2015). During the experiment, the animals were kept in individual stainless-steel cages, under controlled temperature (22 °C ± 2°C), light/dark cycle (12h), and received

deionized water and diets *ad libitum*. The animals were fed a standard diet (AIN-93M; n = 10; initial body weight 156.0 ± 17.0 g) (Reeves et al., 1993) and an HFHF diet (n = 20; initial body weight: 156.5 ± 17.9 g), for 8 weeks, to induce metabolic disorders (Moreira et al., 2022). The HFHF diet comprised fructose 20%, lard 31%, and soybean oil 4% (Marineli et al., 2015). After 8 weeks, the AIN-93M group (n=10) was maintained, and the HFHF group (n = 20) was randomized into the groups: HFHF (n=10) and HFHF plus chia flour (HFHF + CF): animals fed HFHF diet + 14.7% chia flour, n=10) (**Table 1**). In the HFHF + CF diet, 4% of soybean oil from HFHF was replaced by 4% of lipid from chia flour, equivalent to 14.7% of chia flour, which contains 5.58% of flour's fiber. In the diets, AIN-93M and HFHF, microcristalline cellulose (5.58%) was used as a fiber source.

At the end of the 18$^{th}$ week, the animals were euthanized by cardiac puncture after anesthesia with isoflurane 5% (Isoforine, Cristália®). The cecum content, colon, and adipose tissue were collected, weighed, immediately frozen with liquid nitrogen, and stored at -80°C for subsequent analyses. The ascending large colon was collected and stored in a 10% formalin solution for histological analyses.

The study was approved by the Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA/UFV), protocol N° 31/2018. All experimental procedures with animals were carried out in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation.

**2.3. Food Intake and Biometric Measurements**

Food intake and body weight were weekly measured. The body mass index (BMI) was calculated as body weight/ length$^2$ (Shah; Braverman, 2012). Adiposity percentage was calculated by the sum of epididymal, abdominal, and retroperitoneal tissues, and divided by final weight, multiplied by one hundred (Medina Martinez et al., 2022). The ALA and linoleic

fatty acid (ω-6) intake was calculated by the weekly, multiplied by the omega content in 1 kg of diet, and divided by 1000.

### 2.4. DNA Extraction and Sequencing

The genomic DNA was extracted from cecal content samples by mechanical disruption and phenol/chloroform extraction protocol, and each sample was treated with RNAse (Stevenson & Weimer, 2007). The Illumina MiSeq platform was used to load the samples into an Illumina flow cell, for paired-end sequencing reactions, at the Argonne National Laboratory (Lemont, Illinois, USA) (Caporaso et al., 2012). The PCR amplicon libraries targeted the hypervariable V4 - region of 16S rRNA gene, using 515F (5'GTGYCAGCMGCCGCGGTAA3') and 806R (GGACTACNVGGGTWTCTAAT3') primers and a barcoded primer set for the Illumina MiSeq platform (Illumina, San Diego, California, USA) (Caporaso et al., 2011). The customized sequencing primers were used, and procedures followed the 151bp x 12bp x 151bp MiSeq run parameter (Caporaso et al., 2012).

In this study, the sequences obtained in each sample were submitted to the Sequence Read Archive (SRA) in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA926948, and the data analyses were carried out by the Mothur software (1.47.0) (Schloss et al., 2009). UCHIME was used to detect and remove Chimera sequences (Edgar et al., 2011). The taxonomic classification and alignment of the sequences with the 16S rRNA were performed using SILVA database v.138.1 (Quast et al., 2013).

The Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cut-off. The coverage of all samples was assessed by the Good's coverage estimator (Bacteria > 97%). The samples were normalized for the lowest number of sequences produced by any sample (**Supplementary Table S1**). The standardized data table was used for calculating alpha and beta-diversity and the relative abundance of OTUs. The Chao, Shannon

and Simpson indexes estimate alpha-diversity. Beta-diversity was determined by Principal Coordinate Analysis (PCoA), based on the Jaccard dissimilarity index. The metagenome functional predictive analysis was performed with the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) Software (Douglas et al., 2020). The abundance was normalized by the 16S rRNA gene copy number and identified by Greengenes database (DeSantis et al., 2006). Normalized data were used to run the Kyoto Encyclopedia of Genes and Genomes (KEGG), Linear discriminant analysis Effect Size (LEfSe), and alpha and beta-diversity analyses.

### 2.5. Intestinal Health

#### 2.5.1 Cecum Content pH

The cecal content was diluted with distilled water at a 1:10 (w/v) ratio and vortexed until complete homogenization. Then, the pH was read by glass electrode insertion (Grancieri et al., 2017).

#### 2.5.2 Intestinal Permeability

The animals were fasted for 12 h, and 2 mL of a solution containing 200 mg of lactulose and 100 mg of mannitol was administered by gavage. Subsequently, the animals were placed in metabolic cages and fasted for 5 hours. The urine samples were collected for 24 hours, labeled according to the experimental group, and stored at -80°C (Song et al., 2011). Then samples were centrifuged (4°C, 12,000 x g, 10 min), the supernatants were collected and filtered through 0.45 mm Millipore filters, and 1.5 mL was placed in vials for high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) at 55°C and pressure of 1920 psi under isocratic conditions. Chromatography consisted of the degasser system (Model DGU-14A), pump (Model LC-10AT), automatic injector (Model SIL-20A), column oven (Model CTO-10AS), and UV-Vis detector (model SPD-10AV) connected in series with a refractive index detector (model RID-10A). An Aminex HPX-87H analytical column (300 cm x 8.7 mm

- brand BIO-RCalifornia, USA) was used (Grancieri et al., 2017). Sulfuric acid (0.005 mM) was used in the mobile phase with a flow of 0.6 ml/min, and 20 µL of sample was injected (De Sá et al., 2011). The lactulose and mannitol (Sigma-Aldrich, São Paulo/SP, Brazil) were used as internal standards. The peak areas were obtained and converted into g/L according to the percentage of urinary excretion of lactulose and mannitol and the ratio of lactulose/mannitol (De Sá et al., 2011).

**2.5.3 Short-Chain Fatty Acids Concentration (SCFAs)**

Cecal content (400 mg) was homogenized in Milli-Q water (1 mL), vortexed and centrifuged at 12,000 x g for 10 min, and the supernatant was collected and evaluated according to the methodology proposed by Siegfried et al. (1984).

The samples were analyzed by HPLC, using a 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA), coupled to a Shodex RI-101 refractive index (IR) detector, maintained at 40ºC and Rezex ROA ion exclusion column, 300 × 7.8 mm (Phenomenex Inc. Torrance, CA, USA), maintained at 40°C. Sulfuric acid (5 mM) was used at a flow rate of 0.7 mL/min. Acetic, propionic, and butyric acids were used as standard curves (Sigma-Aldrich, São Paulo/SP, Brazil).

**2.5.4 Histological Analyses**

Semi-serial histological sections of 3 µm thick ascending large colon fragments were obtained using an automatic microtome (Reichert Jung®, Germany) and stained using the eosin/hematoxylin technique. The slides were analyzed under a light microscope (Olympus AX 70 TRF, Tokyo, Japan) with a 10× objective. Twenty random fields per animal were selected for longitudinal muscle layer width and quantifying the number of goblet cells from the crypt (Da Silva et al., 2019). The values were obtained using the Image Pro Plus® (version 4.5) (Media Cybernetics, Rockville, USA) and ImageJ® (National Institutes of Health, USA) software.

### 2.5.5 Immunoglobulin A (IgA)

Mucosal immunity was assessed based on the concentration of IgA in the cecum content. Briefly, cecal content (200 mg) was added in 800 µl of phosphate-buffered saline solution and vortexed, and the concentration was measured using an ELISA kit (Cat.N° EA0032Ra). The absorbance was read at 450 nm (Multiskan Microplate Photometer, Thermo Fisher Scientific, MA, USA). and the values were expressed in ng/ml (Vaz-Tostes et al., 2014).

### 2.6. Statistical Analyses

Data of food consumption, body weight, intestinal permeability, cecal pH content, colonic morphometric characteristics, and SCFAs concentrations were analyzed using the Kolmogorov-Smirnov normality *test*, followed by one-way analysis of variance (ANOVA) and *post hoc* Newman-Keuls *test*. Non-parametric and independent samples were submitted to Kruskal-Wallis and *post hoc* Dunn's *test*. Pearson correlation test assessed correlations among biological markers and gut microbial. The analyses were performed in GraphPad Prism (version 9.0).

The Chao, Shannon, and Simpson indexes were used for alpha-diversity. Beta-diversity was assessed by PCoA based on the Jaccard dissimilarity index in the Past software (version 4.12). Differences among beta-diversity values were analyzed by the Pairwise Permutational multivariate analysis of variance (PERMANOVA) *test*. Microbiota data were corrected for multiple comparisons using the false discovery rate (FDR) by Benjamini–Hochberg in the Statistical Analysis of Metagenomic Profiles (STAMP) software (version 2.1.3). The statistical analyses were performed by IBM SPSS Statistics (version 20.0). The data were analyzed by one-way ANOVA and Duncan *post hoc test*. Non-parametric data were submitted to Kruskal-Wallis with Bonferroni correction. The graphics of Phylum, Genera, Firmicute/Bacteriodetes ratio, Pearson Heatmap, and Chao, Shannon, and Simpson indexes were obtained by GraphPad Prism (version 9.0). The KEGG heatmap was generated by Excel. The figure LEfSe was

produced by Galaxy (https://huttenhower.sph.harvard.edu/galaxy/). The significance level was $p < 0.05$.

## 3. Results

### 3.1 Effect of chia flour on body weight gain and food intake

The groups HFHF and HFHF + CF presented lower food intake and higher caloric density ingestion ($p < 0.05$) than the AIN-93M group. Body weight, BMI, and ω-6 intake ($p < 0.05$) presented lower values in groups fed with HFHF + CF and standard diet (AIN-93). The adiposity increased ($p < 0.05$) in the HFHF group compared to AIN-93M. Although the HFHF + CF group did not differ from the HFHF group, it was similar to the AIN-93M group ($p > 0.05$). Chia flour increased the ALA consumption compared to the AIN-93M and HFHF groups ($p < 0.05$) (**Table 2**).

### 3.2 Effect of chia four on intestinal health

Chia flour consumption (HFHF + CF group) positively upregulated the butyric acid and IgA concentrations when compared to the AIN-93M and HFHF groups ($p < 0.05$). Chia flour (HFHF + CF) increased the acetic acid content, the number of goblet cells, crypt thickness, crypt depth, longitudinal muscle layer width, and cecum weight compared to the HFHF group ($p < 0.05$), and it was similar to AIN-93M (**Figure 1**). Furthermore, chia consumption reduced cecal pH content compared to the control groups ($p < 0.05$). The propionic acid content, circular muscle layer width, and mannitol/lactulose ratio ($p > 0.05$) were similar among the three experimental groups (**Table 3**).

### 3.3 Chia flour changes the intestinal microbiota pattern promoted by the HFHF diet.

The sequencing of the 16S rRNA gene generated 810.021 raw sequences, with lengths ranging from 151 bp to 300 bp. After filtering and cleaning the sequences, 611.323 good quality sequences were obtained. The Good's coverage obtained in the samples was > 99%, which indicates good coverage of the sequencing.

The PCoA represented approximately 48.91% of the dissimilarity in bacterial species composition. The Permutational Multivariate Analysis of Variance (PERMANOVA) presented statistical differences in the distance metrics among treatments (PERMANOVA, $p < 0.05$) (**Figure 2A**). The clustering of the bacterial community differed among the three groups ($p < 0.05$).

The microbial richness estimated by the Chao index did not differ among the groups ($p>0.05$) (**Figure 2B**). The Shannon index indicated an increase in species diversity in the HFHF + CF group ($p < 0.05$) compared to the HFHF and AIN-93M groups (**Figure 2C**). The AIN-93M and HFHF groups showed an increase in the Simpson index, while the HFHF + CF group was able to reduce the dominance of species in their bacterial community ($p < 0.05$) (**Figure 2D**).

The samples presented 19 phyla, 30 classes, 70 orders, 108 families, and 204 genera. The Firmicutes/Bacteroidetes ratio was similar among the groups ($p > 0.05$) (**Fig. 3A**). All groups presented eight predominant phyla, including Firmicutes (HFHF + CF: 73%; AIN-93M and HFHF: 69%), Bacteroidetes (HFHF + CF and AIN-93M: 16%; HFHF: 17%), Desulfobacterium (HFHF + CF: 4.2%; AIN-93M: 4.4%; HFHF: 4.9%), Actinobacteria (HFHF + CF: 2.9%; AIN-93M: 3.7%; HFHF: 3.4%) and Proteobacteria (HFHF + CF: 1.9%; AIN-93M: 2.7%; HFHF: 2.4%) (**Fig. 3B**).

The groups presented 17 predominant genera, including *Muribaculaceae sp.* (HFHF + CF and AIN-93M: 7.5%; HFHF: 8%), *Bacteroides sp.* (HFHF + CF: 6.7%; AIN-93M: 5.8%; HFHF: 8.3%), *Lachnospiraceae NK4A136 sp*. (HFHF + CF: 3.5%; AIN-93M: 2.6%; HFHF: 2.3%), *Christensenellaceae R-7 sp.* (HFHF + CF: 2.8%; AIN-93M: 3%; HFHF: 2.6%) and *Oscillibacter sp.* (HFHF + CF and HFHF: 2.8%; AIN-93M: 2.4%) (**Fig. 3C**).

The HFHF + CF group exhibited a higher abundance of *Monoglobus sp.* and *Lachnospiraceae sp.* genera ($p < 0.05$) than the AIN-93M and HFHF groups. Further, the

HFHF + CF group presented a high abundance of *Prevotellaceae sp.* genus ($p < 0.05$) relative to the HFHF group and low Patescibacteria phylum, *Alphaproteobacteria sp*. genus ($p < 0.05$) abundance relative to the AIN-93M group. In addition, the HFHF + CF group presented the lowest abundance of Spirochaetota Phylum and *Treponema sp.* genus ($p < 0.05$), compared to the AIN-93M and HFHF groups and *Erysipelatoclostridium sp.* genus ($p < 0.05$), compared to HFHF.

**3.4. Correlation analysis**

The Pearson correlation assessed the relationship among changes in intestinal microbial abundance, intestinal health markers, and adiposity parameters. *Monoglobus sp.* was positively correlated with IgA ($r = 0.68$; $p < 0.05$). *Prevotellaceae sp.* was positively correlated with crypt thickness ($r = 0.71$; $p < 0.05$). *Lachnospiraceae sp.* was positively correlated with butyric acid concentration ($r = 0.50$), crypt depth ($r = 0.54$), and longitudinal muscle layer width ($r = 0.58$) ($p < 0.05$), but inversely correlated with cecal pH content ($r = 0.50$; $p < 0.05$). IgA presented a positive correlation with acetic acid concentration ($r = 0.50$; $p < 0.05$), which was positively correlated with butyric acid content ($r = 0.46$; $p < 0.05$). The number of goblet cells was inversely correlated with BMI ($r = -0.66$) and positively correlated with crypt thickness ($r = 0.77$) and longitudinal muscle layer width ($r = 0.73$) ($p < 0.05$). Longitudinal muscle layer width was inversely correlated with BMI ($r = -0,66$; $p < 0.05$). Total body weight was positively correlated with BMI ($r = 0,59$; $p < 0.05$) (**Figure. 4**).

**3.5. Chia flour effect on the dominant cecal microbiota**

All OTUs were analyzed to identify dominant cecal microbiota and intestinal biomarkers using the taxonomy. There were 18 dominant OTUs with an effect size > 4 %. The HFHF + CF group exhibited higher bacterial taxa relative to control groups, with a larger effect size of the *Lachnospiraceae sp.* genera. The *Selimonas sp*. was the dominant genus in the

microbiota of the HFHF group. *Allobaculum sp.* (**Figure 5**) was the dominant community in the AIN-93M group.

### 3.6. Chia flour effect on Kyoto Encyclopedia of Genes and Genomes (KEGG)

347 metabolic pathways were observed in all bacterial communities of the experimental groups and 17 pathways presented significance ($p < 0.05$) (**Figure 6**). The HFHF + CF was significant in 15 pathways, such as demethylmenaquinol-9 biosynthesis; menaquinol-9 biosynthesis; 4-hydroxyphenylacetate degradation; menaquinol-6 biosynthesis I; chitin derivatives degradation; demethylmenaquinol-6 biosynthesis I; menaquinol-10 biosynthesis; methylphosphonate degradation I; TCA cycle VII compared with the AIN-93M and HFHF groups. The thiamin salvage II, (Kdo) 2-lipid A biosynthesis, L-methionine biosynthesis III, enterobacterial common antigen biosynthesis, L-arginine, putrescine, and 4-aminobutanoate degradation, and L-arginine and L-ornithine degradation compared with the AIN-93M group ($p < 0.05$) (**Figure 6**). The HFHF + CF and AIN-93M groups were significant in demethylmenaquinol-6 biosynthesis II. The AIN-93M was significant in glycolysis V (Pyrococcus).

## 4. Discussion

Our study investigated the effects of chia flour on gut health in adult *Wistar* rats with metabolic disorders caused by the consumption of a HFHF diet. Chia flour reduced food intake, body weight, BMI, and cecal pH content and increased cecal weight. It also increased SCFAs concentration, IgA content, probiotic bacterial strains, and reduced pathogenic strains. Further, chia flour enhanced the number of goblet cells, thickness, and depth crypt, besides the longitudinal muscle layer width in the cecum.

The groups that received the HFHF diet presented lower food intake and higher energy intake than the AIN-93M group. This fact can be due to the high energy density observed in the HFHF diet, which could have to promote high satiety (Enes et al., 2020; Medina Martinez

et al., 2021; Moreira et al., 2022). The HFHF + CF diet offered 14.7g of chia flour/100g diet. This amount replaced 100% of the recommended oil and dietary fiber for rodents (AIN93-M) (Reeves et al, 1993). Considering the recommendation of 14g of dietary fiber/1000 kcal for human consumption (Dahl et al., 2015), to supply 100% of the human dietary fiber recommendation, it would be necessary to consume 73.8 g of chia flour/day, which is a high amount for human consumption. However, other studies must test different doses to validate the consumption of chia flour for humans.

The HFHF + CF diet reduced body weight gain and BMI. It could be explained by chia chemical composition due to its dietary fiber, bioactive peptides, and alpha-linolenic acid (ALA) (Da Silva et al., 2017; Enes et al., 2020; Grancieri et al., 2022; Moreira et al., 2022). These compounds may contribute to body weight loss (Enes et al., 2020; Fonte-Faria et al., 2019; Kobyliak et al., 2020; Moreira et al., 2022). These data were different from those of Miranda et al. (2019), in which male Swiss mice fed with a high-fat diet with 3% chia flour presented similar body weight compared to the high-fat group.

The beta-diversity analysis presented differences in the intestinal bacterial community in the HFHF + CF group without a change in the Firmicutes/Bacteroidetes ratio. These results indicated that the dietary fibers and ALA present in chia flour may increase intestinal fermentation, probiotic bacteria proliferation, and microbial species diversity. Mishima, et al., (2022b) observed differences in beta diversity in young male rats fed with a high-fat diet (64% fat), with or without chia flour (41.6% flour). Further, no changes in Firmicutes/Bacteroidetes ratio or alpha diversity were reported in these animals.

The HFHF + CF group improved the cecal pH content, cecal weight, butyric and acetic acids content, and the histomorphometry variables. These factors promoted the proliferation of probiotic strains, such as *Monoglobus sp*., *Lachnospiraceae sp*., and *Prevotellaceae sp*. The bioactive compounds from chia flour, such as dietary fibers, and phenolics compounds,

including rosmarinic, ferulic, and caffeic acids (Da Silva et al., 2017; Moreira et al., 2022), may be a substrate to fermentation in the colon, besides providing energy for colonocytes and enabling probiotic strain proliferation (Markowiak-Kopeć & Śliżewska, 2020). Further, person analysis demonstrated a correlation between *Lachnospiraceae sp.* and the content of acetic and butyric acids and between crypt depth and longitudinal muscle layer, thus evidencing the probiotic activity of these bacteria and the synergy among SCFAs. It also indicated the abundance of *Prevotellaceae sp.* correlated with crypt thickness, highlighting the functional effect of these bacteria on intestinal morphology. Similar results were observed in ovariectomized adult female *Wistar* rats fed a high-fat (51% fat) and 23.2% chia flour diet (Mishima et al., 2022a).

The intake of HFHF + CF reduced the richness of pathogenic bacteria, such as Patescibacteria, Spirochaetota, *Treponema sp., Erysipelatoclostridium sp.* and *Alphaproteobacteria sp.* It is an important health effect since chia flour increased IgA concentration, which may promote humoral immune response mediation (Ma et al., 2021; Tang et al., 2022). Furthermore, the bioactive peptides in chia flour may act in the anti-inflammatory pathway and inactivate pro-inflammatory cytokines (Grancieri et al., 2022), which favors IgA production. The Person's correlation in our study demonstrated that *Monoglobus sp.* correlated with IgA and IgA correlated with acetic acid, which indicates a prebiotic effect of chia flour.

The LEfSe indicated a predominance of *Sellimonas sp*. in the HFHF group, since it acts in the transport of amino acids and carbohydrates, as well as energy production and conversion, thus promoting intestinal homeostasis (Muñoz et al., 2020; Shen et al., 2022). This can be justified by the presence of ALA in the HFHF diet, which may act as an energetic substrate that enables the production of SCFAs and the proliferation of this bacterial strain (Kim et al., 2020). The HFHF + CF group presented *Lachnospiraceae sp.* predominance, which assisted to reduce primary bile acids through conversion into SCFAs and/or secondary bile acids, could

lead to intestinal homeostasis (Wan et al., 2022). The AIN-93M group presented the predominance of *Allobaculum sp.*, a bacterium that has a negative correlation with inflammation, insulin resistance, and obesity (Thomaz et al., 2020; Wang et al., 2020; Zhao et al., 2023).

The KEGG microbial metabolic pathway shows that the HFHF + CF upregulated the pathways associated with Thiamin (vitamin B1) salvage, which is fundamental in energy metabolism, being a cofactor of several enzymes (Jenkins et al., 2007; H. J. Kim et al., 2020; *Thiamine Salvage II | Pathway - PubChem*, n.d.); L-methionine biosynthesis III is an essential proteinogenic amino acid, which helps in protein synthesis, DNA methylation, rRNA and xenobiotics, besides promoting the cysteine, phospholipid, and polyamine biosynthesis (*L-Methionine Biosynthesis III | Pathway - PubChem*, n.d.; Mota-Martorell et al., 2021); TCA cycle VII (acetate-producers) is a catabolic pathway by aerobic respiration. Acetic acid bacteria oxidize ethanol into acetate (Kim et al., 2022; TCA Cycle VII (Acetate-Producers) | Pathway - PubChem, n.d.).

The limitation referred to the DNA sequencing analysis, performed only after treatment, since the sequencing performed both before and after the treatment is important for obtaining more reliable data about the bacterial community. Then, new studies should be conducted to elucidate that gap.

## 5. Conclusion

Chia flour consumption associated with a HFHF diet improved probiotic bacterial strain proliferation, immune system, SCFAs synthesis, intestinal morphology, and cecal pH content, and reduced body weight. The results obtained in the present study evidence the functional potential of chia flour to modulate intestinal health, and it is an alternative to control metabolic diseases caused by the consumption of unbalanced diets.

## Declarations of interest

None.

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The authors have no acknowledgement to declare.

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**Authors' Contributions**

**Violeta Nunes de Morais**: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft, Software, Visualization, Writing review & editing. **Mariana Juste Contin Gomes**: Data curation, Formal analysis, Writing review & editing. **Mariana Grancieri**: Data curation, Investigation, Methodology, Validation, Writing review & editing. **Luiza de Paula Dias Moreira**: Methodology, Formal analysis, Writing review & editing. **Renata Celi Lopes Toledo**: Funding acquisition, Methodology, Project administration, Resources. **Neuza Maria Brunoro Costa**: Resources, Methodology, Funding acquisition. **Bárbara Pereira da Silva**: Data curation, Investigation, Methodology, Validation Writing review & editing. **Hércia Stampini Duarte Martino**: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration, Resources, Supervision, Validation, Writing – original draft, Writing review & editing. All authors have read and agreed to the published version of the manuscript.

**Conflicts of Interest**

The authors declare to have no conflict of interest. The funders presented no role in the study design; in the collection, analysis, or interpretation of data; in the manuscript writing; or in the decision to publish the results.

**Data Availability Statement**

The raw data (paired-end files) are accessible in the NCBI Sequence Read Archive under accession number PRJNA926948.

**Institutional Review Board Statement**

The animal study protocol was approved by the Ethics Committee on Animals Use of the Federal University of Viçosa (CEUA/UFV, protocol no. 31/2018, date of approval: April 26th, 2018).

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## Figures Legends

**Fig. 1**. Effects of chia flour intake on colonic histomorphometry characteristics in adult *Wistar* rats fed HFHF diet. n=10 animals/group. AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, high-fat and high-fructose with chia flour diet; CML, circular muscle layer; LML, longitudinal muscle layer. Black arrows refer to goblet cells in the crypt. Black brackets refer to the crypt's depth and width. Staining was carried out with hematoxylin and eosin. Bar: 100 μm.

**Fig. 2**. Effect of chia flour intake on beta and alpha diversity. (**A**) Beta diversity estimated by PCoA based on Jaccard similarity distance of cecal microbial communities in adult *Wistar* rats fed a high-fat and high-fructose diet. Each dot refers to one animal and the colors refer to the experimental groups. The *p*-value among the groups was obtained by PERMANOVA. Alpha diversity was estimated by the indices: (**B**) Chao, (**C**) Simpson and (D) Shannon, by one-way ANOVA, followed by the Ducan *post-hoc* test ($p < 0.05$). AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, HFHF with chia flour (n = 9).

**Fig. 3**. Effect of chia flour intake on cecal microbiota relative abundance at phylum and genera levels, at the end of treatment (10 weeks). (**A**) Firmicutes to Bacteroidetes ratio; (**B**) Bacterial composition at phylum level; (**C**) Bacterial composition at the genera level. n=10 animals/group. Phyla with abundance > 0.3% and genera with abundance > 0.96%. The data were analyzed by the Dunn's test with FDR and Bonferroni corrections. AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, HFHF with chia flour diet.

**Fig. 4**. Heatmap of Pearson correlation among microorganisms (gender level) and intestinal health markers. Blue is a positive correlation and red is a negative correlation. Correlation matrix *($p < 0.05$). n=10 animals/group. BMI, body mass index; IgA, immunoglobulin A. The specific bacteria, such as *Monoglobus sp., Lachnospiraceae sp.* and *Prevotellaceae sp.* were selected due to their abundance increase in the chia flour group.

**Fig. 5**. Histogram of linear discriminant analysis effect size (LEfSe) method to compute linear discriminant analysis (LDA) scores of differences in dominant microorganisms between groups. AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, HFHF with chia flour diet (n = 9). Significant differences were considered with $p < 0.05$.

**Fig. 6**. Heatmap of the effect of chia flour consumption on the relative significance of differentially enriched microbial metabolic pathways in cecal microbiota in mice fed a high-fat and high-fructose diet. Different lower-case letters in the same line: groups are significantly different ($p < 0.05$) by SPSS. Used Kruskal Wallis with false discovery rate (FDR) by Benjamini–Hochberg. Green color indicates higher functional bacterial capacity (FBC); yellow indicates medium FBC; red indicates lower FBC. AIN-93M, standard diet (n = 9); HFHF, high-fat and high-fructose diet (n = 8); HFHF + CF, high-fat and high-fructose with chia flour diet (n = 9); FBC, functional bacterial capacity.

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## Tables

**Table 1** - Nutritional composition of diets.

| **Ingredients** (g/kg) | **AIN-93M** | **HFHF** | **HFHF + CF** |
|---|---|---|---|
| Albumin* | 136.4 | 136.4 | 101.8 |
| Maize starch | 463.5 | 135.0 | 116.8 |
| Dextrinized starch | 155.0 | 45.0 | 45.4 |
| Sucrose | 100.0 | 28.6 | 29.3 |
| Soybean oil | 40.0 | 40.0 | - |
| Cellulose | 55.8 | 55.8 | - |
| Mineral Mix | 35.0 | 35.0 | 35.0 |
| Vitamin Mix | 10.0 | 10.0 | 10.0 |
| Choline Bitartrate | 2.5 | 2.5 | 2.5 |
| L-cystine | 1.8 | 1.8 | 1.8 |
| Lard | - | 310.0 | 310.0 |
| Fructose | - | 200.0 | 200.0 |
| Chia flour | - | - | 147.3 |
| **Macronutrients** | | | |
| Carbohydrate (%) | 77.4 | 30.1 | 31.0 |
| Protein (%) | 12.9 | 9.1 | 9.2 |

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| | | | |
|---|---|---|---|
| Lipid (%) | 9.7 | 59.8 | 60.4 |
| Caloric density (kcal/g) | 3.7 | 5.3 | 5.2 |
| **Fatty acids** (g/kg) | | | |
| ALA | 3.3 | 10.2[#] | 31.8[#] |
| ω-6 | 20.2 | 58.8[#] | 46.5[#] |
| Ratio ω-6:ω-3 | 6.12:1 | 5.77:1[#] | 1.46:1[#] |

*The amount was calculated based on the protein content equal to 88% to provide 12 g of protein. Chemical composition of chia flour (g/100g) used to calculate the diet: carbohydrates: 3.2g; total dietary fiber: 37.9g; lipids: 27.7g; protein: 20.7g; moisture: 6.9g; ALA: 2.49g; and ω-6: 0.79 g (Moreira et al., 2022). # (Fonseca; Gutierrez, 1974). AIN-93M (Reeves et al., 1993), standard diet; HFHF (Marineli et al., 2015), high-fat and high-fructose diet; HFHF + CF, HFHF with 14.7% of chia flour diet; ALA, Alpha-linolenic acid.

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**Table 2** - Chia flour effect on food intake and adiposity in *Wistar* rats (n =10) for 10 days.

| **Variables** | **AIN-93M** | **HFHF** | **HFHF + CF** |
|---|---|---|---|
| Total food intake (g) | 1367.00 ± 69.07[a] | 1046.00 ± 130.20[b] | 1052.00 ± 65.01[b] |
| Caloric intake (kcal.g$^{-1}$) | 4.85 ± 0.24[b] | 5.50 ± 0.68[a] | 5.53 ± 0.34[a] |
| Final body weight (g) | 390.00 ± 31.36[b] | 461.80 ± 29.78[a] | 411.00 ± 28.58[b] |
| Total adiposity (%) * | 5.48 ± 0.89[b] | 7.30 ± 1.57[a] | 6.36 ± 0.62[ab] |
| Body mass index (kg/m$^2$) | 0.61 ± 0.05[b] | 0.71± 0.07[a] | 0.62 ± 0.03[b] |
| Weekly ω-3 intake (g) | 0.45 ± 0.02[c] | 1.05 ± 0.10[b] | 3.24 ± 0.24[a] |
| Weekly ω-6 intake (g) | 2.77 ± 0.10[c] | 6.06 ± 0.63[a] | 4.74 ± 0.35[b] |

AIN-93M, standard diet; HFHF, high-fat and high-fructose diet; HFHF + CF, HFHF with chia flour diet. *The total adiposity was determined by the sum of the epididymal, abdominal, and retroperitoneal tissues, divided by the final weight, multiplied by one hundred. Different lower-case letters in the same line: groups were significantly different ($p < 0.05$). Data analyzed by ANOVA with Newman-Keuls *post-hoc* or Kruskal Wallis with Dunn *test*.

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**Table 3** - Effect of chia flour intake on intestinal health in *Wistar* rats (n =10) for 10 days.

| **Variables** | **AIN-93M** | **HFHF** | **HFHF + CF** |
|---|---|---|---|
| SCFAs (mM) | | | |
| *Acetic acid* | 21.36 ± 5.11[a] | 13.71 ± 2.90[b] | 20.56 ± 4.10[a] |
| *Propionic acid* | 4.58 ± 1.13 [a] | 4.35 ± 1.55 [a] | 6.46 ± 2.45[a] |
| *Butyric acid* | 2.51 ± 0.77[b] | 3.60 ± 1.01[b] | 5.96 ± 1.50[a] |
| Cecal faeces pH | 9.01 ± 0.40[a] | 9.17 ± 0.25[a] | 7.54 ± 1.17[b] |
| Cecum weight (g) | 4.66 ± 0.83[a] | 3.58 ± 0.60[b] | 4.39 ± 0.71[a] |
| IgA (ng/ml) | 851.50 ± 106.20[b] | 786.50 ± 89.50[b] | 1126.00 ± 145.90[a] |
| Number of goblet cells | 24.80 ± 0.88[a] | 19.55 ± 2.66[b] | 24.57 ± 2.76[a] |
| Crypt thickness (µm) | 38.62 ± 3.15[a] | 22.94 ± 3.22[b] | 34.37 ± 5.86[a] |
| Crypt depth (µm) | 201.20 ± 16.27[a] | 167.10 ± 4.52[b] | 215.30 ± 23.19[a] |
| CMLW (µm) | 125.70 ± 38.28[a] | 101.50 ± 43.10[a] | 158.30 ± 15.49[a] |
| LMLW (µm) | 49.61 ± 7.86[a] | 33.88 ± 4.02[b] | 48.11 ± 5.04[a] |
| Mannitol/Lactulose (%) | 1.79 ± 0.24[a] | 1.85 ± 0.68[a] | 1.90 ± 0.29[a] |

AIN-93M: standard diet; HFHF: high-fat and high-fructose diet; HFHF + CF: HFHF with chia flour diet; LMLW: Longitudinal muscle layer width; CMLW: Circular muscle layer width. Different letters in the same line: groups are significantly different ($p < 0.05$). Data analyzed by ANOVA with Newman-Keuls *post-hoc* or Kruskal Wallis with Dunn *test*. Histomorphometry measurements were performed on ascending large colon crypt.

**Authors' Contributions**

**Violeta Nunes de Morais**: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft, Software, Visualization, Writing review & editing. **Mariana Juste Contin Gomes**: Data curation, Formal analysis, Writing review & editing. **Mariana Grancieri**: Data curation, Investigation, Methodology, Validation, Writing review & editing. **Luiza de Paula Dias Moreira**: Methodology, Formal analysis, Writing review & editing. **Renata Celi Lopes Toledo**: Funding acquisition, Methodology, Project administration, Resources. **Neuza Maria Brunoro Costa**: Resources, Methodology, Funding acquisition. **Bárbara Pereira da Silva**: Data curation, Investigation, Methodology, Validation Writing review & editing. **Hércia Stampini Duarte Martino**: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration, Resources, Supervision, Validation, Writing – original draft, Writing review & editing. All authors have read and agreed to the published version of the manuscript.

**Highlights**

- Chia improve colon morphology and increase IgA levels, and cecum weight.
- Chia increase acetic and butyric acids content.
- Chia was able to decrease intraluminal pH *in vivo*.
- Chia flour increases the genera *Monoglobus sp., Lachnospiraceae sp.* and *Prevotellaceae sp.*

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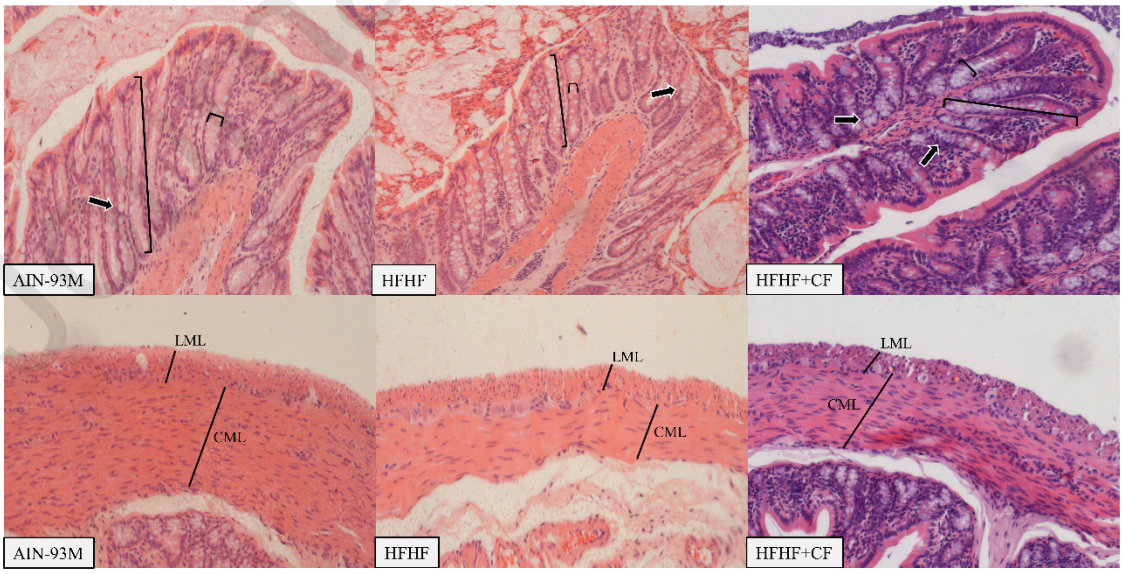
**Conflicts of Interest**

The authors declare no conflict of interest.

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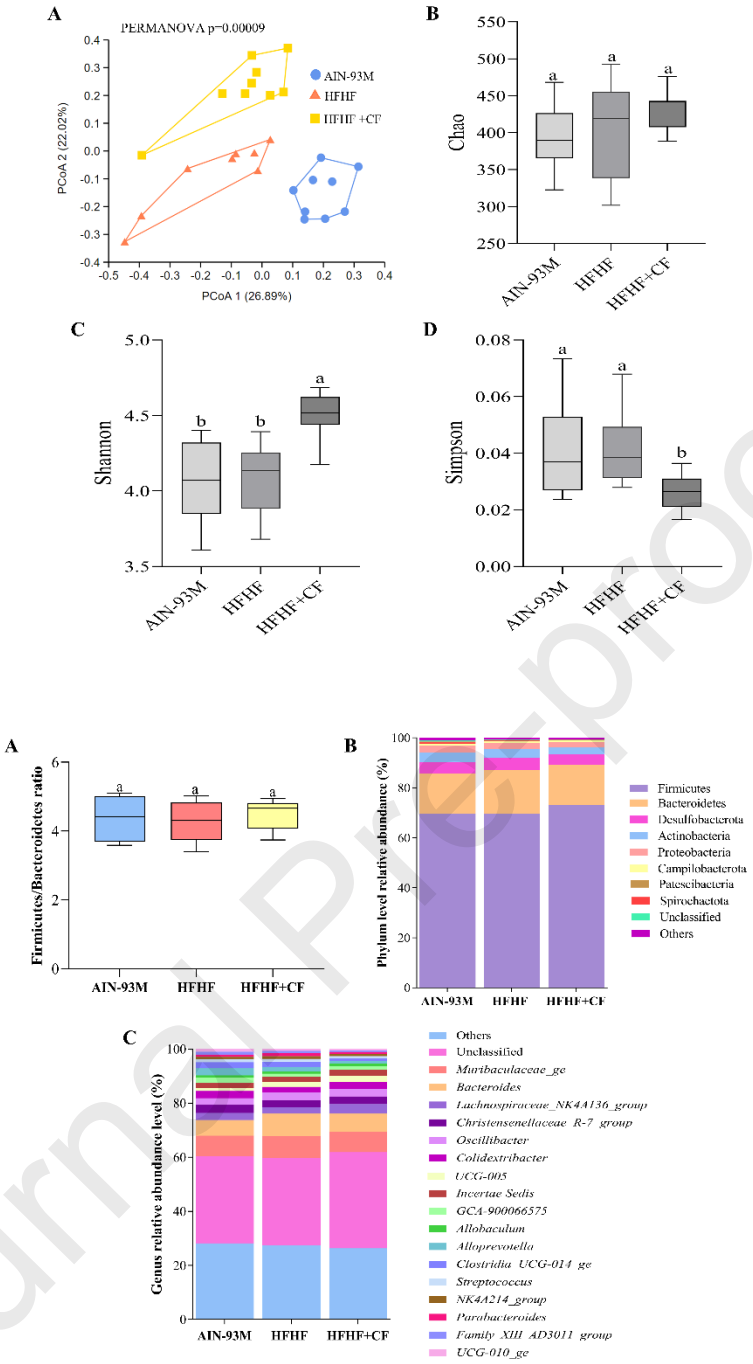


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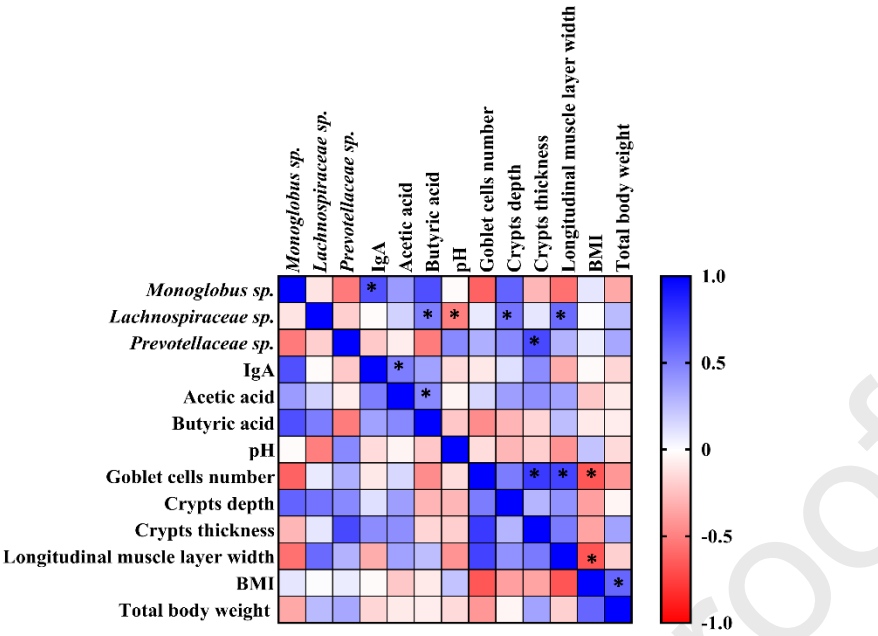


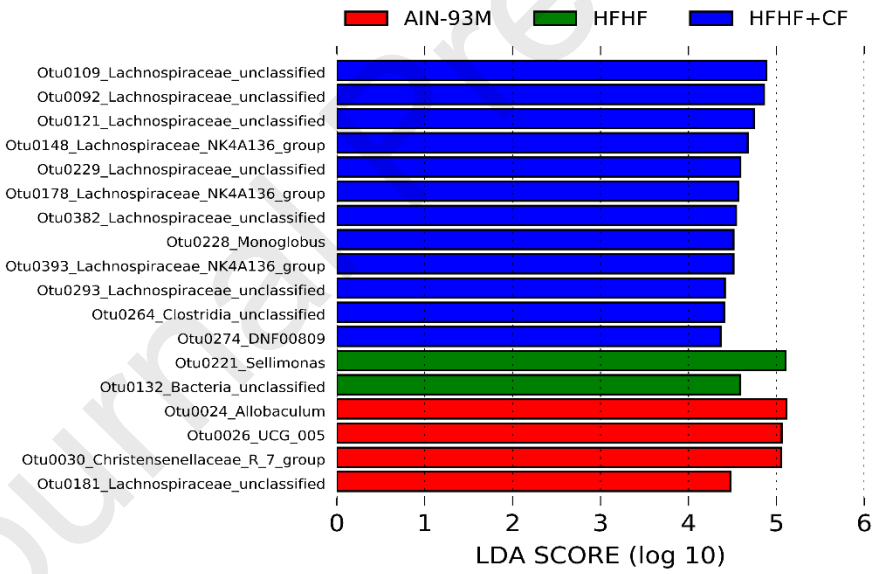


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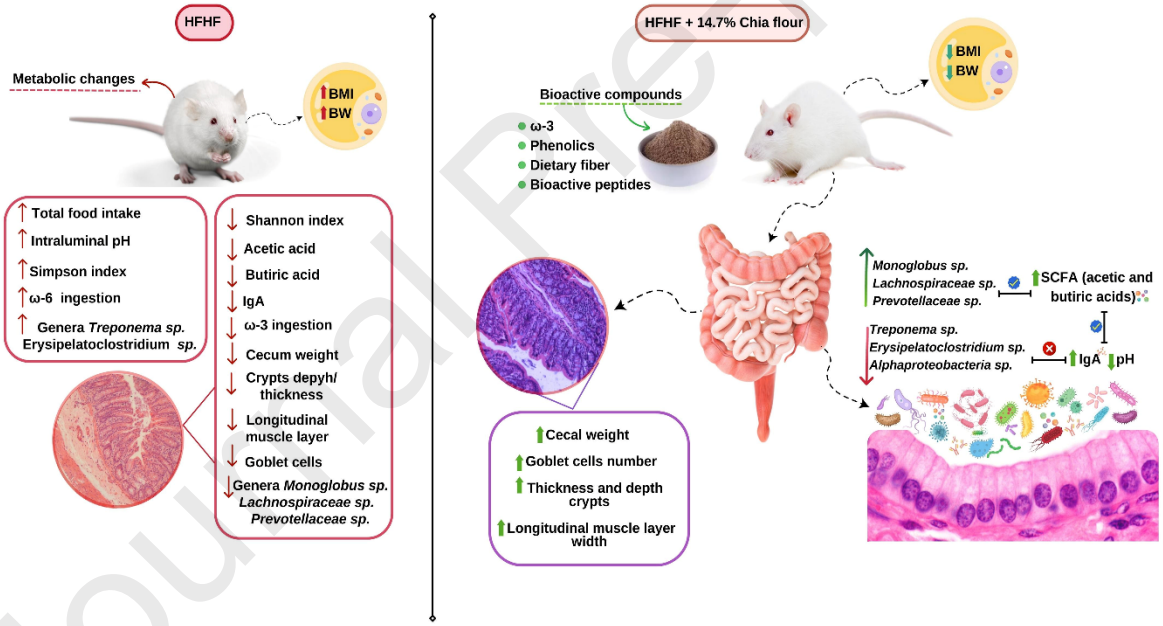
| | AIN-93M | HFHF | HFHF +CF |
|---|---|---|---|
| Demethylmenaquinol-9 biosynthesis | 0.006 ± 0.003 b | 0.005 ± 0.002 b | 0.011 ± 0.002 a |
| Menaquinol-9 biosynthesis | 0.009 ± 0.004 b | 0.008 ± 0.003 b | 0.017 ± 0.003 a |
| Glycolysis V (Pyrococcus) | 0.002 ± 0.002 a | 4.86E+04 ± 44.77 ab | 9.95E+04 ± 7.14 b |
| Thiamin salvage II | 0.393 ± 0.043 b | 0.474 ± 0.052 ab | 0.479 ± 0.035 a |
| 4-hydroxyphenylacetate degradation | 2.37E+06 ± 4.73 b | 3.15E−04 ± 7.77 b | 0.001 ± 0.001 a |
| Menaquinol-6 biosynthesis I | 0.009 ± 0.004 b | 0.008 ± 0.003 b | 0.017 ± 0.003 a |
| Kdo2-lipid A biosynthesis | 7.69E+04 ± 6.91 b | 0.003 ± 0.003 ab | 0.009 ± 0.005 a |
| L-methionine biosynthesis III | 0.182 ± 0.035 b | 0.301 ± 0.059 ab | 0.258 ± 0.053 a |
| Chitin derivatives degradation | 6.76E−05 ± 6.46 b | 9.15E−06 ± 0.00 b | 8.79E+04 ± 4.90 a |
| Demethylmenaquinol-6 biosynthesis II | 0.015 ± 0.013 a | 0.003 ± 0.003 b | 0.022 ± 0.008 a |
| Demethylmenaquinol-6 biosynthesis I | 0.006 ± 0.003 b | 0.005 ± 0.002 b | 0.011 ± 0.002 a |
| Enterobacterial common antigen biosynthesis | 1.31E+06 ± 2.94 b | 4.12E+04 ± 0.001 ab | 0.001 ± 0.001 a |
| Menaquinol-10 biosynthesis | 0.009 ± 0.004 b | 0.008 ± 0.003 b | 0.017 ± 0.003 a |
| Methylphosphonate degradation I | 0.001 ± 0.001 b | 0.001 ± 0.001 b | 0.008 ± 0.011 a |
| L-arginine/ putrescine/4-aminobutanoate degradation | 1.57E+06 ± 3.30 b | 4.63E+04 ± 0.001 ab | 0.001 ± 0.001 a |
| L-arginine and L-ornithine degradation | 1.57E+06 ± 3.30 b | 4.63E+04 ± 0.001 ab | 0.001 ± 0.001 a |
| TCA cycle VII (acetate-producers) | 0.024 ± 0.014 b | 0.015 ± 0.006 b | 0.043 ± 0.021 a |

Low FBC — High FBC

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