INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGÍA MOLECULAR

MODULATION OF INTESTINAL MICROBIOME IN OBESE MICE ASSOCIATED WITH ADMINISTRATION OF AMARANTH OR SOYBEAN PROTEIN ISOLATES

Tesis que presenta

Diana Olguín Calderón

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Director de la Tesis:

Dra. Ana Paulina Barba de la Rosa

San Luis Potosí, S.L.P., mes de año

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Constancia de aprobación de la tesis

La tesis ***"Modulación del microbioma intestinal en ratones con obesidad asociado a la administración de aislados proteínicos de amaranto o soya"*** presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por Diana Olguín Calderón y aprobada el **veintiuno de agosto de dos mil diecisiete** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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a fin de efectuar el examen, que para obtener el Grado de:

MAESTRA EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó la C.

Diana Olguín Calderón

sobre la Tesis intitulada:

Modulación del microbioma intestinal en ratones con obesidad asociado a la administración de aislados proteínicos de amaranto o soya

que se desarrolló bajo la dirección de

Dra. Ana Paulina Barba de la Rosa

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Abbreviations

AMA-HF	Amaranth globulins supplement with high-fat diet group
AMA-RD	Amaranth globulins supplement with regular diet group
ANOVA	Analysis of Variance
BCFA	Branched-Chain Fatty Acids
Ctrl-HF	PBS control supplement with high-fat diet
Ctrl-RD	PBS control supplement with regular diet group
DNA.	Deoxyribonucleic acid
FFAR2	Free Fatty Acids Receptor 2
FIM.	Foundation for Innovation in Medicine
GC-MS	Gas Chromatography- Mass Spectrometry
GLP-1	Hormone Glucagon-Like Peptide
GPR43	G-Protein Coupled Receptors 43
OTU´s	Operational Taxonomic Units
PBS	Phosphate Buffer Solution
PYY	Peptide YY
RDP	Ribosomal Data Project
SCFAs.	Short Chain Fatty Acids
SOY-HF	Soybean globulins supplement with high-fat diet group
SOY-RD	Soybean globulins supplement with regular diet group

Resumen

Modulación del microbioma intestinal en ratones con obesidad asociado a la administración de aislados proteínicos de amaranto o soya

La obesidad se define como la acumulación anormal o excesiva de grasa corporal que puede representar daños en la salud. Diferentes estudios sugieren que en condiciones de obesidad, la modificación de la dieta produce cambios en la microbiota intestinal mejorando así los perfiles bioquímicos. El amaranto, es un alimento reconocido por sus propiedades benéficas a la salud, pero sus efectos en el perfil microbiano aún se desconocen. El objetivo del presente trabajo fue evaluar el efecto del consumo de proteína de amaranto o soya sobre la microbiota intestinal en ratones con obesidad inducida por dieta. Ratones C57BL/6 machos fueron alimentados por 8 semanas con dieta regular o dieta alta en grasa. Se añadió a la dieta el aislado de proteína de amaranto o el de soya. La microbiota fue aislada de las heces y se secuenció el ADN del 16S ribosomal. Los cambios morfológicos del cecum fueron medidos en cortes histológicos teñidos con hematoxilina y eosina. Con el fin de evaluar la actividad metabólica se cuantificaron los ácidos grasos de cadena corta (SCFA) mediante cromatografía de gases. El aislado de soya tuvo efectos indeseables en la pérdida de grasa corporal. El suplemento de amaranto incrementó las familias bacterianas *Prevotellaceae* y *Ruminococaceae*, así como los niveles de propionato como su producto de fermentación, perfiles similares al grupo de peso normal. Además, la incorporación de la proteína de amaranto promovió la elevación de la profundidad de la cripta y el número de células caliciformes del cecum, sustentando su efecto benéfico a la salud. Este análisis comparativo indicó que la administración de la proteína de amaranto restaura la actividad microbiana alterada en el estado de obesidad, sugiriendo así un mecanismo de acción del amaranto sobre la salud metabólico del hospedador.

PALABRAS CLAVE: ÁCIDOS GRASOS DE CADENA CORTA, AMARANTO, MICROBIOMA INTESTINAL, OBESIDAD, SUPLEMENTO PROTEÍNICO.

Abstract

Modulation of intestinal microbiome in obese mice associated with the administration of amaranth or soybean protein isolates

Obesity is an abnormal or excessive body fat accumulation that it may have negative effects on health. Several studies have suggested that diet modification disrupt intestinal microbiota improving the biochemical profiles in the obese condition. Amaranth is well known because its beneficial properties on health but the effects on microbiota profile are still unknown. The aim of this work was to assess the effect of the gut microbiota composition due to amaranth or soybean protein complementation in diet-induced obese mice. Male C57BL/6 mice were fed for 8 weeks with a regular or high-fat diet. Diet was complemented with amaranth or soybean protein isolates. Microbiota was isolated from faeces and 16S ribosomal DNA was sequenced. The morphological changes in caecum ultrathin sections were measured after hematoxylin/eosin staining. To assess metabolic activity, a quantification of Short Chain Fatty Acids (SCFA) was achieved by gas chromatography. The soybean protein isolate had undesirable effects on fat weight. Amaranth supplement increased the *Prevotellaceae* and *Ruminococcacea* families, also the levels of propionate as their fermentation product was similar to regular diet. Amaranth protein also increased cecal crypt depth and calceiform cell number sustaining its beneficial effect on health. This comparative analysis indicated that the administration of amaranth protein restored the altered microbial activity in the obesity state, thus suggesting a mechanisms of action by which amaranth exerts the metabolic health of the host .

KEYWORDS: SHORT-CHAIN FATTY ACIDS, AMARANTH, GUT MICROBIOME, OBESITY, PROTEIN SUPPLEMENT.

1. Introduction

Obesity is a positive imbalance of energy intake and expenditure with excessive weight gain and is related to comorbidities as diabetes mellitus, cardiovascular or metabolic diseases [1–3]. Furthermore, obesity has increased the international morbidity rate and has become one of the most disquieting health problems of the 21st century with over 1.9 billion overweight adults and approximately 600 million within these are obese [4]. Moreover, this increase in obesity has been observed in both developed and developing countries and is not exclusively for adults, more than 41 million children under five are overweight or obese. Mexico is an example of obesity impact in economical of developing countries. The International Association for the Study of Obesity reported that, just in Mexico, it has been an increase of obese adults economically actives, from 26.8% in 2000 to 37.5% in 2012. This rates are placing Mexico as the second country with the highest rates, after the United States, and the first place with child overweight and obesity ratio [5].

There are a set of treatments for the obese condition, which lead from lifestyle modification, pharmacological or psychological therapy, surgery and even the implementation of new procedures as a fecal transplant [6, 7]. Nervertheless, drugs and surgery are not appropiate for each patients and represent the last options due to side effects, while a lifestyle modification have limitations and low efficienty. Diet modification has always had the greatest impact in preventing the development of comorbidities and improving of life quality in patients who present an excessive weight gain. On the pursuit of health-beneficial foods, incorporating diets

containing nutraceutical foods such as, soybean, garlic or tea, have become a popular reaseach topic due to the favorable effects on hypertension, cardiovascular and metabolic diseases [8, 9]. The founder of Foundation for Innovation in Medicine (FIM), describes the nutraceutical term as, “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of disease” [10]. Amaranth is another example of a nutraceutical food with reported health benefits [11]. In addition, high content of dietary fibers and unsaturated fatty acids, this pseudocereal possesses proteins rich in essential amino acids (lysine, tryptophan, and sulfur amino acids) as well as bioactive peptides which efficacy in differents diseases has been demonstrated [12–15].

Interactions between nutraceutical food and microbiota are extremely associated. Dietary components are susceptible to be metabolized by the bacteria according to their metabolic capacity during the gastrointestinal passage for their subsequent absorption [16]. The intestinal microbiota is composed of a large amount of microorganism, approximately 10 to 100 trillion, exceeding up to ten times the human body cells. Thus, microbiota is considered as a “superorganism” that is comprised by one tenth of human cells, and the remaining are microbial organisms [17]. Furthermore, it has been noticed that the intestinal microbiota is susceptible to be modulated through the modification of diet. According to this, the bacterial changes by nutritional therapy may generate a similar profile to healthy individuals indicating a restoration on bacterial profiles in stool samples [18].

Bacteroidetes and *Firmicutes* are the principal phyla that suffer alterations in abundance and diversity when feeding habits and compounds of diet are modified in obesity individuals, having a greater ratio of Firmicutes to Bacteroidetes in obese

conditions than their lean controls [19]. In the distal colon, these phyla are important for dietary fibers fermentation in order to create ends product such as Short-Chain Fatty Acids (SCFAs), that not only improve the microbial environment but also regulate several processes in the host [20]. In lean individuals, intestinal SCFAs concentration is mainly constituted by acetate, followed by propionate and butyrate in a molar ratio of 60:20:20. Principal members of Bacteroidetes are acetate and propionate producers whereas, in Firmicutes, butyrate producers are abundant [21]. Differents functions in intestinal tissue or whole organism are attributed to SCFAs, among them are the inhibition of histone deacetylases related to modulation of inflammatory processes and their recognition by specialized G-protein coupled receptors (GPR43-41) [22]. It is also known that this recognition by GPR43 or GPR41 in colon or adipose cells estimulates the secretion of the anorexigenic molecule, intestinal peptide YY (PYY) and the hormone glucagon-like peptide 1 (GLP-1), key molecule of the increase of plasmatic insulin [23].

Effects in microbial communities by whole digestible compounds from pseudocereals, such as quinoa or amaranth, have an impact in the production of SCFA, principally due to butyrate producer communities that has been observed in increased amount during whole amaranth seeds treatments [24]. Also, consumption of protein from this grains increases leptin levels, molecule positively involved in appetite control, on the other hand, these formulations reduce concentrations of the ghrelin orexigenic hormone, principally exposed by amaranth protein [11, 25].

The majority of SCFA are products from the fermentation of resistant starch, but also their colonic generation is attributed to all oligosaccharides of

monosaccharides units, such as fructooligosaccharides and galactooligosaccharides in the proximal colon [26]. In a lesser extent, amino acids are substrates of fermentation processes that create Branched-Chain Fatty Acids (BCFA) in the distal colon that have similar effects in host metabolism regulation as SCFA [27].

To date, there are no reports of how amaranth proteins, which contain a well balance of essential amino acids, can modify the profile of the intestinal microbiota and hence the generation of SCFA, and whether this change could be responsible of the health benefits attributed to the amaranth consumption. Due to the above, the aim of this work was to assess the effect of the intestinal microbiota profile on diet-induced obese mice due to a daily amaranth or soybean protein administration.

2. Materials and Methods

2.1 Animals and diets

Male 6-week old C57BL mice were obtained from Unidad de Producción y Experimentación de Animales de Laboratorio UPEAL (UAM, Xochimilco, Mexico). Mice were housed in controlled conditions at 21± 2 ºC and 50% ± 15% humidity with a 12 h light/dark cycle in groups of four to five mice in a standard stainless steel cage with free access to water. Animals were randomly assigned to six groups containing eight mice per group. In the two first groups, the control groups, animals were feed with regular diet (Ctrl-RD) and high-fat diets (Ctrl-HF). Third and fourt groups mice with regular diet or high-fat diets were supplemented with amaranth protein isolate (AMA-RD or AMA-HF, respectively). The fith and sith groups diets were supplemented with soybean protein isolate (SOY-RD or SOY-HF, respectively).The commercial Teklad 2018S with 18% kcal from fat (RD) and the Teklad TD 06414 with 60.3% kcal (HF) from fat (Envigo, Huntingdon, UK). were used. All groups were given the same amount of feed for 8 weeks. The food consumption and mice weight were recorded every week. Supplements were obtained from amaranth or soybean flour as described in a parallel study [28], the administration was achieved at a dose of 10 mg/Kg.

Procedures for the animal housing and care were assessed according to Mexican regulatory standard (NOM-062-ZOO-1999) and the animal experiments were approved by the Institutional Research Bioethics Committee at IPICYT Code:LPBM-AMA-C57/002 and ratified by the Ethics Research Committee Code: DIX.UC-EB-17-001 (Registration code: CONBIOETICA24CEI00320130722).

At the end of the experiment, mice were euthanized, whole intestinal tissue was collected, placed in liquid nitrogen, and stored at -80 ºC until microbial analysis.

2.2 Bacterial characterization analysis.

2.2.1 Sequencing and analysis of bacterial 16S DNA

Faeces were obtained by extrusion from each thawed intestinal tissue. DNA extraction was achieved using the DNeasy UltraClean microbial kit (Qiagen, Hilden, Germany) from 200mg of intestinal content following to the manufacturer´s instructions. Obtained DNA was quantified by spectrophotometry with NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and stored at -80 ºC until use. The assay was made from four intestinal contents measured equally and pooled as a representative major sample.

Paired-end sequencing with a read length of 2×250 pb was performed using the Illumina Miseq platform by the molecular sequencing laboratory Research and Testing Laboratory (Lubbock, Texas, USA.). The V3-V4 regions of 16S ribosomal DNA were amplified by PCR using the universal primers containing Illumina adapter sequences (357wF/785R). The obtained sequences were merged and filtered by quality using Pear from the free online software Galaxy v 0.9.6.0 (http://www.usegalaxy.org). A specify P value of 0.01, 35 as minimum overlap size, 200 pb as the minimum possible length of the assembled sequences and a Phred score of 25 were applied in the analysis [29]. The filtered sequences were dereplicated and cleaned of chimeric sequences with UCHIME with the reference database GoldFasta using USEARCH-Tool-Suite. Resulted data were grouped into operational taxonomic units (OTU's) with 97% of identity using the database from

Ribosomal Data Project (RDP) available online in GALAXY VGL 4.0.1 (http://galaxy-qld.genome.edu.au accessed APRIL/2017). Explicet v2.10.5 software was used to make the OUT Stacket Bar plot to represent bacterial relative abundances between groups.

2.2.2 Measurement of Short-Chain Fatty Acids (SCFAs) from feces.

The intestinal content was used for SCFAs quantification following the protocol described by Hun et al. [30]. Briefly, stool samples (100 mg) were homogenized in one mL of ultrapure MilliQ® water (Merck, Darmstadt, DE), and mixed on a vortex mixer for 2 min. The homogenized sample was incubated for 20 min in an ice-water bath and centrifuged at 4 ºC for 20 min at 4800*g.* The supernatant was recovered and this procedure was repeated two times for clarifying. The sample was filtered trough 0.22 µm Millipore filter (Merck, Darmstadt, Germany) before injection in the chromatographic system.

Analysis of SCFAs was performed on an Agilent 6890 N GC system using a 30 m X 0.25 mm I.D. with a film thickness of 0.5 µm HP-INNOwax GC capillary column (Agilent Technologies Inc, CA, USA). Helio was used as carrier gas at a flow rate of 1.5 mL/min with a split ratio of 1^{e+001}:1 and the set temperatures for the injector and flame ionization detector (Agilent Technologies Inc.) were 220 and 250 ºC, respectively. The flow rates of hydrogen and air were 30 and 300 mL/min, respectively. The volume for injected sample was 1 µL, and the running time was 20.5 min. The determinations were performed on three individual samples for each group. Calibration curves were performed from 1.5 to 100 mg/L for acetic acid, 3.1

to 50 mg/L for propionic acid and 1.87 to 30 mg/L for butyric acid. (5 concentration levels, 3 replicated for each level).

The additional GC-MS confirmatory analyses were carried out in similar conditions in a TRACE™ 1300 gas chromatograph (Thermo Fisher Scientific™, MA, USA.) coupled to an ISQ™ Series Single Quadrupole Gc-MS System (Thermo Fisher Scientific™) with 30m × 0.25 mm I.D. with a film thickness of 0.25 µm DB-wax GC capillary column (Agilent Technologies Inc.). The chromatographic conditions were 30 °C for 8 min, increased at 15 °C/min to 210 °C and maintained at this temperature for a final time of 5 min. Helium was used as carrier gas at a flow rate of 1.0 mL/min and the injector and detector temperatures were 250 and 230 °C, respectively. The MS ionization potential was 70 eV, transfer line temperature was 220 °C, and the scan mode was 30–200 m/z. The assigned of compounds were performed comparing their mass spectra with the NIST library of the MS database.

2.3 Effects on bacterial-host interaction

2.3.1 Histomorphometry appearance of the cecum

Frozen cecum was dissected into 7 mm sections cutting 0.6 mm approximately from the ileum for each randomly selected guts. Samples were immersed in 10% formaldehyde in PBS (pH 6.4), de-hydrated, clarificated and embedded in a paraffin. Each block was sectioned into 7 µm thick and stained with hematoxylin-eosin. The morphometrical analysis was achieved with the light microscope Zeiss Axio Imager M2 (Carl Zeiss Co. Oberkochen, German).

Crypt depth measurements were adapted from a previous report by De Conto

et al. [31], by triplicate in 10 well-oriented Lieberkühn crypts by field from the base to the highest point still visible with 40-fold magnification using ImageJ software v 1.46r.

2.4 Statistic analysis.

Quantitive data was evaluated using SigmaPlot 12.3 software (Systat Inc., Illinois, USA), through a Kolmogórov-Smirnov normality test followed by a one-way analysis of variance (ANOVA) with a post hoc Tukey test ($p<0.05$) with a desired statistical power of 0.8 and a Kruskal-Wallis with a post hoc Dunn test for non-parametrical data.

3 Results

3.1 Physiognomic parameters measurements

Several studies suggest that body weight, food intake and gut microbiota composition may presents variations depending on interactions with sex [32, 33]. To avoid gender as a variation factor, only male C57BL mice were used. In assessing physiognomic parameters, it was noted a major proportion on weight gain through period treatment in all mice with HF (high fat) diets than with mice with RD (regular diets), even with protein supplementation. On the other hand, HF diet with additional protein from grains gained weight in less proportion than the Ctrl-HF diet. Nevertheless, soy proteins caused a similar increase in body weight regardless of diet (Table 1).

Food consumption at the beginning of the experiment (Table 1) was similar in both control (Ctlr) groups, while mice from a SOY-RD or AMA-RD ate more than the Ctrl-HF group ($P<0.001$). At the end of the experiment period, all mice ingested a major feed amount compared with SOY-HF, which was a group with the least consumption of all diets ($P<0.001$ in relation to Ctrl-HF).

Total weight of extracted intestinal tissue from SOY-HF mice was lower and statistically different in respect to regular diet groups ($P<0.05$). Only mice supplemented with protein isolate were different on intestinal weight by the kind of administrated diet (AMA-RD vs AMA-HF $P<0.05$ and SOY-RD vs SOY-HF $P<0.05$) (Table 1).

Table 1. Physiognomic parameters at different nutrimental conditions in all groups at the beginning and after eight weeks of protein consumption.

	Ctrl-RD	Ctrl-HF	AMA-RD	AMA-HF	SOY-RD	SOY-HF
Body weight (%)	14.56 ± 6.01^{a}	41.21 ^{b} ±20.4	19.33 ±7.76 ^{a}	36.19 ±13.63 ^{b}	25.12 ± 8.28 ^{a,b}	35,71(10.66) ^{b}
Food at T_o (g)	3.21^{a,b,c,d}	2.31^{a,c}	4.10 ^{b,d}	2.26^{c}	5.46^{d}	3.15^{a,b,c,d}
Food at T_8 (g)	2.93^{a}	2.33^{a,c}	3.35^{a,b}	2.22^{a,c}	3.51^{a,d}	1.36^{c}
Intestinal weight (g)	1.88 ±0.22 ^{a,b}	1.70 ±0.30 ^{a,b,d}	2.02 ±0.22 ^{b}	1.66 ±0.29 ^{a,d}	1.88 ±0.15 ^{a,b,c}	1.47(0.27) ^{d}
Epididymal fat tissue weight (g)	0.38 ± 0.17 ^{a,d}	1.3 ± 0.56 ^{b,c,e}	0.38 ± 0.12 ^{a,d}	1.38 ±0.49 ^{c,e}	0.61 ± 0.18 ^{d}	1.2(0.27) ^{e}

To=values at the beginning .of the experiment. T_0=after 8 week. Values represent the mean values of total population in each group ± standard deviation (SD). Grams (g). Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. SD=Statistic deviation. Superscript letters along rows indicate statistical differences at $P<0.05$ Kruskal-Wallis with a post hoc Dunns test .

Epididymal fat tissue weight (Table 1) was higher with HF diet in respect to the RD provided. Furthermore, there were no statistical differences among groups with similar kind of diet, nevertheless, SOY-HF tended to increase the amount of fat tissue (P>0.05). Macroscopically appearance showed that intestinal tract was mainly coated by fat on a HF-SOY diet (Figure 1).

3.2 Cecum histomorphometric appearance with and without globulin administration

Intestinal mucosa in both RD-AMA and HF-AMA behaves similarly in terms of crypt depth. However, there were more epithelial cells by crypt in the AMA-HF group and the number and size of calceiform cells on this diet therapy closely resembled Ctrl-RD (Figure 2A). Furthermore, independently of treatments, a HF-diet decreased Lieberkhün crypt depth in a statistically significant way (P<0.001). Nevertheless, the amaranth protein therapy had the minor impact with similar values in both sort diets (P>0.05) (Figure 2B). On the other hand, SOY-RD increased the size and number of calceiform cells with a high relative crypt depth with respect to SOY-HF, which tended to lose crypt order and definition as well as tissue preservation.

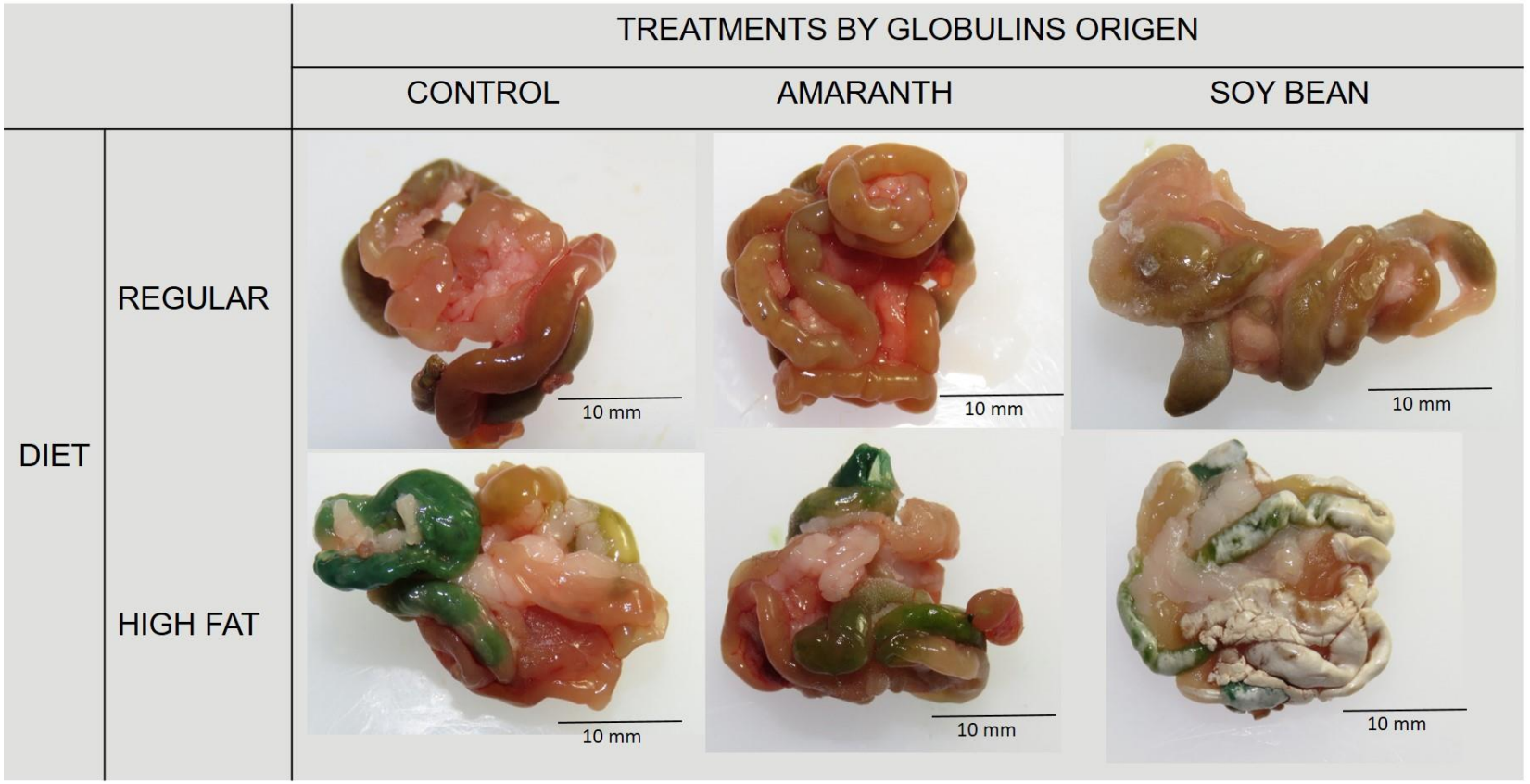

Figure 1. Macroscopical appearance of intestinal tissue from mice with different diets. Digital images indicate the amount of fat accumulation coating the gut structures. Intestinal tissues from mouse were selected randomly from each group to display as representative samples.

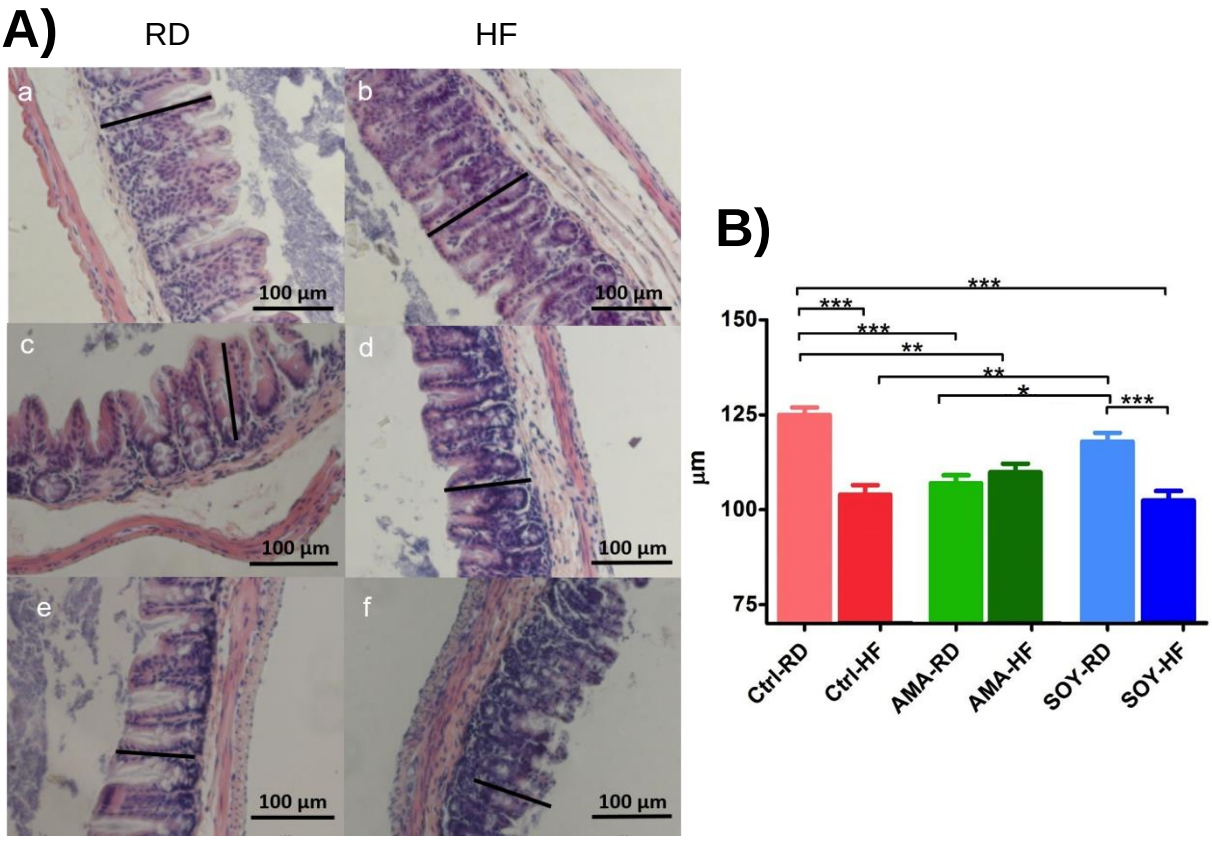

Figure 2. Morphometric analysis of cecum from control and protein isolate diets. (A) Representative micrographics for control (a and b), amaranth globulins (c and d) and soybean globulins (e and f) groups with regular (RD) or high-fat (HF) diet. H&E staining 40X. **(B)** Crypt depth modification of intestinal cecum. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Bars indicate mean and SEM values of each ultrathin slide. Asterisks denote significant differences (*P<0.05, **P<0.01 and ***P<0.001 Kruskal-Wallis with a post hoc Dunns test).

3.3 Sequence analysis of the intestinal bacteria population.

Relative abundance of the sequences obtained showed the microbial profile in the samples. It was observed the abundance of the *Firmicutes* as the main phylum in

the entire population and an increase of *Bacteroidetes* and *Actinobacteria* phyla in AMA-RD group (Figure 3).

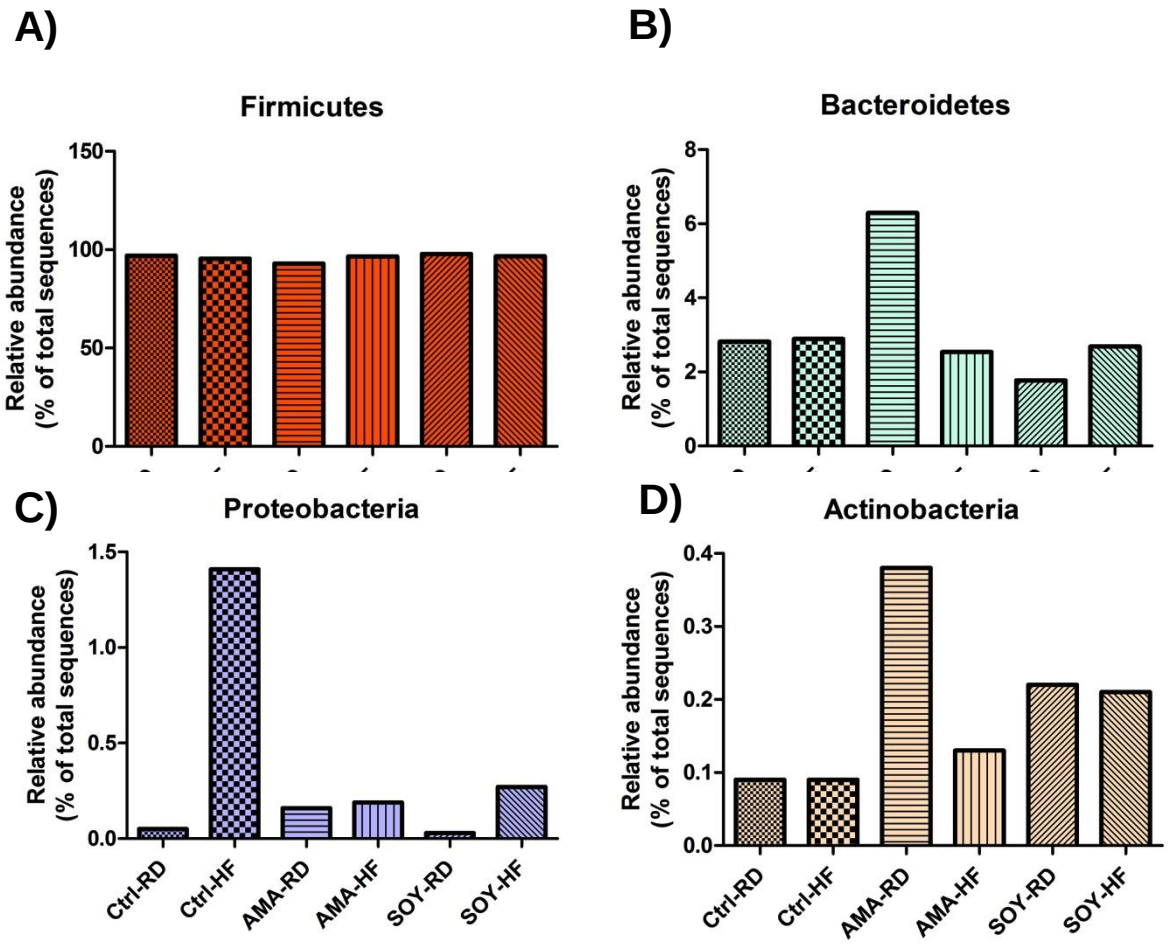

Figure 3. Relative abundance (%) of microbiota at the phylum level. Four more relative abundant phyla with values higher than 0.10% are shown in decreasing order: (**A)** Firmicutes, (**B)** Bacteroidetes, (**C)** Proteobacteria and (**D)** Actinobacteria. Bars indicate relative abundance from four mice pooled for each group. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet.

However, looking at this phylum independently at a lower taxonomic level, *Ruminococcaceae* family was reduced in HF diet mice with both protein treatments in respect to Ctrl-HF group. *Lachnospiraceae* increased in both kind of treatments, regardless of diet but mainly showed by soy proteins administration (Figure 4).

In addition to the *Firmicutes* phylum, there was a notable difference in *Helicobacteraceae* family, which was decreased on HF-diet treatments. *Prevotellaceae* and *Porphyromonadaceae* were identified with a light increment in AMA-RD in comparison to Ctrl-RD. *Bacteroidaceae* was decreased in both treatments in contrast to Ctrl-HF, being similar to Ctrl-RD. Finally, HF-AMA increased the *Verrucomicrobia* family, while soy treatment did not modify the profiles (Figure 5).

3.4 Protein administration generates similar values of Chain Fatty Acids (SCFAs) determination in diets.

Fecal concentration of SCFAs showed that the most abundant acid was acetate, followed by butyrate and propionate (Figure 6). Values of total acids were decreased in both treatments groups with lower values in HF diets. Mice in the Ctrl-RD group have the higher acetate quantities compared with HF diets ($P>0.05$).

The mice fedd with SOY-HF showed the lowest propionate or butyrate concentrations in relation to Ctrl-RD ($P<0.05$). Meanwhile, the propionate concentration showed low differences among diets with protein treatments ($P>0.05$).

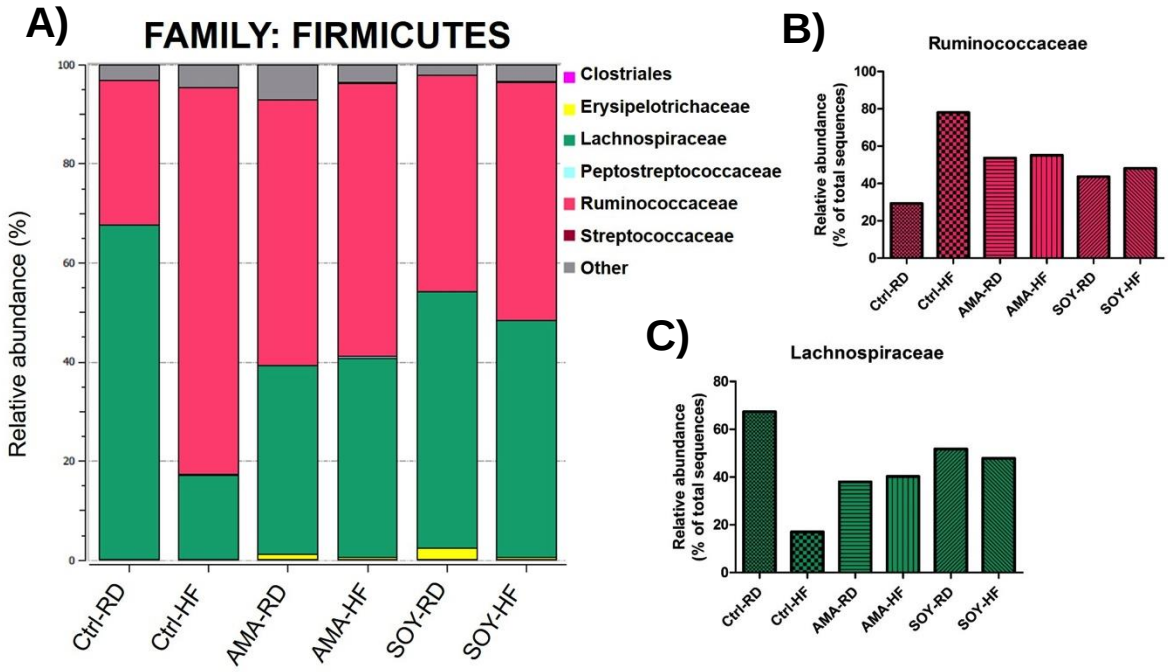

Figure 4. Relative abundance (%) of Firmicutes phylum at the family level. (A) The population of total bacteria. Other groups represent the proportion of all bacterial families but Firmicutes. (B) and (C) indicate the relative abundance or the most abundant families, Ruminococcaceae and Lachnospiraceae, respectively. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet.

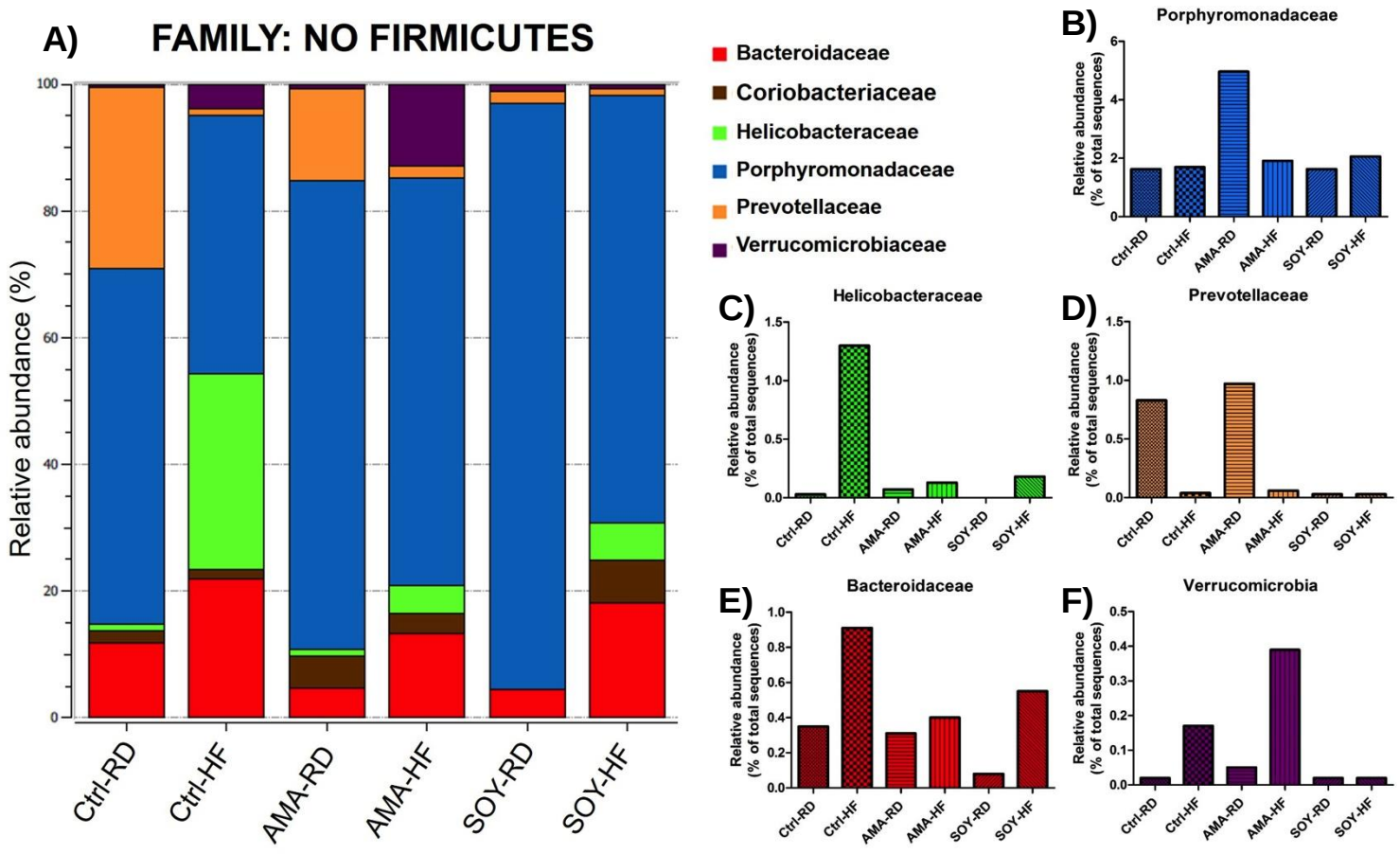

Figure 5. Relative abundance (%) of no Firmicutes phyla at the family level. (A) The total population of non-Firmicutes families. (B) to (F) indicate the relative abundance for families with more than 0.10% of abundance. Graphics show family values in decreasing order. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet.

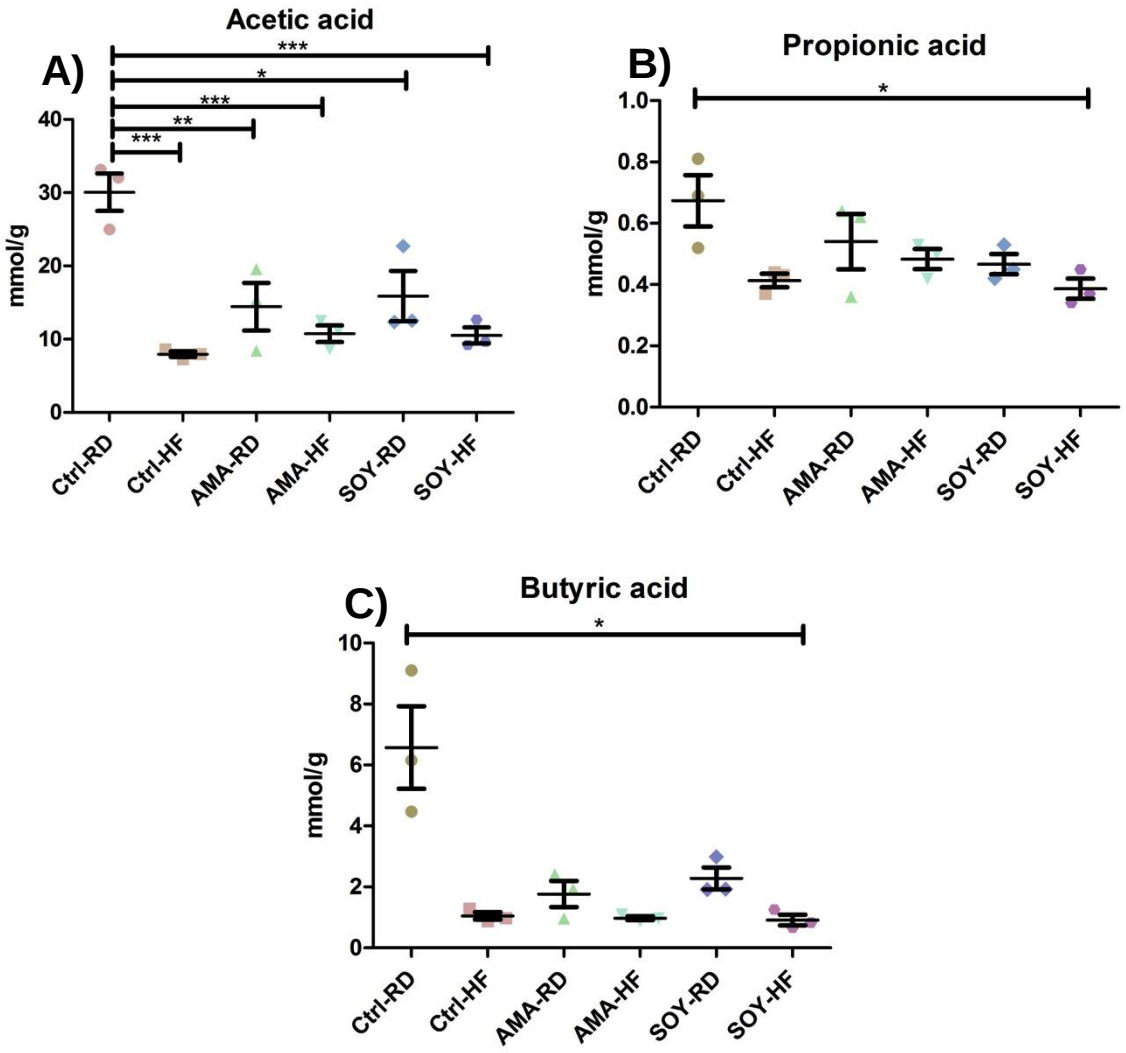

Figure 6. Short-Chain Fatty Acids analysis in mice intestinal contents with different diets. The concentration of **(A)** acetic acid, **(B)** propionic acid and **(C)** butyric acid. A and B distributions present means with SEM for each group and C median with interquartile range. Dots show individual samples. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Asterisks indicate statistical significance (**P*<0.05, ***P*<0.01, ****P*<0.001 Tukey or Dunns posthoc tests).

4 Discussion

Consumption of ancestral whole grains such as amaranth and quinoa, because of their prebiotic or nutraceutical beneficial effects, have been widely studied [10, 24, 34, 35]. Although the consumption of the whole amaranth seeds has had an impact as a nutraceutical food, due to its multiple benefits to health, to date the molecules responsibles by which amaranth exerts its biological functions are not fully known [13]. Therefore, the use of a reduced amount of components from their seeds will allow a better understanding of the health benefits of each component [36, 37]. It has been reported that the consumption of amaranth proteins, which contains an excellent balance of essential amino acids, decreases the accumulation of body fat and the inhibition of HMG-CoA, a key enzyme in cholesterol synthesis [12, 15]. On the other hand, soybean is the main plant source of dietary protein [38, 39]. Soybean proteins are comprised by globulins in approximately 80% of total protein and its consumption is reported to ameliorate biochemical profiles on a hypercholesterolemic diet [40].

Recently, the modifications in microbiota population involved in processing of general non-metabolized proteins by host have been studied. Because portion of not digested components are substrates for microbial metabolism that generates products that affect the overall health [27, 41].

In the present work, we analysed the microbiome profile present at bacterial phyla in mice subjected to a HF or RD diets and the changes of this microbiome due to the diet supplementation with protein isolates from amaranth and soybean. With this feed regimens, mice exhibited a similar body weight gain, intestinal

weight and epididymal fat content dependent on fat percentages on diet. However, the values of weight gain and fat from the epididymal region were highest with soy protein isolates treatment among RD diet groups, which have a comparable behave to SOY-HF (Table 1).

Previous studies suggest that the high consumption of soybean protein decrease the food intake and body weight compared to others protein sources, as whey [42, 43]. Withstanding this, the current study shows the notable differences on food consumption by SOY groups. At the end of the experiment, SOY-RD mice change from being the most feed consumers to regulate their intake to normal levels. Furthermore, with the inclusion of 60% of calories from fat, SOY-HF had the lowest eating levels thus explaining the low intestinal weight on this group. Despite limited food ingest, macroscopical differences shown the excessive cover of fat, while on an amaranth protein the cover is comparable to controls, supporting previous ideas that are not only the amount of protein but also source of protein is important to have positive effects [43].

Regarding the modulation of microbiota profiles, results exposed by population showed a similar *Firmicutes* abundance in all diets (Figure 1A). Some authors have related this to an obese or overweight status [44], while others have had associating it with higher values to lean population [45, 46]. Variability is remaining unknown but could be attributed to different model species, kind of diet, laboratory conditions, and also the design of the study [47]. According to above, this project differs from others by the fact that mice were submitted to a starvation period before euthanasia, this in order to evaluate prediabetes signs in a parallel study, as well as impaired fasting glucose [28]. The stress generated by

deprivation of sleep or forced exercise can promote the abundance of *Firmicutes*, more specifically the abundance of *Ruminococcaceae* family [48, 49], as well as starve as indicated in the present report (Figure 2B). *Ruminococcaceae* and *Lachnospiraceae* families are associated with the increase of genes involved in inflammation process, such as *angpl4,* in diet-induced obese mice [46]. The present *Ruminococcacea* data sustain this statement, raising values on a HF-diet and being similar among treatments (Figure 2B), but not *Lachnospiraceae* behave, revealing that this family could be implicated in another metabolic mechanism. Furthermore, families aside of *Firmicutes*, like *Helicobacteraceae* from *Proteobacteria* phylum were incremented due to a HF-diet, but with protein administration, the family growth is limited. Similarly, in a strain of C57LB/6 mice on HF feed regimen, this family reasonably increased with emphasis within *H. hepaticus and H. bilis* species [50], whose activity mitigation is related to ameliorate inflammation on colitis and bowel disease [51, 52]. Families belonging to *Bacteroidetes* phylum, *Porphyromonadaceae* and *Prevotellaceae*, were markedly increased on AMA-RD (Figure 1B, 3B and D). Zhu et al. (año) demonstrated the compensatory effect of *Porphyromondaceae, Prevotellaceae, Bifidobacteraceae* and *Peptostreptococcaceae* growth through restoring protein consumption to normal on a low protein diet, along with the increase of SCFAs, majorly butyrate, and tight junction molecules, as ocludin and ZO-1, indicating an improvement of barrier function in the colon [53]. Due to the poor knowledge of *Verrucomicrobiaceae* family, it is difficult to perceive the overall activity of this clade because, within the few studies on this bacterial group, one indicates the importance of *Akkemansia miciniphila* activity on intestinal mucus layer

maintenance, which increases even on a HF-diet [54]. Relative abundances of this family on the current study have showed arise on HF diets except on those with soybean proteins, opposing to Ctrl-RD, suggesting that the keeping mucus barrier mechanisms involved are more complex and not exclusively depends on one specie.

Except for acetate, values of intestinal SCFAs exposed a considerable but not statistically significant decrease between treatments, only SOY-HF diet was different in all acids with respect to Ctrl-RD (Figure 5). According to this fact, the principal substrate to SCFAs generation are carbohydrates [26] and, the regular diet used in the present study had these molecules as major macronutrients. However, the restriction of carbohydrates was lightly compensated by protein intake in propionate production on AMA-RD as Ctrl-RD (Figure 5B). These acid levels have a similar tendency as their producer family *Prevotellaceae* on these groups (Figure 3D). SCFAs production by this family are expressed from polysaccharide fermentation [55], but the propionate and acetate observations have already been reported in a study where the restoration of protein intake on low protein diet rats, increased similarly to normal protein diet [53]. Also in faecal cultures from healthy individuals with amaranth or quinoa after *in vitro* digestion as a source of carbon, increased SCFAs, *Prevotellaceae*, and families from phylum *Firmicutes* in relation to control [24], an effect that may be attributed to amaranth proteins as shown in this analysis. In addition to above, butyrate is the principal energy substrate used by epithelial cells from colon tissue and is associated with maintained epithelial barrier of gut on optimal metabolic and immunological conditions [56]. Differences on acetate values could be explained by the elevation

of families from *Clostridia* class, which is known as butyrate producers when acetate is used as a substrate in co-colonization with *Bacteroidetes* acetate producers [57]. Alongs *Eubacteriaceae*, *Ruminococcaceae* within the *Clostridia* class was also found, and it was the increased family among treatments (Figure 2B), exposing their participation in similar butyrate production processes.

Evaluating the bacterial effect on the overall status of gut mucosa, the microscopical observation of cecum section from HF-diet mice confirm the previous description about the effect of limited carbohydrates consumption on epithelial barrier maintenance. Differentially, soy globulin therapy on a regular diet induces mucin production by calceiform cells on cecal intestinal portion, this could be observed by the increased size of this secretory cell lineage, calceiform cells, on SOY-RD (Figure 6A,C), which may explain the increment of crypt depth on this feed regimen (Figure 6B). Furthermore, on SOY-HF (Figure 6A,F) is appreciable the destruction in general epithelial structure, even when crypt depth is evaluated in reason to Ctrl-HF. Due to the extremely degradation observed, there exist stimulation on proliferative cell process, described as a compensative mechanism on a colitis infection-induced model [58]. SOY-HF exhibits an abnormal morphology with a considerable increase of epithelial cells density. Thus, this appearance suggests that a proliferative activity combined with an unappropriated microbiota would allow an abnormal differentiation process that, confer to toxins be absorbed due to barrier laxity [59, 60] and may permit the lipids that coat guts from SOY-HF mice form the fat cover described above.

There are exist reports that demonstrate SCFAs contribute in intestinal mucosa health, as a source of energy of epithelial cells from colon, promoting cell

proliferation. Nevertheless, while this study does not demonstrate significant values of these due to a probable carbohydrates reduction, there is a bunch of products from amino acids fermentation, such as BCFA, that belong to the same group of fatty acids with a similar recognition by FFAR, as iso-butyrate. Other products are biogenic amines, as putrescine, which are generated via *Actinobacteria* metabolism, that could regulate the immunological and morphometrical parameters [27].

Within the evaluation of SCFAs, chromatogram confirmation profile of a representative sample from each group, displayed differential peaks between diets (Supplementary Figure S1A). Meanwhile high-fat diets had similar profiles, among regular diets SOY-RD intestinal content showed peaks comparable to a high-fat diet. In a posterior analysis by mass spectrometry, abundant peaks represented complex cycled molecules, like paromomycin (34.27% of probability) or I-Gala-I-ido-octosa (31.75% of probability) (Supplementary Figure S1B). These findings indicate a potential group of molecules involved in epididymal fat accumulation and, therefore, an increased body weight gain in relation to Ctrl-RD and AMA-RD.

5 Conclusions

The supplementation of the daily diet with seeds protein isolates from amaranth and soybean induced the reduction of the abundance of Helicobacteracea and Ruminococcaceae families in the gut of mice feed with a diet rich in fat or obese mice. However, differences in microbiota profile were observed depending of the protein source. Soybean protein isolates caused undesirable results, due that although the mice showed a loss weight, the intestines macroscopic analysis showed an increase of adipose tissue and microscopically it was observed a damage of the epithelial barrier even when the consumption of the high fat diet decreased. By other hand, amaranth protein isolates, which are proteins with an excellent essential amino acid balance, consumed with a regular diet increased the abundance of the Prevotellaceae family as well the SCFA and propianate levels, which are fermentation products characteristics of these family. Also the *Actinobacteria* phylum was increased including bacteria containing aminopeptidases suggesting a probable increase of molecules such as BCFA that participate in the intestinal barrier recovery even when high fat diet were consumed. Amaranth then, is an excellente source of protein supplementation that exerts benefical health effects in obese mice.

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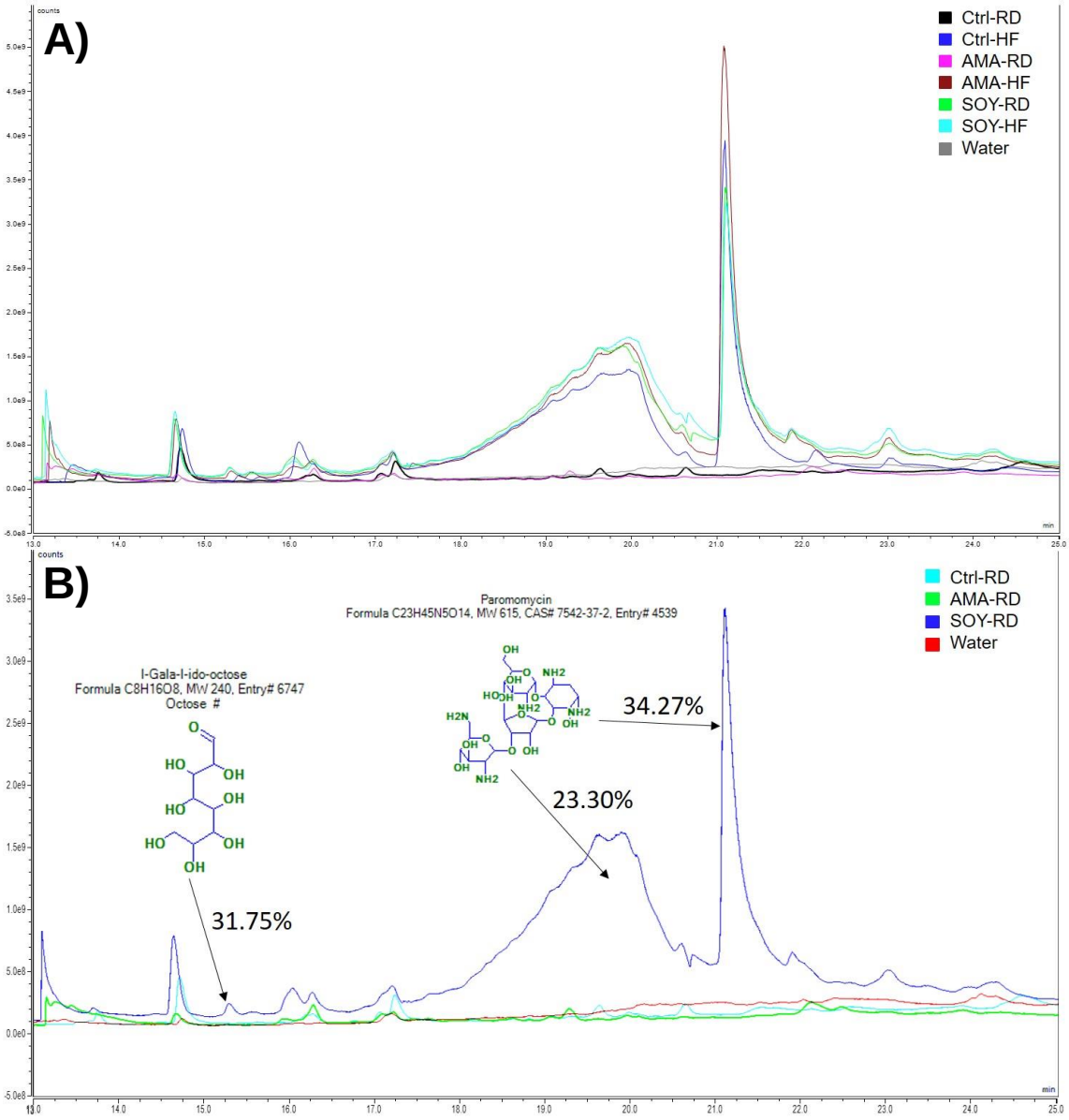
Supporting information



Supplementary Figure S1. Gas chromatograms of colonic contents at different treatments. (A) Representative individual mouse sample for all groups. **(B)** Samples from mice with globulins administration and regular diet. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Percentages indicate the probability of potential molecules for characteristic peaks. Chromeleon chromatography studio was used for determination of probability.