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Modulation of Caecal Microbiome in Obese Mice Associated with Administration of Amaranth or Soybean Protein Isolates

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Obesity is defined as abnormal or excessive body fat accumulation that may have negative effects on health. Healthy diet induces a balance of gut microbiota, helping in turn to combat this metabolic disorder. Amaranth is well known because of its beneficial properties on health, but its effects on microbiota profile are still unknown. The aim of this study was to analyse the changes of gut microbiota in diet-induced obese mice due to amaranth protein consumption and to compare them with the changes due to soybean protein intake. Male C57BL/6 mice were fed for 8 weeks with regular (RD) or high-fat (HF) diet, without or with complementation with amaranth or soybean protein isolates. Morphological changes in caecum ultra-thin sections were measured after hematoxylin/eosin staining. Microbiota was isolated from the caecum and *16S rRNA* gene was sequenced. Caecal Short Chain Fatty Acids (SCFAs) were quantified by gas chromatography. The consumption of soybean protein induced the ectopic deposition of fat in the whole intestine while amaranth proteins increased caecal crypt depth and calceiform cells number sustaining its beneficial effect on health. The count of Ruminococcacea family bacteria was increased in mice fed with HF diet, but amaranth proteins intake reduced its abundance. In turn, Lachnospiraceae bacteria abundance decreased in mice fed the Control-HF and amaranth HF diets, but increased in mice fed the soybean diets. In mice fed the RD diets, amaranth induced the abundance of Prevotellaceae, an acetate-producing bacteria. Study results indicate that the modulation of caecal microbiota could be one of the mechanisms by which amaranth exerts its beneficial effects on health.

INTRODUCTION

Obesity is a positive imbalance of energy intake and expenditure with excessive weight gain and is related to comorbidities as diabetes mellitus, cardiovascular, and metabolic diseases. 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 from which approximately 600 million are obese [WHO, 2016]. The increase in obesity condition has been observed in both developed and developing countries and does not exclusively afflict adults; more than 41 million children under five are overweight or obese. Reports indicate that Mexico is the second country with the highest obesity rates, after the United States, and first one regarding child overweight and obesity ratios [OECD, 2015].

There are several treatments for the obese condition that range from lifestyle modification, pharmacological or psychological therapy, surgery, and even the implementation of new procedures as a faecal transplant [Marotz & Zarrinpar, 2016]. Drugs and surgery are not appropiate for each patient and represent the last options due to their side effects, hence diet modification will have the greatest impacts in preventing the development of comorbidities and improving the quality of life of patients who present an excessive weight gain. In the pursuit of health-beneficial foods, the incorporation of nutraceuticals in diets is gaining increased attention [Houston, 2013].

Amaranth is considered a nutraceutical and evidences of its health benefits have been reported [Gómez-Cardona *et al*., 2017]. Amaranth seeds have high dietary fiber contents, are rich in unsaturated fatty acids, and their proteins are rich in essential amino acids (lysine, tryptophan, and sulfur amino acids) [Bressani *et al*., 1987]. It is known that the amaranth lipid fraction has hypocholesterolaemic properties due to its high quantities of squalene [Martirosyan *et al*., 2007], while amaranth protein consumption induces the accumulation of antioxidant proteins such as paraoxonase/arylestereas 1 (PON1) [Velarde-Salcedo *et al*., 2017]. It has also been observed that the consumption of amaranth proteins increases leptin levels, a molecule that is involved in appetite control. Moreover, these proteins reduce concentrations of ghrelin, an orexigenic hormone [Gómez-Cardona *et al*., 2017].

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Nutraceutical food and microbiota are extremely associated with the nutritional and health status of the host. Dietary components are susceptible to be metabolized by the bacteria during the gastrointestinal passage for their subsequent absorption [Laparra & Sanz, 2010]. Bacteroidetes and Firmicutes are the principal phyla that suffer alterations in abundance and diversity when feeding habits and compounds of diet are modified in obese individuals, but results are still unclear. Reports using mice models have indicated a significant increase in the Firmicutes/Bacteroidetes ratio [Turnbaugh *et al*., 2008], while Jiao *et al*. [2018] reported no differences when comparing obese with lean rodents. Others works have indicated that Bacteroidetes increase in human fecal microbiota in overweight subjects compared with lean ones [Schwiertz *et al*., 2010]. Nonetheless, in the distal colon these phyla are important for dietary fibers fermentation in order to produce end products such as Short-Chain Fatty Acids (SCFAs), that not only improve the microbial environment but also regulate several biochemical processes in the host [Kasubuchi *et al*., 2015].

To date, there are no reports regarding how amaranth proteins, which have a good balance of essential amino acids, can modify the profile of the caecal microbiota and influence the generation of SCFAs, and whether this potential change could have positive effects on human health. Therefore, the aim of this study was to assess the effect of the gut microbiota profile on diet-induced obese mice due to a daily amaranth protein intake and to compare it with the effects due to soybean protein consumption.

MATERIALS AND METHODS

Animals and diets

Male 6-week old C57BL/6 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°C and dry air humidity at 50% ± 15 g H_2O/g with a 12 h light/dark cycle in groups of four to five mice in a standard stainless steel cage. Animals were randomly assigned to six groups containing eight mice per group. In the two first groups, the control groups, animals were fed with regular diet (Ctrl-RD) and high-fat diets (Ctrl-HF). In the third and fourth groups, mice were fed with regular diet or high-fat diets supplemented with amaranth protein isolate (AMA-RD and AMA-HF, respectively). In the fifth and sixth groups, the mice diets were supplemented with soybean protein isolate (SOY-RD and SOY-HF, respectively).

The regular diet groups received the commercial Teklad (Envigo, Huntingdon, UK) diet 2018S containing (in 100 g diet): 18.6 g protein (from wheat, corn, soybean), 44.3 g carbohydrates, and 6.2 g fat. High-fat diet was supplied with Teklad TD 06414 containing (in 100 g diet): 23.5 g protein (casein), 27.3 g carbohydrates, and 34.3 g fat. The food consumption and mice weight were recorded every week. Protein isolates from amaranth and soybean, with a purity of 76.5% and 85.5%, respectively, were obtained as described elsewhere [Escobedo-Moratilla *et al*., 2017]. Amaranth or soybean protein was administered once daily using a stainless steel oral gavage at a dose of 10 mg protein/kg BW (body weight). Food and water were provided *ad libitum* during the study.

Procedures for animal housing and care were assessed according to the Mexican regulatory standard (NOM-062-ZOO-1999) and 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, animals were fasted for 6 h on paper bedding before euthanized; all segments after stomach were collected, placed in liquid nitrogen, and stored at -80°C until microbial analysis.

Histomorphometry appearance of the caecum

Frozen caecum sample was dissected into 7 mm sections cutting 0.6 mm approximately from the ileum for each randomly selected gut. Samples were immersed in 10 mL PBS/100 mL formaldehyde (pH=6.4), de-hydrated, clarificated, and embedded in paraffin. Three serial sections of 7 μm from each animal were collected in silanized slides at one location of caecum and stained with hematoxylin-eosin. The analysis was carried out blind to the treatment to evaluate the histological changes and the Lieberkühn cryps depth. The morphometrical analysis was achieved with the light microscope Zeiss Axio Imager M2 (Carl Zeiss Co. Oberkochen, German). Crypt depth measurements were done 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 v1.46r.

Sequencing and analysis of bacterial *16S rRNA* gene

Caecal contents were obtained by extrusion from each thawed intestinal tissue. DNA extraction was achieved using the DNeasy UltraClean microbial kit (Qiagen, Hilden, Germany) from 200 mg of intestinal digesta following the manufacturer's instructions. The obtained DNA was quantified by spectrophotometry with NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and stored at -80°C until use.

Paired-end sequencing with a read length of 2×250 bp 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 rRNA* gene 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 bp as the minimum possible length of the assembled sequences, and a Phred score of 25 were applied in the analysis [Kautz *et al*., 2013]. The filtered sequences were dereplicated and cleaned of chimeric sequences with UCHIME with the reference database GoldFasta using USEARCH-Tool-Suite. Resulting 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 OTU Stacket Bar plot to represent bacterial relative abundances between groups. OTU table was analyzed to survey the relationship of metadata characteristics

for multiple diversity measurements. Shannon, Good's coverage, and Rarefaction were calculed using iNEXT [Hsieh *et al*., 2016] and Spade (http://chao.stat.nthu.edu.tw/wordpress/software).

Measurement of caecal Short-Chain Fatty Acids (SCFAs)

Caecal digesta were used for SCFAs quantification. Briefly, 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 4ºC and 4800×*g* for 20 min. Supernatant was recovered and this procedure was repeated two times for clarifying. The sample was filtered through 0.22 μm Millipore filter (Merck, Darmstadt, Germany) before injection into 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. HP-INNOwax GC capillary column with a film thickness of 0.5 μm (Agilent Technologies Inc, CA, USA). Helium 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 250ºC, respectively. The flow rates of hydrogen and air were 30 and 300 mL/min, respectively. The volume of the 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 replicates for each level).

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.

RESULTS AND DISCUSSION

The incidence of obesity-related diseases has increased and currently the intake of a high protein diet is attracting particular attention due to its impact on gut microbiota. Amaranth proteins, which present a good balance of essential amino acids as well as an excellent digestibility [Bressani *et al*., 1987], are the ideal protein source for food supplementation. Therefore, we explore the effects of amaranth protein on modulation of intestinal microbiota in obese mice. However, several studies suggest that body weight, food intake, and gut microbiota composition may vary depending on interactions with sex [Yang *et al.*, 2014] and then only male C57BL/6 mice were used in the present study.

Physiognomic parameters measurements

The physiognomic parameters showed that at the end of the experiment, the mice fed the high-fat (HF) diet had the highest weight gain. No changes in body weight were observed in mice treated with the regular diet supplemented with amaranth (AMA-RD), but mice fed with soybean (SOY-RD) showed a tendency to increase weight (Table 1).

At the beginning of the experiment, the highest feed intake was observed in the mice fed the SOY-RD diet, but in all HF diets, the mice feed intake was similar in all groups ($p<0.001$). At the end of the experiment, it was observed a similar amount of feed ingested by the mice treated with AMA diets, but not in the mice fed the SOY-HF diets, which showed a significant decrease ($p<0.05$) (Table 1). The weight gain of the whole intestine was correlated with the feed intake, where SOY-HF diet-fed mice showed the lowest intestinal weight. With regard to epididymal fat tissue, it was similar among groups fed either with RD or HF diet (Table 1). Overall, the physiognomic parameters showed slight changes as it has been observed in previous studies with HF diet [Tomas *et al*., 2016]. However, an anatomically important observation of intestines in mice displayed that samples from the SOY-HF diet-fed mice were coated with fat (Figure 1). These results potentially indicate that fat accumulation is linked with the consumption of soy protein because mice fed amaranth protein presented morphology comparable to control groups (Figure 1).

Studies have suggested that the high consumption of soy protein decreases food intake and body weight compared to other protein sources, like whey [Li *et al*., 2016], which is also in agreement with the present results. However, an excessive ectopic deposition of fat in intestine was observed in the SOY-HF diet-fed mice, in spite of a lower feed intake. Interestingly, other studies have already detected the deposi-

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

Parameters	Ctrl-RD	Ctrl-HF	AMA-RD	AMA-HF	SOY-RD	SOY-HF
Body weight (g)	14.6 ± 6.01^{b}	41.2 ± 20.4^{a}	19.3 ± 7.76^{b}	36.2 ± 13.6^{a}	$25.1\pm8.28^{a,b}$	35.7 ± 10.7^{a}
Feed intake at T_o (g)	$3.21\pm0.30^{c,d}$	2.30 ± 1.50^{d}	$4.10\pm0.28^{b,c}$	2.30 ± 0.29^{d}	5.46 ± 0.14^{a}	$3.15\pm0.23^{c,d}$
Feed intake at T_8 (g)	2.93 ± 0.49^{b}	$2.30\pm0.79^{b,c}$	$3.35\pm0.51^{a,b}$	$2.22\pm0.38^{b,c}$	$3.51\pm0.38^{a,b}$	1.36 ± 0.49^{c}
Intestinal weight (g)	$1.88\pm0.22^{a,b}$	$1.70\pm0.30^{b,c}$	2.02 ± 0.22^{a}	$1.66\pm0.29^{b,c}$	$1.88\pm0.15^{b,c}$	1.47 ± 0.27^{d}
EFT weight (g)	$0.38\pm0.17^{b,c}$	1.30 ± 0.56^{a}	$0.38\pm0.12^{b,c}$	1.38 ± 0.49^{a}	0.61 ± 0.18^{b}	1.20 ± 0.27^{a}

T_0=values at the beginning of the experiment. T_8=values after 8 weeks of protein intake. EFT= Epididymal fat tissue. Values represent the mean of total population in each group (n=8) ±standard deviation (SD). Grams (g). Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters along rows indicate statistical differences at $p<0.05$ in a Kruskal-Wallis with a post hoc Dunn test.

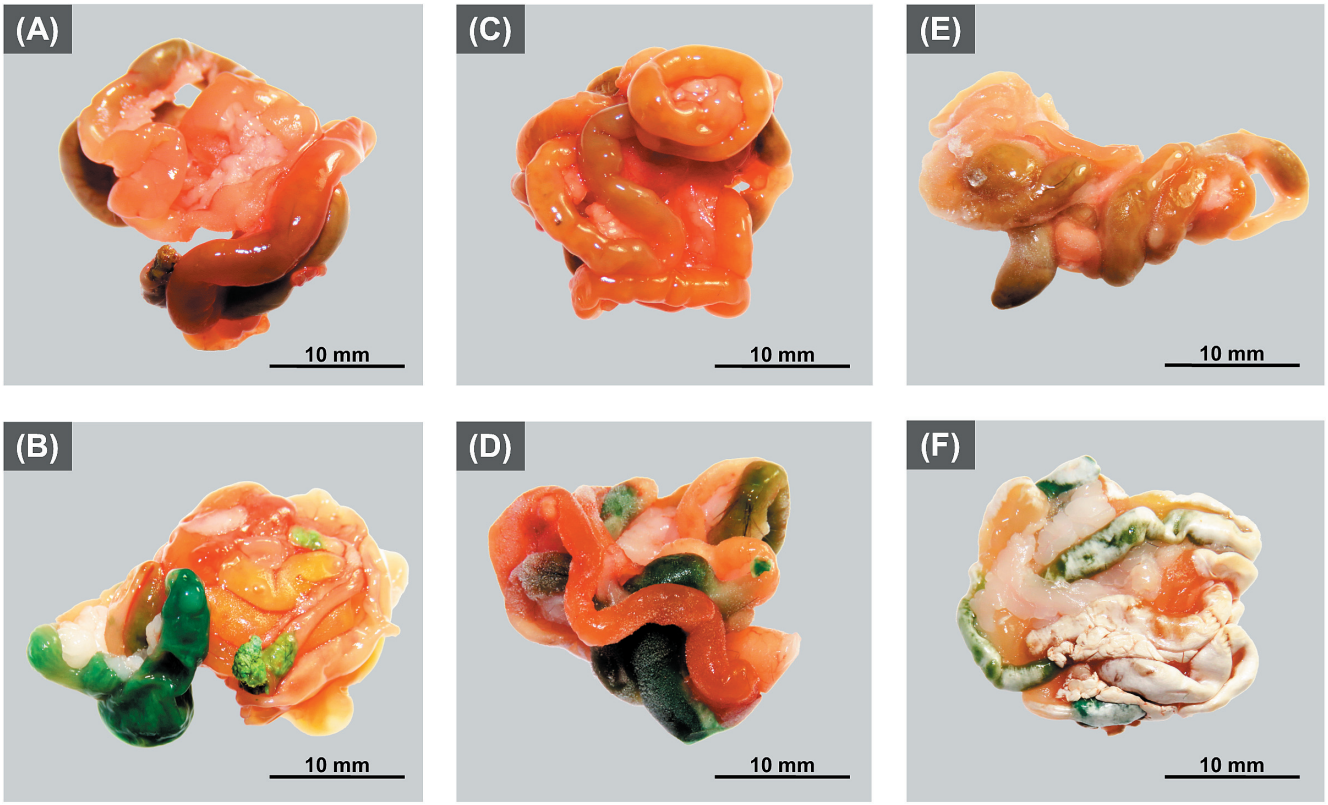

FIGURE 1. Macroscopical appearance of the intestinal tissue of mice fed with different diets. Digital images show fat coating the gut structures: (A) Ctrl-RD; (B) Ctrl-HF; (C) AMA-RD; (D) AMA-HF; (E) SOY-RD; and (F) SOY-HF. Ctrl=control, RD=regular diet, HF=high fat diet, AMA=amaranth proteins, SOY=soybean proteins.

tion of fat in the intestinal mucosa by triacylglycerol accumulation in mice fed a high fat diet compared to mice fed a low fat diet [Douglass *et al*., 2012]. This abnormal fat accumulation could be the result of metabolic and endocrine changes that may trigger both oxidative stress and inflammation at tissue and systemic levels with the development of obesity, lipotoxicity, and insulin resistance [Barazzoni *et al*., 2018]. This is in agreement with previous results that have shown that amaranth protein supplementation was able to reduce the insulin levels in mice plasma but mice fed soy proteins tended to generate insulin resistance [Escobedo-Moratilla *et al*., 2017].

Caecum histomorphometric appearance

The morphological changes of the caecum due to the diet are shown in Figure 2A, and the crypt depth dimensions are shown in Figure 2B. A significant decrease in crypt depth was observed in mice fed the Ctrl-HF diet when compared with samples from mice fed the Ctrl-RD diet (Figure 2Aa and 2b). Amaranth diets caused also a significant decrease in crypt depth, when compared with Ctrl-RD diets (Figure 2B). However, the crypt of mice fed the AMA-HF showed more epithelial cells, as well as the number and size of calceiform cells by crypt (Figure 2Ac and 2d), which resemble those observed in the Ctrl-RD fed-mice group. Alike, SOY-RD diet (Figure 2Ae) elicited a similar effect as Ctrl-RD, but SOY-HF diet reduced significantly crypt depths (Figure 2Af) even more than the Ctrl-HF diet did (Figure 2Ab). Furthermore, it was observed the loss of the intestinal epithelial structure in the mice fed the SOY-HF diet, which may be related to the inflammatory state (Figure 2Af).

The gut epithelium is broadly folded into crypts and villi, which increase the contact surface for secretory, absorptive, and digestive activities. These functions contribute to homeostatic regulation that impacts mucosal defences, but also the dynamics of intestinal microbiota [Jakobsson *et al*., 2015]. Therefore, structural changes or alterations in gut epithelium are extremely linked with dysbiosis and permeation of luminal noxious molecules, triggering the dysregulation of inflammation that promotes the pathogenesis of intestinal and systemic diseases [Battson *et al*., 2018].

Our results revealed that the caecum appearance was altered not only by the HF diet, but also by the type of proteins in diet. The SOY-HF diet had a greater impact on the mice epithelial tissue integrity with a considerable increase of epithelial cells density. This effect of soy protein consumption in conjunction with the previously reported insulin resistance [Escobedo-Moratilla *et al*., 2017], could be a consequence of a grade of tissues inflammation. In addition, the accumulation of fat observed in mice intestines (Figure 1) could be accompanied by secretory products and inflammatory cytokines that promote expansion of tissues as reported elsewhere [Magnuson *et al*., 2015]. Therefore, these modifications of the epithelium potentially suggest that the HF treatments caused a degree of dysbiosis, and that the soy protein does not help to mitigate the effects caused by the HF diet as the amaranth proteins do.

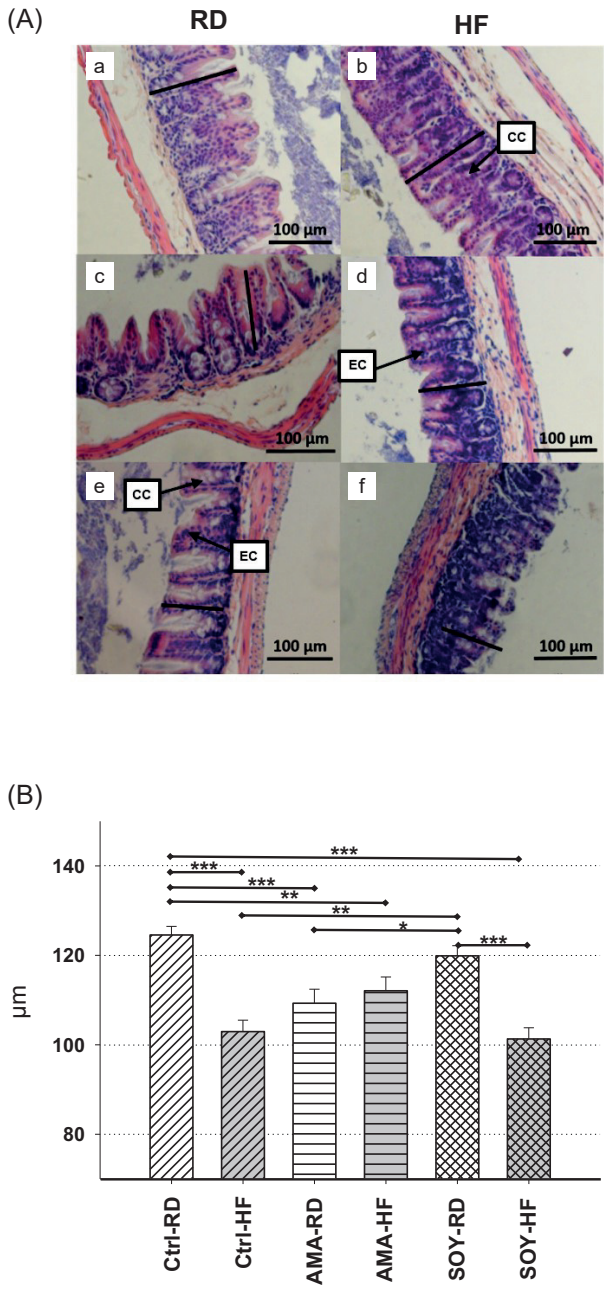

FIGURE 2. Morphometric analysis of the caecum of mice fed with different diets. (A) Representative micrographics of hematoxylin/eosin stained cuts of caecum samples from: a and b=control animals; c and d=from animals feed with amaranth proteins; e and f=from animals feed with soybean proteins. RD=regular diet or HF=high-fat diet. Stained cuts were observed in a Zeiss Axio Imager microscope. (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. Black lines in A) represent a crypt depth measure in each treatment. CC=calceiform cells; EC=epithelial cells. Asterisks denote significant differences (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ in the Kruskal-Wallis with a post hoc Dunn test).

Sequence analysis of the caecum bacteria population

Obesity-related changes in the gut microbiota have been linked to the decrease in species diversity. However, recent works have reported an increase of gut bacteria diversity in mice fed high fat diets [Zeng *et al*., 2016]. Our results showed that AMA and SOY protein diets also increased species diversity in the mice caecal microbiota. Ctrl-HF diet intake was characterized by a trend to increase numbers of OTUs compared with Ctrl-RD (Table 2). Mice fed the AMA and SOY diets did not differ significantly concerning numbers of OTUs, but a significant increase was observed in OTUs count compared with the mice group fed the Ctrl-RD diet (Table 2). These changes in bacterial diversity are associated with the relative abundance of sequences, but not with the OTUs distribution as observed in Good's coverage and Shannon index (Table 2).

It is known that gut microbiota in the vertebrates is dominated by Firmicutes and Bacteroidetes, which constitute 80–90% of the total resident bacteria [Cani & Knauf, 2016]. Some authors have related the presence of Firmicutes to an obese or overweight status [Turnbaugh *et al*., 2008], while others have had associated them to the lean population [Schwiertz *et al*., 2010; Ravussin *et al*., 2012]. Jiao *et al*. [2018] reported that there were no significant differences in Bacterioidetes to Firmicutes (B/F) ratio between obese and lean rodents. Although this relationship is still not well understood, this could be attributed to different model species, kind of diet, laboratory conditions, and also study design [Aguirre & Venema, 2015]. However, a notable change in gut microbial ecology has been observed in periods of nutrient deprivation or fasting [Beli *et al*., 2018]. We observed high levels of Firmicutes (over 90%) and low levels of Bacteroidetes (about 8%), which could be associated with the fasting period (6 h) before mice euthanasia (Figure 3A and 3B). A similar microbial profile was reported not only in mice fed with regular diet (control) but also in mice fed with high-fat diet fasted for 16 h [Zarrinpar *et al*., 2018]. This tendency is related to the ability to harvest energy from endogenous substrates in the absence of food during extended fasting; in this situation the gut microbiome could affect the generation of metabolites such as bile acids and SCFAs [Beli *et al*., 2018].

The phylum Firmicutes was mainly composed by Ruminococcaceae and Lachnospiraceae families (Figure 3A), while in the non-firmicutes the main family was Porphyromonadaceae with a notable reduction of Helicobacteraceae when protein isolates were included in diets (Figure 3B). Mitigation of Helicobacteraceae family abundance is related to the improvement of inflammation in colitis and bowel diseases [Rooks *et al*., 2014]. Regarding to Ruminococcaceae family, an increase in abundance was observed in caecal contents in mice fed with Ctrl-HF and SOY-diets compared with AMA-diets fed mice (Figure 4A). High abundance of Ruminococcaceae has been observed in other studies following HF diets [Zhang *et al*., 2010]. It has stated that Ruminoccocaceae induced expression of genes involved in inflammation process, such as *Angpl*4, in diet-induced obese mice [Ravussin *et al*., 2012]. These bacteria have been also observed in leptin-resistant obese and diabetic mice; this correlates with a previous work, which showed that mice fed with SOY protein tended to have insulin resistance [Escobedo-Moratilla *et al*., 2017]. The Lachnospiraceae family showed the same abundance levels in mice fed with Ctrl- and AMA diets but in caecal digesta of mice fed with SOY-diets an increase in abundance was observed (Figure 4B).The Lachnospiraceae have been detected in infants of overweight mothers [Tun *et al*.,

TABLE 2. Relative abundance, number of OTUs, and alpha diversity measures of mice caecal microbiota.

	Ctrl		AMA		SOY	
	RD	HF	RD	HF	RD	HF
Total sequence	11,733±2252^{c}	20,167±1020^{a}	15,237±21.0^{b}	15,176±1251^{ab}	15,824±1624^{ab}	21,050±7295^{a}
Total OTUs[1]	6152±503^{c}	7324±700^{b}	8468±413^{a}	7976±1449^{ab}	9757±1588^{a}	9710±1655^{a}
Rarefaction*	9150±3039^{a}	7047±4373^{a}	9650±4551^{a}	8701±2459^{a}	9775±3653^{a}	7660±1450^{a}
Shannon index	8.80±2.81^{a}	9.17±3.55^{a}	8.07±3.13^{a}	8.75±2.73^{a}	8.37±2.41^{a}	9.05±3.41^{a}
Good's coverage	0.67±0.05^{ab}	0.64±0.01^{b}	0.75±0.14^{ab}	0.65±0.07^{ab}	0.72±0.02^{a}	0.63±0.09^{b}

[1]Data was grouped into operational taxonomic units (OTUs) with 97% identity. Values represent the mean of total population ±standard deviation (SD). Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters along rows indicate statistical differences at $p<0.05$ in a Kruskal-Wallis with a post hoc Dunn test. *Sample-size-based rarefaction was computed with an extrapolation extends up to the maximum size of each sample.

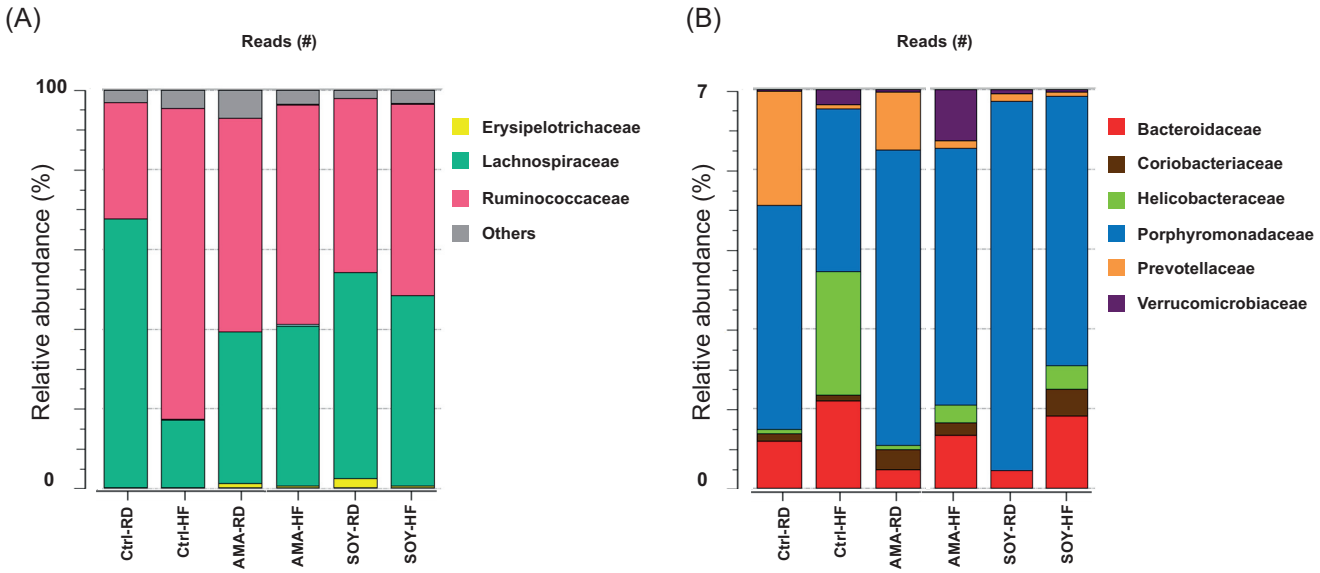

FIGURE 3. Modulation of the composition of mice caecal microbiota by different diets. The average relative abundance was expressed as a percentage of the total population at the family level in each group. (A) Families belonging to the Firmicutes phylum, and (B) families belonging to the phyla of Bacteroidetes, Verrucomicrobia, and Proteobacteria.

2018], the tendency to an increased abundance of Lachnospiraceae in SOY diets is potentially linked with the obesity status observed in those SOY- groups.

Within the Bacteroidetes families, Prevotellaceae showed higher relative abundance in caecal contents of mice fed with AMA-RD (Figure 4C), while Bacteroidaceae were abundant in mice fed with Ctrl-HF diet (Figure 4D). *Prevotella* belonging to the Prevotellaceae family possess the enzymatic machinery involved in sensing and hydrolysing complex carbohydrates and proteinaceous compounds with acetate as a product of fermentation [Hahnke *et al*., 2015]. Among the Bacteroidaceae family, *Bacteroides thetaiotaomicron* has been associated with the damage of epithelial barrier. According to Wrzosek *et al*. [2013], this bacterium together with bacteria that consume acetate and produce butyrate can interfere goblet cell differentiation, glycosylation, and mucin production in the colonic epithelium. Moreover, the increase of *Bacteroidales* has been detected in overweight women [Tagliabue & Elli, 2013]. These data could explain the occurrence of Bacteroidaceae family in mice fed with Ctrl-HF diet as a potential indicator of the epithelial damage caused by dysbiosis.

Protein administration generates similar values of caecal Short Chain Fatty Acids (SCFAs)

Gut microbiota uses products of fermentation of dietary fiber and in lesser extent dietary, endogenous proteins, to produce Short Chain Fatty Acids (SCFAs), which have emerged as important signalling molecules with a diverse physiological effects including stimulation of ileal motility and mucus production [Battson *et al*., 2018].

Acetic acid, propionic acid, and butyric acid are the most abundant SCFAs and constitute 95% of all those found in the body. In lean individuals, intestinal SCFAs concentration is mainly constituted by acetic acid, followed by propionic acid and butyric acid in a molar ratio of 60:20:20, respectively. Our results showed that mice fed the Ctrl-RD presented a SCFAs ratio of 75:2:23 (acetic, propionic, and butyric acids, respectively), ratio that was significantly decreased in caeca

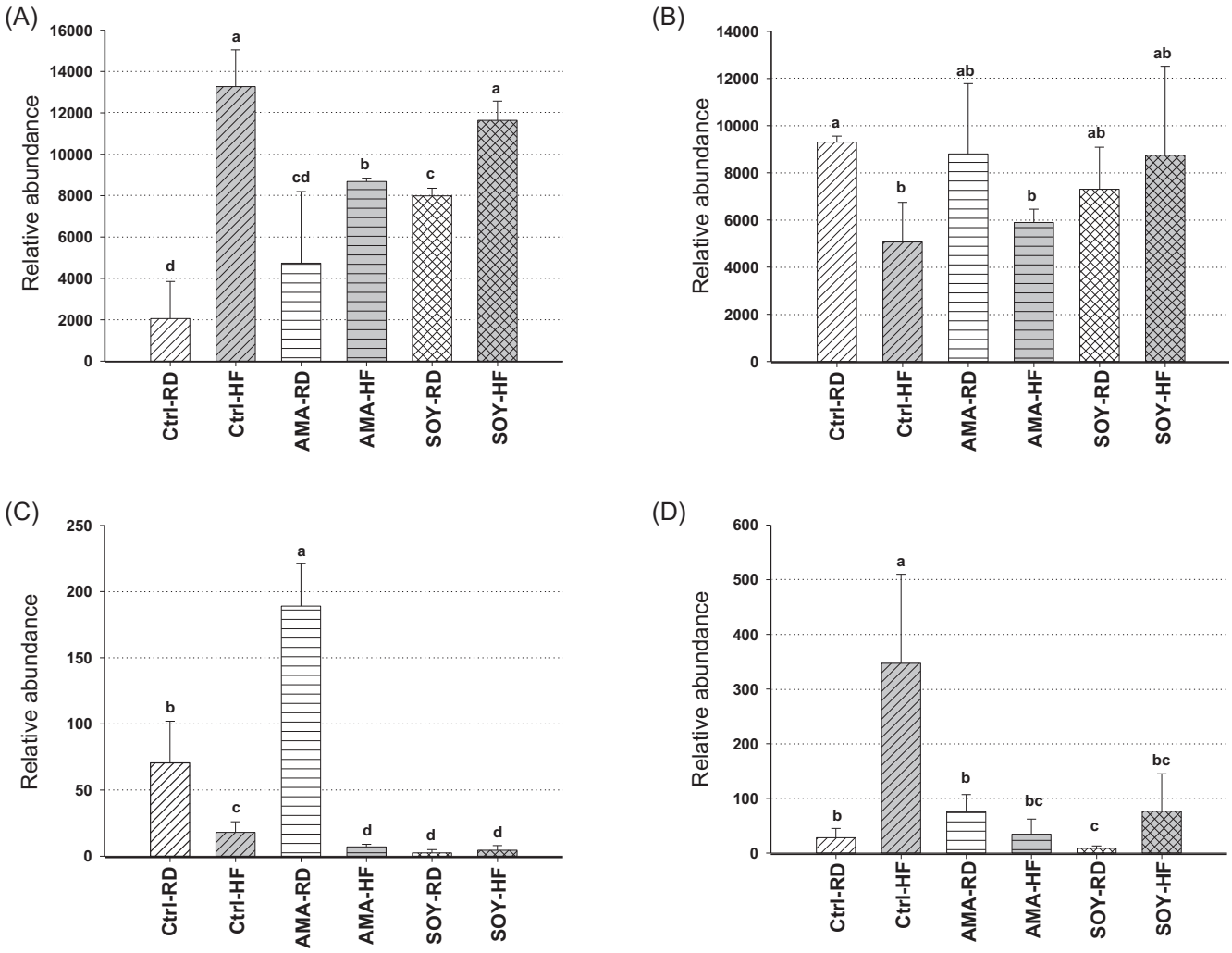

FIGURE 4. Family-level distributions of the microbial communities. Average of the relative abundance of microorganism family in each mice group. (A) Ruminococcaceae, (B) Lachnospiraceae, (C) Prevotellaceae, and (D) Bacteroidaceae. Different superscript letters indicate statically significant differences at $p<0.05$ in the Kruskal-Wallis with a post hoc Dunn test.

of mice fed the following diets: Ctrl-HD (80:5:15), AMA-RD (82:4:14), AMA-HD (84:5:11), SOY-RD (80:3:17), and SOY--HD (86:4:10) (Figure 5). This marked difference was observed due to a statistical increase in acetic acid and a decrease in propionic acid contents (Figure 5B and C) that potentially are associated with the consumption of proteins. Hedemann *et al*. [2009] reported a decrease in propionic acid in the caecum and colon of rats fed with cellulose, in a molar ratio of 83:6:11 and 80:4:6, respectively.

Gullan *et al*. [2016] reported that faecal cultures from healthy individuals fed with amaranth or quinoa diet showed an increase of SCFAs and Prevotellaceae family, as observed in this work. Prevotellaceae and Lachnospiraceae families are acetate-producing bacteria [Ferrario *et al*., 2017], their presence could be associated with acetic acid content increase (Figure 4B and 4C). Bacteroidetes richness has been correlated with faecal propionic acid levels in humans [Salonen *et al*., 2014]; in this context the decrease of propionic acid could be linked to the lack of those bacterial groups in the microbial composition (Figure 3B). However, Vital *el al*. [2015] indicate that there is no clear association between the butyric acid production with the abundance of Ruminococcaceae and Lachnospiracea that exhibit alternative, protein-fed, butyrate-synthesis pathways.

CONCLUSION

Not only the amount of protein is important, but also the source of protein has a significant impact on health. Although soybean meal has been reported to provide benefits to health, basically for the contents of isoflavones, proteins alone could have undesirable results. Our results have shown that soybean proteins consumption tends to increase fat accumulation, in contrast to amaranth proteins. The macroscopic analysis of the caecum also showed that amaranth consumption, under HF diets, had a tendency to increase the number and size of calceiform cells by crypt, similarly to the control diets.

The consumption of both amaranth and soybean proteins caused a reduction of Helicobacteracea, which is related with a decrease in pathogen families. But the intake of amaranth proteins, which have an excellent essential amino acid balance and good digestibility values, led to an improved microbial profile of the Prevotellaceae and Ruminococcaceae families,

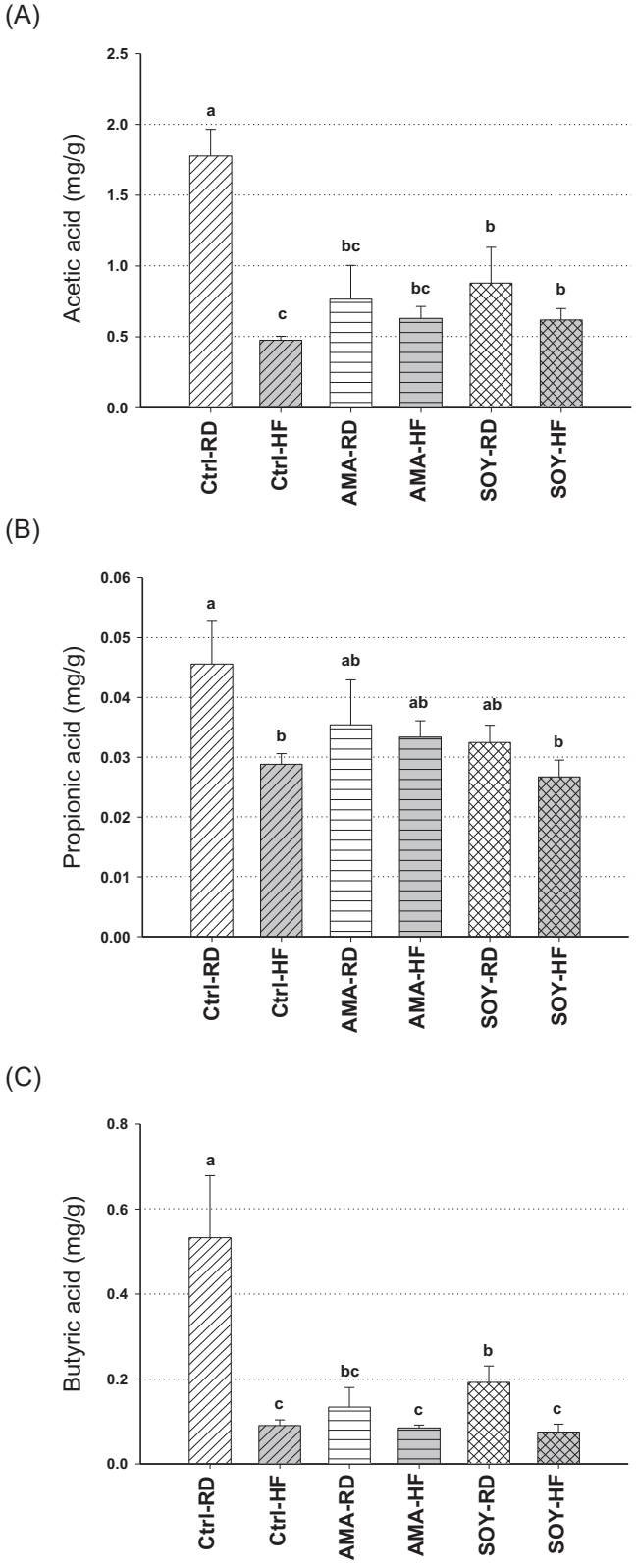

FIGURE 5. Short-chain fatty acid accumulation in caecal digesta of mice fed with different diets. (A) acetic acid; (B) propionic acid; and (C) butyric acid. Means values with SEM for each group. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters indicate statically differences at $p<0.05$ in the Kruskal-Wallis with a post hoc Dunn test.

which are microorganisms related with the intestinal barrier recovery. Results of our study suggest that amaranth could exert a modulation of caecal microbiota, which could be a new mechanism of action by which it exerts its health benefits. This finding opens new avenues to future research on amaranth mechanisms of action.

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CONFLICT OF INTEREST

Authors declare no conflict of interests.

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