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**Oral administration of *Lactobacillus fermentum* CRL1446 improves biomarkers of Metabolic Syndrome in mice fed a high-fat diet supplemented with wheat bran**

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## Abstract

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This work aimed to evaluate the effect of oral administration of probiotic *Lactobacillus (L.) fermentum* CRL1446, with feruloyl esterase (FE) activity, on metabolic biomarkers and intestinal microbiota of mice with high fat diet-induced Metabolic Syndrome (MS) and supplemented with wheat bran as a source of esterified ferulic acid. Six-week-old male Swiss albino mice developed the components of MS when fed with high fat diet supplemented with wheat bran (HFD+WB) for 14 weeks. Positive impact of *L. fermentum* CRL1446 administration on these animals was reflected in a decrease in body weight gain and adiposity index compared to the animals that did not receive the probiotic strain. In addition, a decrease in plasma leptin levels, improvement of inflammatory profile, reduction of fatty infiltration in hepatocytes and modification of lipid profile (increased HDL-cholesterol and decreased LDL-cholesterol and triglyceride levels) were observed. On the other hand, *L. fermentum* CRL1446 reduced fasting glucose and insulin levels, improving the HOMA index in mice with MS. Postprandial glucose levels were also reduced in the oral glucose tolerance test. Consumption of *L. fermentum* CRL1446 with HFD+WB (HFD+WB-Lf mice group) had a great impact on host metabolism, modulating intestinal microbiota, with an increase in Bacteroidetes and a decrease in Firmicutes abundance being observed. Increased intestinal FE activity, improved oxidative status and increased abundance of 3-hydroxyphenylpropionic acid and butyric acid concentration in colonic content, were also demonstrated in HFD+WB-Lf mice. Results obtained suggest that supplementation with *L. fermentum* CRL1446 enhances beneficial effects of a bran diet, attenuating the risk factors associated with MS.

**Keywords:** Lipid lowering effect, hypoglycemic effect, feruloyl esterase, postbiotic, metabolic syndrome.

## 1. Introduction

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Metabolic syndrome (MS) is characterized by the presence of at least three of the following risk factors: central obesity, hyperglycemia, atherogenic dyslipidemia, and hypertension. Its prevalence in recent years has increased mainly due to changes in lifestyle, which include a greater sedentary behaviour and the consumption of diets rich in fats and sugars (Western diet). The MS is also associated with a high incidence of cardiovascular diseases and type 2 diabetes mellitus (T2DM), with significantly increased morbidity and mortality rates in the population<sup>1</sup>.

Regarding public health, the medical costs due to diseases related to MS are continuously increasing, and the effort required to reduce the percentage of body fat in individuals has become a global concern<sup>2</sup>. There are several strategies to reduce body weight, such as physical activity, calorie-restricted diets, use of medications, surgery, etc., but most of them involve high treatment costs, and in some cases, can generate adverse side effects. For this reason, the development of dietary strategies that include natural food products with probiotics and/or prebiotics has emerged as an alternative that could help to reduce the risk factors associated with MS, without generating adverse side effects on health<sup>3</sup>.

In recent years, the interest in using probiotics, postbiotics or paraprobiotics, has emerged as an alternative for the prevention and treatment of pathologies related to obesity and MS, improving the balance of intestinal microbiota, decreasing the sensation of hunger and central adiposity, and improving the lipid profile and integrity of intestinal mucosa with a decrease in the inflammatory degree<sup>4</sup>. Some probiotic bacteria synthesize products or metabolic byproducts (postbiotics) which are secreted by live cells, such as enzymes and organic acids, that might also offer physiological benefits to the host by providing additional bioactivity<sup>5</sup>. Among the physiological functions of postbiotics, immunomodulation, anti-inflammatory, hypocholesterolemic, anti-obesogenic, and antioxidant effects are included<sup>6, 7</sup>. As probiotic microorganisms, mainly lactic acid bacteria (LAB) belonging to *Lactobacillus* genus is used. Several LAB strains can exert beneficial effects on the host based on enzyme activities. In this sense, LAB with feruloyl esterase (FE) activity allow

increasing the bioavailability of ferulic acid (FA) at the intestinal level, which could induce metabolic changes that are positive in individuals who develop MS<sup>8</sup>. The consumption of FA, a type of phenolic compound, has been associated with the reduction of several risk factors present in chronic non-communicable diseases, such as T2DM, cardiovascular, neurodegenerative pathologies, etc.<sup>9</sup> Its beneficial properties are attributed to multiple bioactive functions (antioxidant, antimicrobial, anti-inflammatory, etc.), which depend on FA intake. However, the bioavailability of FA is limited because it is present in vegetable matrices in esterified form (hydroxycinnamates), which regulates its bioefficacy<sup>10</sup>. Therefore, FEs play an important role in the hydrolysis of ester bonds and the subsequent release of FA, thus constituting a fundamental step required for its bioavailability and metabolism.

In rodents, the intestinal FE activity is provided by both intestinal microbiota and epithelial cells<sup>11, 12</sup>. On the other hand, Filannino *et al.*<sup>13</sup> affirm that bacterial FE enzymes present in the colon are primarily responsible for increasing the bioavailability of FA in humans. When FA is ingested in the dietary fiber, 90% of it reaches the colon in the form of hydroxycinnamates (ferulates). Thus the colonic microbiota is the main responsible for the release of FA from its conjugated forms, because of the breakdown of ester bonds by action of FE and its subsequent metabolism<sup>14</sup>.

This work aims to evaluate the effect of oral administration of probiotic *L. fermentum* CRL1446, with FE activity, on metabolic biomarkers and intestinal microbiota of mice with high fat diet-induced MS and supplemented with wheat bran as a source of esterified FA.

## 2. Materials and Methods

### 2.1. Bacterial strain, culture conditions and cell suspension preparation

*L. fermentum* CRL1446 was obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). This strain has FE activity and is capable of releasing FA from wheat bran<sup>15</sup>. *L. fermentum* was maintained in de Man, Rogosa and Sharpe

(MRS) broth (Britania, Buenos Aires, Argentina)<sup>16</sup> containing 20% (v/v) glycerol at  $-80^{\circ}\text{C}$  and it was propagated three times in MRS broth before each experimental use. For cell suspension preparation, *L. fermentum* CRL1446 was inoculated at 2% (v/v) in 50 mL of MRS broth and incubated at  $37^{\circ}\text{C}$  for 12 h. Cells were harvested by centrifugation (10,000 g 10 min) and washed twice with sterile phosphate-buffered saline (PBS) pH7. Cells were resuspended in sterile drinking water to achieve a concentration of  $10^9$  cells/mL for animal assays.

## 2.2. Animal protocol

Six-week-old male Swiss albino mice ( $n=24$ ) were obtained from the closed random-bred colony maintained at CERELA. They were housed in individual cages and were acclimated to a temperature of  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  with a 12-h light/dark cycle. Mice were then separated into three groups containing 8 mice each. The groups were as follows:

1. Control group, mice receiving by gavage 100  $\mu\text{L}$  of drinking water and fed daily with a normal diet;
2. MS group, mice receiving by gavage 100  $\mu\text{L}$  of drinking water and fed daily with a high-fat diet supplemented with wheat bran to 7% (w/w) (HFD+WB);
3. MS+Lf group, mice receiving by gavage 100  $\mu\text{L}$  of *L. fermentum* CRL1446 suspension (resulting in a dose of  $10^8$  cells/day) and fed daily with HFD+WB.

The control group mice received a normal diet (3.1 Kcal/g, with 6.5% vegetable oil-derived Kcal) and drinking water *ad libitum* for 14 weeks. Animals from the MS and MS+Lf groups were acclimated for 5 days to *ad libitum* normal diet, after which they were fed daily with HFD+WB (5.1 Kcal/g, with 60% lard-derived Kcal) for 14 weeks. Both diets supplied approximately 0.60 mg of hydroxycinnamates/day/mouse which are in their ester-linked form in wheat bran. The nutritional composition of normal diet and HFD+WB are listed in Supplementary Table S1.

The food intake and body weight of each mouse were measured every week. Body weight gain was expressed as follows: body weight at week 14 (g) – initial body weight (g). The food efficiency ratio was expressed as follows: body weight gain (g)/food consumed (g) at week 14.

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Oral glucose and sucrose tolerance tests were performed after week 14 of treatment. Mice were fasted for 12 h, and glucose or sucrose was orally administered by gavage at a dose of 2 g/Kg. Blood samples were obtained by saphenous vein puncture before and after 30, 60, 90 and 120 minutes of glucose or sucrose administration, according to Tomaro-Duchesneau *et al.*<sup>16</sup>. Glucose levels were analyzed with glucose test strips using a Glucometer from Accu-Chek Active (Roche, Germany). The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using insulin and glucose plasma values obtained at week 14 of the experimental trial<sup>17</sup>.

At the end of the experimental period, the mice were fasted for 12 h and were anesthetized using an intraperitoneal injection of ketamine hydrochloride (100 mg/Kg of body weight) and xylazine hydrochloride (5 mg/Kg of body weight).

The experimental protocol complied with current Argentinean laws and was approved by the Animal Protection Committee of CERELA (CRL-BIOT-EF-2019/1A).

### 2.3. Biochemical assays

Blood samples were obtained from all animals ( $n=24$ ) by cardiac puncture and were transferred into tubes containing the anticoagulant EDTA (Wiener Lab, Rosario, Argentina). Plasma was obtained by centrifugation ( $2500 \times g$ , 10 min) and was used for biochemical assays. Plasma glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, alanine transaminase (ALT) and aspartate transaminase (AST) concentrations were measured by enzymatic methods using commercial kits (Wiener Lab, Rosario, Argentina).

Cardiovascular risk indicators (LDL-/HDL-cholesterol ratio, Triglyceride/HDL-cholesterol ratio and atherogenic index) were calculated according to the protocol described by Millán *et al.*<sup>18</sup> and Vega *et al.*<sup>19</sup>.

Plasma leptin concentrations were determined using an immunoassay kit (ELISA, DuoSet, R & D Systems, MN, USA). Plasma insulin levels were measured using ELISA (Mouse Insulin ELISA Kit, Alpco Diagnostics, USA).

Fasting plasma insulin and glucose levels were used to calculate insulin resistance using the homeostasis model assessment for insulin resistance (HOMA-IR) [(fasting glucose × fasting insulin)/22.5]<sup>17</sup>.

#### **2.4. Determination of plasma cytokines**

Plasma cytokine levels (TNF- $\alpha$ , IFN- $\gamma$ , IL-6, and IL-10) were determined by flow cytometry, using the CBA Mouse Soluble Protein Flex Set kit (BD CA, USA).

#### **2.5. Determination of plasma lipoperoxidation**

Lipoperoxide derivative concentrations in plasma were determined using the thiobarbituric acid reactive substances (TBA) methodology<sup>20</sup>. Results were expressed as nmoles TBARS/g of proteins.

#### **2.6. Animal sample collection, adiposity index and liver histology**

After blood collection, organs (large intestine, liver, mesenteric and epididymal fat) were surgically removed, washed using PBS, wiped with a paper towel and weighted.

The adiposity index was calculated using the following formula: total fat (mesenteric + epididymal) weight/body weight) × 100.

Liver weight was expressed as follows: liver weight (g)/body weight (100 g). Four liver samples from each experimental group were macrodissected, placed into plastic cassettes and fixed with formaldehyde solution (10% v/v in PBS, pH 7.0); the dehydrated samples were embedded in paraffin blocks. The paraffin blocks were sectioned at approximately 3–5  $\mu$ m and were placed on glass slides stained with hematoxylin and eosin for subsequent histological studies.



The size of adipocytes in adipose tissue from each experimental group was determined according to the methodology described by Cano *et al.*<sup>21</sup>, using Carl Zeiss software - Axio Vision Release 4.8. Photomicrographs of adipose tissue sections from each group were taken with a 40x objective. The area ( $\mu\text{m}^2$ ) of each adipocyte was determined from a total of 100 adipocytes in two histological sections per mouse of each group. The adipocytes were grouped by size ranges according to their areas (A) as follows:  $500 \leq A \leq 1000 \mu\text{m}^2$ ;  $1000 < A \leq 2000 \mu\text{m}^2$ ;  $2000 < A \leq 3000 \mu\text{m}^2$ ;  $3000 < A \leq 4000 \mu\text{m}^2$ ;  $4000 < A \leq 8000 \mu\text{m}^2$ .

For determination of FE activity, ferulic acid metabolites and short-chain fatty acid levels, eight large intestine samples from each experimental group were aseptically removed and their contents were collected according to the protocol described by Abeijón Mukdsi *et al.*<sup>12</sup> and Lorenzo-Pisarello *et al.*<sup>22</sup>.

## **2.7. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities in liver**

Four liver samples from each experimental group were homogenized (buffer volume: fresh liver weight, 5:1) using a mortar and pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 3 mM DL-dithiothreitol<sup>23</sup>. The homogenate was centrifuged ( $12,000 \times g$  for 30 min at  $4^\circ\text{C}$ ) and the supernatant was aliquoted and stored at  $-80^\circ\text{C}$ , until determination of protein concentration and GPx and GR activities. GPx activities were determined according to the protocol described by Flohé and Günzle<sup>24</sup>. Results were expressed as units (U) of GPx activity/g liver. One unit was defined as the amount of enzyme-producing 1 nmol of oxidized NADP/minute. GR activities were determined according to the protocol described by Esterbauer and Gril<sup>25</sup>, by following the rate of NADPH oxidation at 340 nm. Results were expressed as units (U) of GR activity/g liver. One unit was defined as the amount of enzyme producing 1 nmol of NADP/ minute.

## 2.8. Inhibition of $\alpha$ -glucosidase activity *in vitro*

The inhibitory capacity of  $\alpha$ -glucosidase activity was determined using the method described by Li *et al.*<sup>26</sup>, using *p*-nitrophenyl  $\alpha$ -D-glucopyranoside as substrate.

## 2.9. Protein determination

Protein concentration was estimated by the Bradford method<sup>27</sup>, using a commercial kit (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (Sigma, St. Louis, MO, USA) was used as standard.

## 2.10. Determination of Intestinal FE Activity

Intestinal FE activity was determined in intestinal contents according to the protocol described by Abeijón Mukdsi *et al.*<sup>12</sup>. Results were expressed as units (U) of FE activity/gram of intestinal content. One unit was defined as the amount of enzyme releasing 1 mmol of FA/hour.

## 2.11. Determination of short-chain fatty acid (SCFA) in large intestine contents

Acetic, propionic and butyric acids were determined in large intestine content according to the methodology described by Lorenzo-Pisarello *et al.*<sup>22</sup>. SCFA produced were quantified by HPLC (Knauer system) using an ion-exchange column (BIO-RAD Aminex HPX-87H; 300 × 7.8 mm). The different components were eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL min<sup>-1</sup>. SCFA concentrations were reported as mmol/g of large intestine content.

## 2.12. Detection of ferulic acid (FA) metabolites in large intestine contents

The presence of FA-derived metabolites was determined in large intestine contents of mice fed for 14 weeks. For this purpose, 30% (w/v) intestinal homogenates were prepared in PBS pH 7. To extract metabolites, aliquots of 500  $\mu$ L of homogenate were acidified with 10  $\mu$ L of HCl (37%)

and extracted with 2 mL ethyl acetate. Samples were centrifuged (8000 x g, 10 min), and the organic phase was recovered and subsequently evaporated to dryness in a water bath at 40°C under a stream of N<sub>2</sub>. The dry residues were resuspended in 100 µL of methanol. 20 µL of each sample was injected into an HPLC-UV-MS system (InMetS. A., Rosario, Argentina) to detect the different phenolic compounds derived from FA metabolism. The conditions used were the following: Mobile phase: A: H<sub>2</sub>O MQ / 0.1% v/v formic acid; B: Acetonitrile / 0.1% formic acetic; Flow: 0.2 mL / min; Column: C18 Hypersil-GOLD, Thermo Scientific (50 x 2.1 mm; 1.9 µm particle size); Column temperature: 25°C; Autosampler temperature: 20°C; Detection: UV 320 nm, 265 nm + MS; Run time: 21 minutes.

### 2.13. Bacterial DNA Extraction, Sequencing, and Quantification

At the end of the 14 weeks of feeding and before the animals were slaughtered, stool samples from mice from each group were collected in sterile eppendorf tubes and stored at -20°C (*n*=3).

The DNA extraction was performed using a specific commercial kit for fecal samples (QIAamp DNA Stool Mini Kit, Hilden, Germany).

A 20 µl aliquot of DNA from each sample was used for amplicon sequencing by means of a MiSeq Illumina sequencing platform (Shallowater, TX, USA). The V4 region of the 16S rRNA gene was amplified by PCR using primer pairs 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') according to Illumina Protocol to generate an amplicon size of ~400 bp<sup>28</sup>. Libraries were constructed by ligating sequencing adapters and indexes onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's specifications. Equimolar amounts of each of the libraries were pooled and they were sequenced in an Illumina MiSeq personal sequencer using a paired end-of-reading protocol of 400 bp in length. Quality of sequences control was performed using the QIIME pipeline<sup>29</sup>. Sequences were first quality filtered and screened for chimeras using the UCHIME algorithm implemented in USEARCH (version 6.1544; 21)<sup>30, 31</sup>. The remaining high quality 16S

rRNA gene sequences were clustered into OTUs (operational taxonomic units) at 97% similarity with the de novo reference OTU picking method and USEARCH (version 6.1544). Taxonomy assignment was performed with Bayesian RDP Classifier<sup>32</sup>, using an assignment confidence cutoff of 0.8. For calculation of the richness estimators, Chao 1 and the Shannon diversity indexes were determined using QIIME, with sample rarefaction set at 18,850 sequences per sample based on the sample with the least number of sequences. In addition, QIIME was also used to calculate beta diversity metrics among samples using weighted Unifrac distances and Bray-Curtis similarity<sup>30</sup>.

## 2.14. Statistical Analysis

Results are means of three independent experiments  $\pm$  standard error of the mean (SE). Data were submitted to one-way analysis of variance (ANOVA) using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Tukey's test was used to identify statistically significant differences ( $P < 0.05$ ).

## 3. Results and discussion

### 3.1. Effects of *L. fermentum* CRL1446 administration on body weight gain, food efficiency ratio, adiposity index and leptin levels in MS mice

Body weight gain (BWG) and food efficiency ratio (FER) were augmented by the consumption of HFD+WB, being observed in mice from MS group, an increase of 63% and 86%, respectively compared to control group (Table 1). *L. fermentum* CRL1446 administration (MS+Lf group) caused a significant reduction in both parameters with respect to MS group, reaching values similar to the control. Values of food intake and BWG allows evaluating the ability of an organism to transform grams of food consumed into grams of body mass, which is known as FER. These two parameters are closely related to the composition of intestinal microbiota and diet<sup>33</sup>. Similar results were reported by Park *et al.*<sup>34</sup>, who observed a decrease in FER by administering a combination of two probiotic strains (*L. curvatus* HY7601 and *L. plantarum* KY1032) for 8 weeks to mice fed with

HFD. Previous studies, which analyze the influence of *Lactobacillus* spp. administration on the BWG showed that this effect is dependent on each strain. While some species induce a decrease in weight, others do not affect it or induce increases<sup>35,36</sup>.

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Mice of MS group developed obesity characterized by an increase in mesenteric and epididymal adipose tissue (which was reflected in a 99% higher adiposity index value, compared to control group). The MS+Lf group showed a 39% decrease in the adiposity index compared to MS group (Table 1). These results are in accordance with those of Bhatena *et al.*<sup>8</sup>, who observed a reduction in the adiposity index when *L. fermentum* ATCC11976, strain with FE activity, was administered to hamsters with MS.

Histological studies of epididymal adipose tissue revealed changes in the number and size of adipocytes in mice from the two experimental groups under study (Figure 1A and 1B). In the MS group, a high abundance (57%) of large adipocytes ( $4000 < A \leq 8000 \mu\text{m}^2$ ) was found, while in the control group only 9% of adipocytes were within this range. Besides, a significant decrease in the amount of small-sized adipocytes (ranges between 500 and  $3000 \mu\text{m}^2$ ) was observed in the MS group compared to control group. In MS+Lf group, there was an increase in the number of larger adipocytes ( $4000 < A \leq 8000 \mu\text{m}^2$ ) compared to control group, however, these represented only 20% of the total (3 times less than in the MS group). These results showed that CRL1446 strain could prevent hypertrophy of adipose tissue in animals fed with HFD+WB. Other authors also reported a similar effect of probiotic microorganisms on adipocyte size<sup>21</sup>. The adipose tissue increase is considered a critical determinant of obesity and can occur through the increase in adipocyte size (hypertrophy) or in adipocytes number (hyperplasia)<sup>37</sup>.

Since adipose tissue is primarily responsible for the synthesis and secretion of leptin, plasma levels of this hormone were dosed in mice from all groups (Table 1). Plasma leptin levels were 7-fold higher in animals from MS group than in the control group. In MS+Lf group, leptin values were 3.5-fold lower than in the MS group. Leptin is secreted by adipocytes in levels proportional to the amount of body fat and constitutes a key peripheral signal that indicates the status of body

energy stores<sup>38</sup>. Our results reflected a status of hyperleptinemia in mice from MS group, which was reported as a characteristic of obesity, with a status of resistance to leptin. Oral administration of *L. fermentum* CRL1446 allowed decreasing the hyperleptinemia in animals fed with HFD+WB. The reduction of plasma leptin levels as a consequence of the administration of probiotic microorganisms in diet-induced obesity models has been reported by other authors<sup>21</sup>.

### 3.2. Effect of *L. fermentum* CRL1446 administration on inflammatory status of MS mice

The inflammatory status of the animals was evaluated by determining the plasma levels of cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-6 and IL-10 (Table 2). The proinflammatory cytokine levels (TNF- $\alpha$ , IFN- $\gamma$ , IL-6) showed a significant increase in MS group compared to control group. In MS+Lf group, the levels of these cytokines were significantly reduced with respect to MS group. These results are consistent with those reported by other authors in animal models fed with HFD<sup>39</sup>. Regarding the levels of IL-10 (an anti-inflammatory cytokine), a three-fold decrease was observed in MS group compared to control group. In MS+Lf group, IL-10 levels were 1.5-fold higher than in MS group. Previous studies reported that there is a negative relationship between obesity and IL-10 levels<sup>40</sup>. The administration of *L. fermentum* CRL1446 improved the inflammatory profile of animals that received HFD+WB, a reduction in pro-inflammatory cytokines and an increase in IL-10 being observed.

### 3.3. Effect of *L. fermentum* administration on the accumulation of fat in liver and transaminase activities in MS mice.

A pathology that is usually associated with MS is the accumulation of fat in the liver; therefore, an analysis of liver histology in animals from each group was carried out. The results showed that mice fed with HFD+WB exhibited hepatic steatosis characterized by the presence of abundant vacuoles or lipid droplets of different sizes, as well as cytoplasmic granularity

of hepatocytes. Ballooning and hepatocellular binucleation could also be observed. MS+Lf group mice showed a reduction in fatty infiltration in hepatocytes (Figure 2). The accumulation of liver fat is a sign of the early development of non-alcoholic fatty liver disease (NAFLD) and is associated with the deregulation of lipid and glucose metabolism<sup>41</sup>.

The increased activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is usually associated with liver damage. In MS group, plasma levels of ALT and AST showed a 2-fold increase compared to control group. *L. fermentum* administration decreased ALT and AST activities to values similar to those observed in the control group (Figure 3). Transaminase activities (ALT and AST) are considered clinical biomarkers of liver function and disease. Also, it has been found that the augmentation of ALT and AST levels is associated with a higher body mass index and abdominal obesity, even in the absence of liver disease<sup>42</sup>. Other authors have reported an improvement in the fatty liver when a mix of probiotics was administered to rats with steatosis, associating the lower accumulation of fat with the modulation of the lipid profile, leptin and inflammatory biomarkers<sup>43</sup>. On the other hand, Bhatena *et al.*<sup>42</sup>, have demonstrated the beneficial effect of the administration of a microorganism with FE activity (*L. fermentum* ATCC 11976) in a golden Syrian hamster NAFLD model.

### **3.4. Effect of administration of *L. fermentum* on lipid profile and cardiovascular risk indicators**

The effect of HFD+WB and *L. fermentum* CRL1446 administration on plasma levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were evaluated (Table 3). A 33% increase in total cholesterol level was observed in MS group compared to control group. Total cholesterol level in MS+Lf group showed no significant differences with control animals (Table 3).

The HDL-cholesterol values in MS group decreased by 22% compared to control group. The administration of *L. fermentum* CRL1446 (MS+Lf group) allowed maintaining levels similar to those found in the control group. Regarding to LDL-cholesterol, in MS group a 26% increase in the

levels of this lipoprotein was observed compared to control group, while the administration of *L. fermentum* CRL1446 (MS+Lf) reduced LDL-cholesterol levels to values similar to those detected in the control group. These results were similar to those observed by Bhatena *et al.*<sup>8</sup> in hamsters with MS treated with *L. fermentum* ATCC11976, strain with FE activity.

MS group showed a 56% increase in triglyceride levels compared to control group. In MS+Lf group, hypertriglyceridemia was reduced to values similar to the control group.

Table 3 shows the values of the cardiovascular risk indicators, which showed an approximately 2-fold increase in the MS group compared to control group. In MS+Lf group, values lower than those of MS group or similar to control group were observed. Various cardiovascular risk indicators (CRI) have been proposed, which have greater predictive value than the lipid fractions themselves<sup>18</sup>. Our results showed an increase of CRI in MS group compared to control. Mice from MS+Lf group presented lower values than those from MS group. Tomaro- Duchesneau *et al.*<sup>44</sup> reported similar results in MS rats treated with *L. fermentum* NCIMB 5221, strain with FE activity. Naowaboot *et al.*<sup>45</sup> showed that the administration of ferulic acid in obese mice fed with HFD improved lipid homeostasis, probably through the modulation of lipogenic gene expression (SREBP1c, FAS, ACC) and stimulation of  $\beta$ -oxidation genes (CPT1A, PPAR $\gamma$ ).

### **3.5. Effect of *L. fermentum* CRL1446 administration on Oral glucose tolerance test (OGTT), fasting glucose and insulin levels**

Results show that MS group presented significantly higher blood glucose levels at all points of the curves with respect to control group, with values greater than 200 mg /dL after 120 min of oral overload, indicating insulin resistance (IR). Supplementation HFD+WB diet with *L. fermentum* CRL1446 reduced postprandial glucose levels to values similar to those observed in control group (Figure 4A and 4B).



Fasting plasma glucose and insulin levels in MS group increased by 96% and 45%, respectively, compared to control group, whereas in mice of the MS+Lf group, they decreased to values similar to control group (Table 4).

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The HOMA-IR index to assess insulin resistance was approximately 3-fold higher in MS group than in control group. The administration of *L. fermentum* CRL1446 decreased the HOMA-IR index in MS group, to similar values to those observed in control group (Table 4).

Naowaboot *et al.*<sup>45</sup> have suggested that ferulic acid could act decreasing the expression of hepatic enzymes of gluconeogenesis, (phosphoenolpyruvate carboxylase and glucose-6-phosphatase), thus reducing blood glucose levels. Bhathena *et al.*<sup>8</sup> reported that the administration of *L. fermentum* ATCC11976 (strain with FE activity) to hamsters with MS decreased serum insulin and glucose levels. The results obtained in this work also agree with those reported by Lim *et al.*<sup>46</sup>, who demonstrated that the administration of *L. sakei* OK67 to obese HFD-induced mice improves glucose tolerance.

### 3.6. Evaluation of the antihyperglycemic effect of *Lactobacillus fermentum* CRL1446

To evaluate the antihyperglycemic effect of *L. fermentum* CRL1446, the Oral Sucrose Tolerance Test (OSTT) was performed and the ability of this bacterium to inhibit  $\alpha$ -glucosidase was determined. In the OSTT test, the results showed a significant increase in blood glucose levels in MS group at all points of the curve; while in MS+Lf group, no differences were observed with respect to control group. The area under the curve in MS group showed a 36% increase with respect to control group, while in MS+Lf group it was similar to control group (Figure 5A and 5B). Measuring blood glucose levels after a sucrose intake is an indirect method for assessing the ability of LAB strains to inhibit  $\alpha$ -glucosidase activity. Intestinal  $\alpha$ -glucosidases participate in the cleavage of sucrose with the consequent release of glucose, and its inhibition would allow maintaining lower blood glucose concentrations.

In order to verify whether the effect on glycemia observed *in vivo* could be attributed to the strain administered, an *in vitro* study was conducted to assess its ability to inhibit  $\alpha$ -glucosidase enzyme. Results showed that *L. fermentum* CRL1446 was able to inhibit 90.41% of  $\alpha$ -glucosidase activity. Li *et al.*<sup>26</sup> reported that the administration of *L. plantarum* X1 with  $\alpha$ -glucosidase inhibitory capacity improved risk markers in rats with DT2. Likewise, Malunga *et al.*<sup>47</sup> reported that ferulic acid and mono and oligosaccharides (feruloylated arabinoxylan) were able to inhibit  $\alpha$ -glucosidase enzyme. This information suggests that the antihyperglycemic effect of *L. fermentum* CRL1446 in MS mice could be enhanced by the presence of ferulic acid in dietary bran fibers.

### 3.7. Effect of administration of *L. fermentum* CRL1446 on intestinal FE activity and oxidative status in MS mice

Intestinal FE activity was significantly reduced (42%) in mice from MS group compared to control group. Supplementation of HFD+WB with *L. fermentum* CRL1446, strain with FE activity, increased intestinal FE activity in MS+Lf group by 46%, compared to MS group (Table 5). However, FE activity of MS+Lf group did not reach the values observed in the control group. It should be noted that despite the fact that we administered a probiotic strain with FE activity, mice in the MS+Lf group are animals on the way to develop pathology such as MS due to their diet, while the control group is made up of healthy animals, so the difference in activity intestinal enzyme is understandable. In previous studies, Abeijón Mukdsi *et al.*<sup>12</sup> reported increased FE activity in large intestine content in mice fed normal diet supplemented with *L. fermentum* CRL1446. Our results would indicate that *L. fermentum* CRL1446 could increase the bioavailability of FA from dietary wheat bran at the colon level, to be subsequently absorbed and metabolized, thus exerting its beneficial effects.

The oxidative status of mice was evaluated by determining in liver glutathione peroxidase (GPx) and glutathione reductase (GR) activities and plasma lipoperoxide (TBARS) concentration (Table 5). In MS group, there was a decrease in GPx and GR activities (11% and 15%, respectively) and an

increase in TBARS concentrations (48%) compared to control group. The administration of *L. fermentum* CRL1446 improved oxidative status in mice (MS+Lf group) as evidenced by an increase in GPx (37%) and GR (64%) activities compared to MS group. Besides, plasma TBARS levels in MS+Lf group showed a significant reduction with respect to MS group, maintaining similar values to control group (Table 5). These results indicate that CRL1446 strain is capable to improve the oxidative status of mice with MS.

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FA shows high antioxidant activity *in vitro*; however, its biological effect *in vivo* is questionable due to its low degree of absorption and rapid metabolism within the host<sup>48</sup>. Rondini *et al.*<sup>49</sup> showed that the administration of FA to rats, in its complexed form in a natural product such as wheat bran, increases the half-life of FA in plasma, improving its bioavailability. Recent studies reported that the administration of feruloylated oligosaccharides to rats with T2DM improved the lipid profile of these animals more effectively compared to free FA<sup>50</sup>. The administration of LAB with FE activity in the diet could be a strategy to increase the bioavailability of FA in the gut, since it is gradually released by enzymatic hydrolysis, improving MS biomarkers.

### **3.8. Detection of metabolites derived from FA and quantification of short-chain fatty acids (SCFA) in colon contents**

To verify whether the administration of CRL1446 strain improves the release of FA in the colon, the presence of this acid and its metabolites in colon contents was evaluated. The determination was semiquantitative since the abundance of each metabolite could be estimated according to the size of the spectral peaks (Figure S1 and Table 6). FA metabolism by intestinal microbiota enzymes produce bioactive compounds: dihydroferulic acid (DHF), 3,4-dihydroxyphenylpropionic acid (DHPPA), 3-hydroxyphenylpropionic acid (HPPA) and benzoic acid (BA) that can be absorbed and transported to the liver or part of them can be excreted in the feces<sup>51</sup>. It has been shown that bioactive phenolic compounds reach low concentrations in the plasma (in the order of  $\mu\text{g}$ ), so the role of the microbiota in their metabolism is highlighted<sup>52</sup>.

In all groups of animals dihydroferulic acid (DHF), 3,4-dihydroxyphenylpropionic acid (DHPPA) and 3-hydroxyphenylpropionic acid (HPPA) were detected (Table 6). A lower abundance of HPPA was observed in MS group compared to control, and an increase of this compound was found when *L. fermentum* CRL1446 was administered. These results would indicate that FA was bioavailable and it was metabolized and transformed into each of the above mentioned compounds, which is consistent with the highest intestinal FE activity observed in control and MS+Lf groups. Similarly, Duncan *et al.*<sup>53</sup> found HPPA as the main metabolite derived from FA when evaluating the fermentation of wheat bran by human feces. These authors, based on metagenomic studies, reported that wheat bran promotes an enrichment in butyric acid producing bacteria capable of releasing FA from the fiber (i.e., with FE activity). Thus, they postulated that the increase in bacteria producing butyric acid and releasing FA during the degradation of wheat bran could explain the beneficial health effects attributed to fiber consumption.

SCFA are produced by fermentation of dietary fiber by colonic microbiota. Therefore, SCFA content depends on the composition of the intestinal microbiota and the type of fermented substrate<sup>54</sup>. Acetic, propionic and butyric acid concentrations in large intestine contents of mice from all the groups studied were determined (Figure 6). Results showed a significant reduction in the levels of all SCFA in MS group with respect to control. The MS+Lf group showed no differences in acetic and propionic acid concentrations with respect to MS group, but an increase in the concentration of butyric acid was observed. These results would indicate a lower metabolic activity of colonic microbiota in animals with MS, and that supplementation with *L. fermentum* CRL1446 causes an increase in the concentration of butyric acid with respect to MS group.

The type and concentration of SCFA produced by intestinal microbiota are important for the development or prevention of obesity and MS<sup>55</sup>. The most abundant SCFA in the colon are acetic, propionic and butyric acids, which are normally found in a molar ratio that can range from 3: 1: 1 to 10: 2: 1<sup>56</sup>. Butyrate is a key energy source for human colonocytes; there is also evidence that butyrate can activate intestinal gluconeogenesis through a cAMP-dependent mechanism with

beneficial effects on glucose control and energy homeostasis<sup>57</sup>. Likewise, it was reported that butyrate protects against diet-induced obesity without suppressing food intake<sup>58</sup>. Brinkworth *et al.*<sup>59</sup> reported a decrease in SCFA, especially butyrate, in obese subjects who consumed HFD low in carbohydrates, compared to obese subjects who consumed a diet low in fat and enriched in carbohydrates and fibers.

The complex and dynamic intestinal microbial ecosystem contributes to the metabolism of various compounds from the diet, producing numerous metabolites. The production of specific metabolites, such as SCFA, is closely linked to the host's energy homeostasis<sup>56</sup>.

For this reason, it has been suggested that the modification of intestinal microbiota by the administration of specific LAB could influence metabolic parameters, either to counteract or favor the effects of the diet.

### **3.9. Effect of HFD+WB and *L. fermentum* CRL1446 administration on intestinal microbiota of mice**

To investigate the impact of the administration of HFD+WB and *L. fermentum* CRL1446 on the composition of intestinal microbiota of mice, high performance sequencing technology (HTS) was used. Several indices of alpha diversity were calculated to determine whether dietary supplementation with *L. fermentum* CRL1446 was associated with a difference in the number of OTUs. Interestingly, significant differences were observed in the number of OTUs in the "species" level and in the Chao1 metrics (an accurate estimator of the number of different species or richness) when comparing the MS+Lf group with the control or MS group (Table 7). The results indicate a greater phylogenetic diversity (PD whole tree) in the mice that received *L. fermentum* CRL1446 with respect to the mice to which the probiotic was not administered. No significant differences were observed in the alpha diversity metrics between MS and Control groups, except for the Shannon index that was higher in MS group (Table 7). The analysis of beta diversity using Unifrac

showed a variation of the principal components (PCoA) according to the type of diet administered; the different clustering in the 3 groups of mice evaluated was evident (Figure 7).

The bacterial communities in all groups were dominated by two phyla: Bacteroidetes and Firmicutes (Figure 8A). The relative abundance of Bacteroidetes phylum (which includes all Bacteroidales) was lower in MS group (31.2%) with respect to control group (85.8%), while in mice that received *L. fermentum* CRL1446 (MS+Lf group) Bacteroidetes increased (59.05%) compared to MS group (Figure 8A). On the other hand, the proportion of the phylum Firmicutes (which includes all Clostridiales and Lactobacillales) was 12.75% in control group and it increased with the administration of HFD+WB in the MS group (55.05%); however, when this diet was also supplemented with the probiotic *L. fermentum* CRL1446 (MS+Lf group), it was observed a decreased abundance of Firmicutes (36.05%) (Figure 8A).

The current vision regarding the investigation of metabolic syndrome and associated pathologies focuses on the study of intestinal microbiota. One of the main functions of the microbiota is its ability to extract energy from the diet, which is possible thanks to the role that bacteria play in transforming complex nutrients, such as dietary fiber, into simple sugars and short-chain fatty acids, avoiding their loss in stools<sup>60</sup>. It is interesting to mention that many studies indicate that Bacteroidetes are more abundant when maintaining a low-calorie diet<sup>61</sup>. Mice subjected to fat-rich Western-type diets show an increase in Firmicutes and a decrease in Bacteroidetes<sup>62</sup>. The relative abundance of Bacteroidetes in MS mice was lower than 50%, whereas the Firmicutes were higher than 50% (Figure 8A). Similar results were reported by Ley *et al.*<sup>63</sup> in obese mice. In this study, the administration of *L. fermentum* CRL1446 allowed modifying the intestinal microbiota by counteracting the effects of HFD+WB with an increase in Bacteroidetes and a decrease in Firmicutes abundance. Ma *et al.*<sup>64</sup> reported that FA modulates the intestinal microbiota composition of mice fed HFD by decreasing the proportion of Firmicutes while increasing the proportion of Bacteroidetes, improving non-alcoholic fatty liver disease in animals. This suggests that the observed effect on the microbial communities of mice that received *L. fermentum* CRL1446 may be

due to the increased release of FA in the gut, which would have a positive impact on individuals at risk of MS.

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Firmicutes were dominated by Clostridiales, mainly by Ruminococcaceae family (Figure 8B). Overall, the most abundant group was an unclassified group of Clostridiales (Control: 7.30%; MS: 20.50%; MS+Lf: 20.65%). Within the Ruminococcaceae family the genus *Oscillospira* prevailed in all groups of mice evaluated (Control: 1.70%; MS: 15.85%; MS+Lf: 5.35%). The Lachnospiraceae family was also present, but its abundance was low in all the groups evaluated (Control: 1.25%; MS: 2.60%; MS+Lf: 1.20%). With respect to the genus *Lactobacillus* (Lactobacillales family), a higher proportion was found in the MS+Lf group (1.95%) compared with the Control (0.25%) and MS (0.35%) groups (Figure 8B). In the present study it was found that Lachnospiraceae abundance was approximately 2-fold higher in MS group compared to Control and MS+Lf groups (Figure 8B). Other researches showed that Lachnospiraceae are more abundant in infants of overweight mothers<sup>65</sup> and in obese subjects<sup>66</sup> and it has also been shown that they positively correlate with epididymal adipose tissue<sup>67</sup>. Moreover, enrichment of specific Lachnospiraceae was reported in human studies with diets supplemented with whole grain barley<sup>68</sup> and wheat bran<sup>69</sup>. Regarding the genus *Lactobacillus* (family Lactobacillaceae), previous studies conducted by our research group in a murine model demonstrated that a calorie restricted diet increases its abundance, and that the administration of *L. fermentum* CRL1446 to mice subjected to caloric restriction, would allow this bacteria to colonize the large intestine with the consequent increase in FE activity in intestinal mucosa and content<sup>70</sup>. A similar effect was observed in animals from MS+Lf group, in which the proportion of *Lactobacillus* was higher (Figure 8B), probably because the prolonged administration time of *L. fermentum* CRL1446 allowed intestinal colonization, altering the microbiota and causing increased release of FA from bran fibers; however, more studies of species and strain identification are required. Santacruz *et al.*<sup>71</sup> evaluated the influence of an obesity treatment program (intervention based on an energy-restricted diet and regular physical activity) on the gut microbiota

and body weight of overweight adolescents, observing that in the high weight-loss group, *Lactobacillus* group counts increased.

The phylum Bacteroidetes was dominated by Bacteroidales whose family and genus could not be determined precisely (Control: 29.65%; MS: 15.80%; MS+Lf: 36.85%). It was also found that *Prevotella* genus (family Prevotellaceae) was not present in the MS group, while the abundance in Control and MS+Lf groups was 34.9% and 12.45% respectively (Figure 8B). It is known that increase in the *Prevotella* genus facilitates the fermentation of the carbohydrates that escape intestinal digestion and increases the production of SCFA<sup>72</sup>. Nadal *et al.*<sup>73</sup> reported shifts in microbiota associated with weight loss in obese adolescents which included increased proportions of Bacteroides-Prevotella.

Regarding the phylum Proteobacteria, the genus *Desulfovibrio* was predominant, a much higher relative abundance being observed in the MS group (7.35%) compared to the Control group (1.25%). Administration of *L. fermentum* CRL1446 allowed to maintain lower levels of *Desulfovibrio* in the MS+Lf group (4.05%) (Figure 8 A and B). Larsen *et al.*<sup>74</sup> determined the composition of intestinal microbiota of diabetic people and observed that the occurrence of diabetes correlated with high Proteobacteria levels compared to healthy individuals. Likewise, Zhang *et al.*<sup>55</sup> reported an enrichment of sulfatereductive populations such as *Desulfovibrio* in obese subjects. In a study performed by Parker *et al.*<sup>75</sup>, it was found that Proteobacteria abundance was significantly lower in obese mice group fed with HFD and treated with probiotics (*Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032) compared to mice receiving HFD and placebo.

#### 4. Conclusion

Our results showed that animals fed with HFD+WB and that jointly received *L. fermentum* CRL1446 hydrolyzed bran fiber more efficiently, so that their metabolic status of improved with respect to their MS pairs that did not receive the probiotic strain.



There is a close relationship between the gut microbiota composition, intestinal FE activity, metabolites derived from FA, SCFA and hydrolysis of the fiber contained in foods. The oral administration of *L. fermentum* CRL1446 increased intestinal FE activity improving the biochemical markers of MS and the oxidative status of mice fed with HFD+WB for 14 weeks. Modulation of the intestinal microbiota composition through a diet that includes wheat bran fiber along with a FE producing probiotic strain, such as *L. fermentum* CRL1446, or its metabolic products/byproducts (postbiotics) represent a promising nutritional strategy to prevent or modify the risk of MS and associated metabolic disorders.

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### Conflict of interest

There are no conflicts to declare

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## Figures Legends

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**Figure 1:** **A:** Representative photomicrographs of epididymal adipose tissue at 14 weeks. The histological sections were stained with hematoxylin-eosin and the images were captured with Carl ZEISS microscope with 40x magnification. (A) Control group, (B) MS group, (C) MS + Lf group. **B:** Abundance of adipocytes (%) according to their size ( $\mu\text{m}^2$ ) in mice fed for 14 weeks. Data represent mean  $\pm$  SE (standard error) of  $n=8$  mice per group. The statistical difference was determined using Tukey's test with  $P<0.05$ . Values with different letters differ significantly in each area range

**Figure 2:** Representative photomicrographs of liver of mice at week 14. The histological sections were stained with hematoxylin-eosin and the images were captured with Carl ZEISS microscope with 200x magnification. (A) Control group, (B) MS group, (C) MS+Lf group. The arrows indicate some alterations observed in MS group (lipid drops and binucleation).

**Figure 3:** Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations (U/L). Data represent mean  $\pm$  SE (standard error) of  $n=8$  mice. Statistical differences were determined using Tukey's test with  $P<0.05$ . Values with different letters differ significantly.

**Figure 4:** **A:** Glucose tolerance curves at week 14 in (●) Control group, (●) MS group and (●) MS+Lf group. **B:** Areas under the curve (AUC) at week 14. Data represent mean  $\pm$  SE (standard error) of  $n=8$  mice. Statistical differences were determined using Tukey's test with  $P<0.05$ . (\*) indicate statistical differences with respect to the control group. In figure B, values with different letters differ significantly.

**Figure 5:** **A:** Oral sucrose tolerance curves at week 14 in (●) Control group, (●) MS group and (●) MS+Lf group. **B:** Areas under curve. Data represent mean  $\pm$  SE (standard error) of  $n = 8$  mice. Statistical differences were determined using Tukey's test with  $P < 0.05$ . In figure A the asterisks

(\*) indicate differences statistics regarding the control group. In figure B values with different letters differ significantly.

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**Figure 6:** Concentration of short chain fatty acids (SCFA) produced in colon contents at week 14 of feeding. Mice received normal diet (Control group), HFD+WB (MS group), HFD+WB supplemented with *L. fermentum* CRL1446 (MS+Lf group). The dose of administration of the strains was  $10^8$  CFU/day/mouse. Data represent mean  $\pm$  SE (standard error) of  $n=8$  mice. The statistical difference was determined using Tukey's test with  $P<0.05$ . Values with different letters differ significantly for each acid evaluated.

**Figure 7:** Weighted Unifrac-based PCoA analysis of gut microbiota (beta diversity) via 16S rRNA sequencing of mice fed for 14 weeks with normal diet (Control group), HFD+WB (MS group) and HFD+WB supplemented with *Lactobacillus fermentum* CRL1446 (MS+Lf group).

**Figure 8:** Relative abundance of intestinal bacterial communities in mice fed for 14 weeks with normal diet (Control group), HFD+WB (MS group) and HFD+WB supplemented with *Lactobacillus fermentum* CRL1446 (MS+Lf group). The figures show the taxonomic categories for the different groups evaluated. **A:** phylum. **B:** order, family and bacterial genus.

**Table 1: Body weight gain, food efficiency ratio, adiposity index, and leptin levels.**

	Groups		
	Control	MS	MS+Lf
Body weight gain (g)	12.90±0.77 <sup>b</sup>	20.44±1.38 <sup>a</sup>	13.92±0.42 <sup>b</sup>
Food efficiency ratio	0.03±0.001 <sup>b</sup>	0.05±0.004 <sup>a</sup>	0.03±0.001 <sup>b</sup>
Adiposity index	2.20±0.16 <sup>c</sup>	4.70±0.21 <sup>a</sup>	3.00±0.20 <sup>b</sup>
Leptin (ng/mL)	0.98±0.17 <sup>c</sup>	6.65±1.07 <sup>a</sup>	1.87±0.16 <sup>b</sup>

Data are expressed as mean ± SE (standard error),  $n=8$  per group. Values with different superscript letters in the same row are significantly different ( $P<0.05$ ) as assessed by Tukey's test. Control, mice receiving normal diet; MS (Metabolic syndrome), mice receiving HFD+WB diet; MS+Lf, mice receiving HFD+WB diet and *L. fermentum* CRL1446.

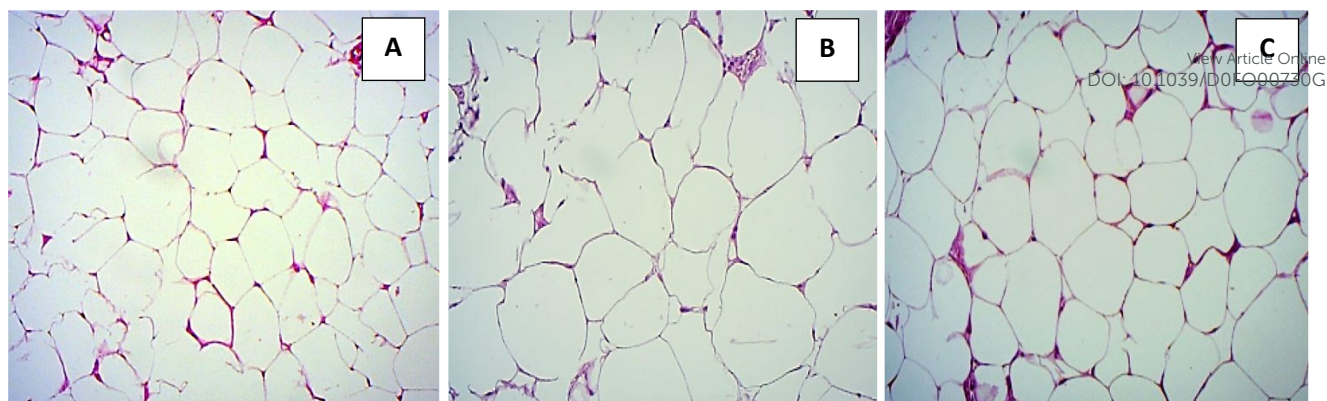


Figure 1A

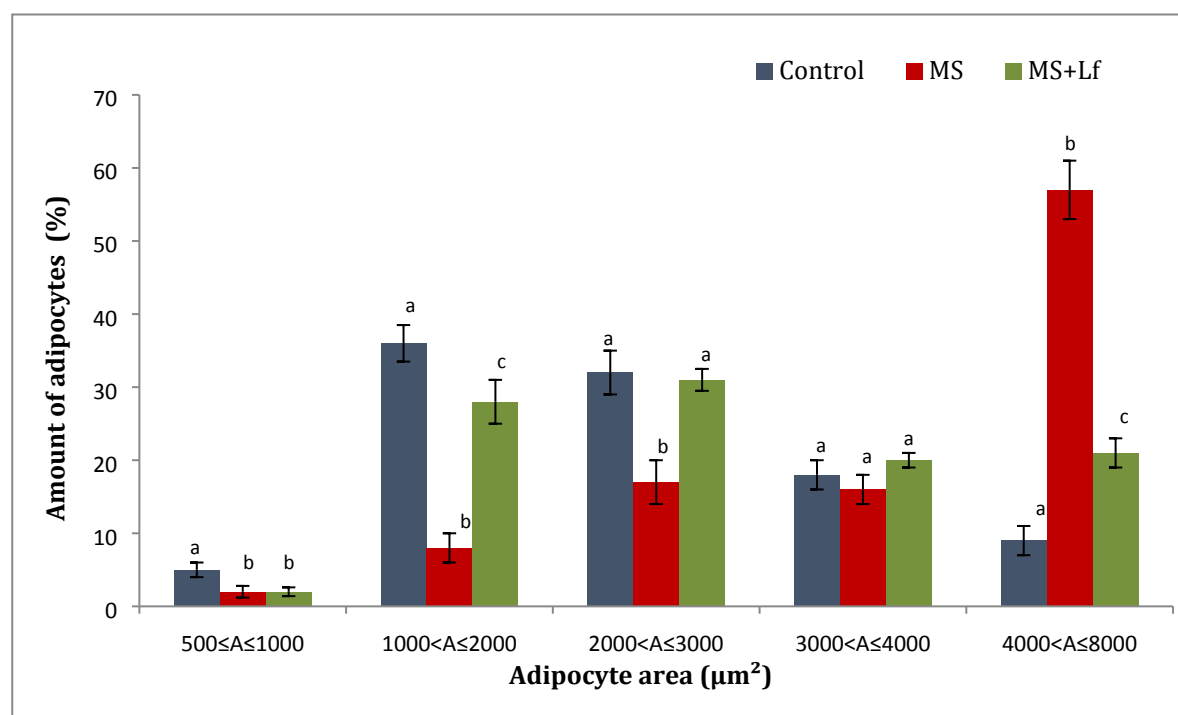
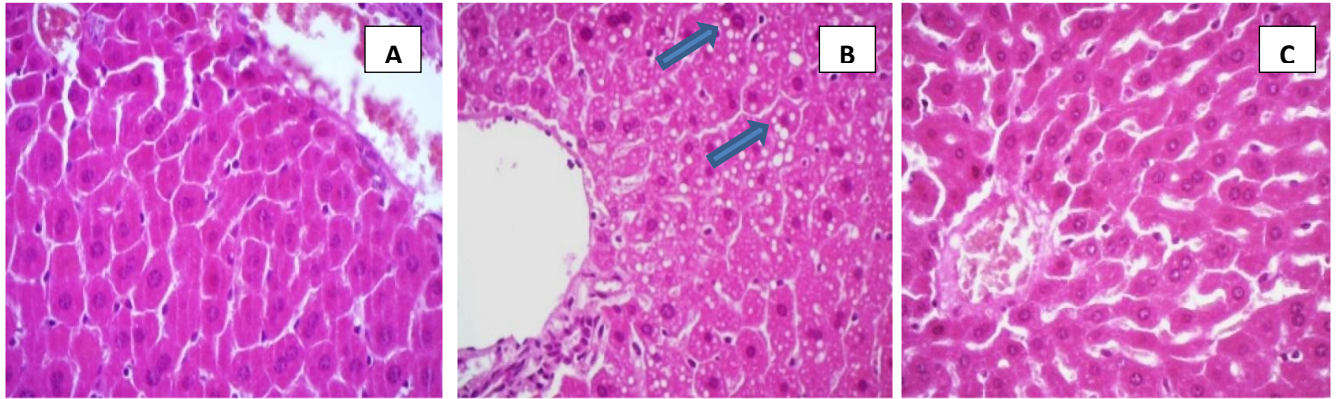


Figure 1B

**Table 2: Inflammatory status of mice with metabolic syndrome**View Article Online  
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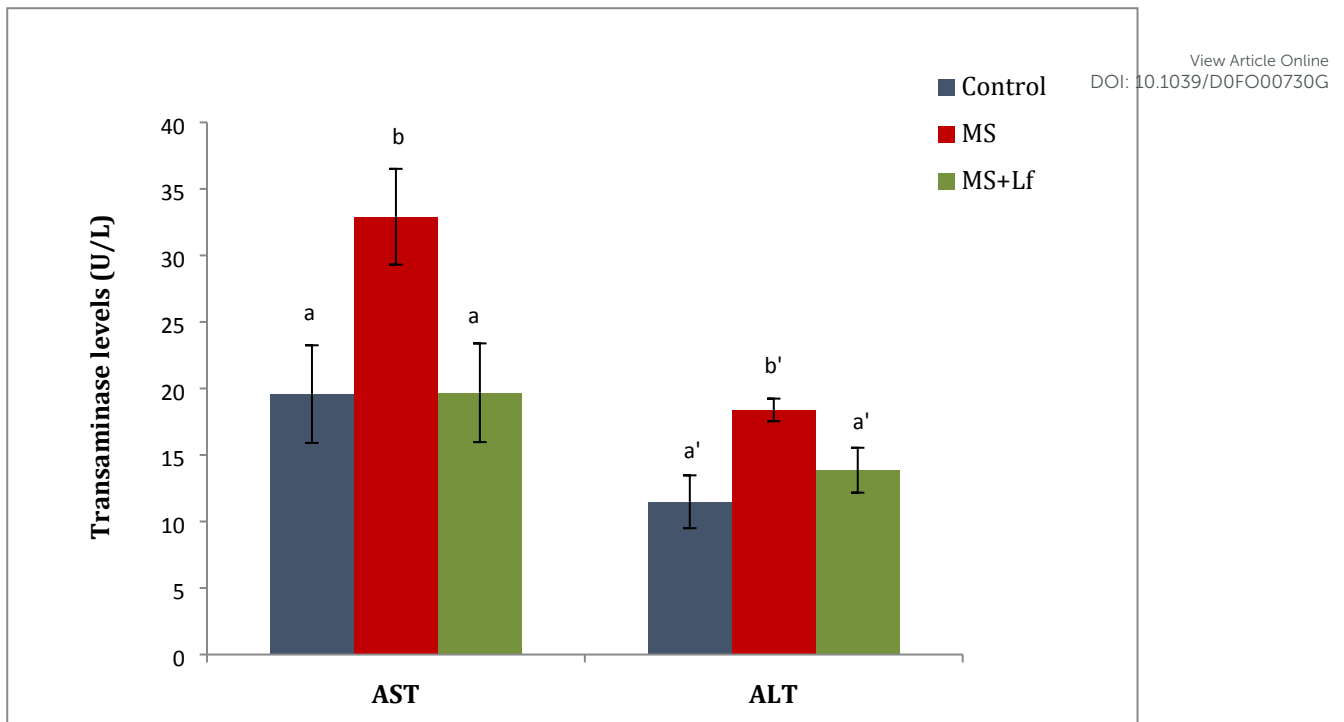
Cytokines (pg/mL)	Groups		
	Control	MS	MS+Lf
TNF- $\alpha$	5.42 $\pm$ 1.69 <sup>c</sup>	36.56 $\pm$ 6.60 <sup>a</sup>	19.80 $\pm$ 1.50 <sup>b</sup>
IFN- $\gamma$	1.34 $\pm$ 0.41 <sup>a</sup>	14.34 $\pm$ 2.91 <sup>a</sup>	5.68 $\pm$ 0.78 <sup>c</sup>
IL-6	2.98 $\pm$ 1.09 <sup>c</sup>	18.02 $\pm$ 2.06 <sup>a</sup>	10.86 $\pm$ 1.79 <sup>b</sup>
IL-10	223.50 $\pm$ 43.60 <sup>a</sup>	75.81 $\pm$ 14.56 <sup>c</sup>	114.60 $\pm$ 15.14 <sup>b</sup>

Data are expressed as the mean  $\pm$  SE (standard error),  $n=8$  per group. Values with different superscript letters in the same row are significantly different ( $P<0.05$ ) as assessed by **Tukey's test**. Control, mice receiving normal diet; MS (Metabolic syndrome), mice receiving HFD+WB diet; MS+Lf, mice receiving HFD+WB diet and *L. fermentum* CRL1446.



**Figure 2**





**Figure 3**

**Table 3: Plasma lipids and cardiovascular risk indicators**

Plasma lipids (g/L)	Groups		
	Control	MS	MS+Lf
Triglyceride	0.27±0.02 <sup>c</sup>	0.43±0.03 <sup>a</sup>	0.35±0.02 <sup>b</sup>
Total cholesterol	0.66±0.02 <sup>b</sup>	0.88±0.02 <sup>a</sup>	0.65±0.03 <sup>b</sup>
LDL-cholesterol	0.56±0.02 <sup>b</sup>	0.71±0.01 <sup>a</sup>	0.52±0.02 <sup>b</sup>
HDL-cholesterol	0.16±0.01 <sup>b</sup>	0.12±0.01 <sup>a</sup>	0.16±0.01 <sup>b</sup>
<b>Cardiovascular risk indicators</b>			
LDL/HDL	3.57±0.34 <sup>b</sup>	5.81±0.25 <sup>a</sup>	3.34±0.22 <sup>a</sup>
Triglyceride/HDL	1.73±0.11 <sup>c</sup>	3.49±0.15 <sup>a</sup>	2.26±0.21 <sup>b</sup>
Atherogenic index	3.18±0.43 <sup>b</sup>	6.19±0.52 <sup>a</sup>	3.16±0.64 <sup>b</sup>
AIP	0.24±0.04 <sup>c</sup>	0.54±0.06 <sup>a</sup>	0.35±0.06 <sup>b</sup>

Data are expressed as the mean ± SE (standard error),  $n=8$  per group. Values with different superscript letters in the same row are significantly different ( $P<0.05$ ) as assessed by **Tukey's test**. Control, mice receiving normal diet; MS (Metabolic syndrome), mice receiving HFD+WB diet; MS+Lf, mice receiving HFD+WB diet and *L. fermentum* CRL1446. Atherogenic index: (total cholesterol – HDL-cholesterol)/ HDL-cholesterol. AIP: Atherogenic Index of Plasma = Log (TG/HDL).

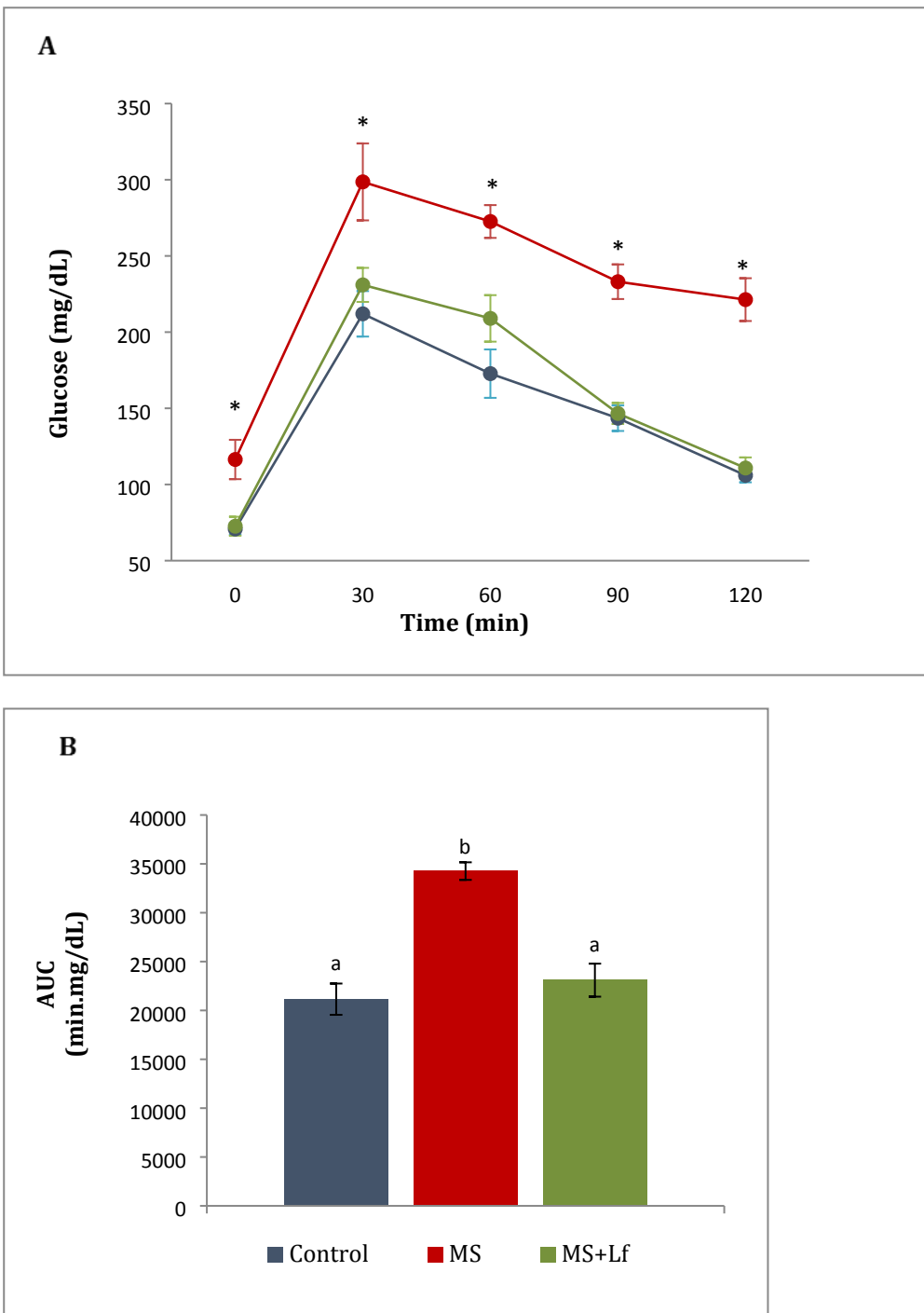


Figure 4

**Table 4: Plasma glucose, insulin levels and HOMA-IR**View Article Online  
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	Groups		
	Control	MS	MS+Lf
Glucose (mmol/L)	3.96±0.46 <sup>b</sup>	7.79±0.91 <sup>a</sup>	4.12±0.23 <sup>b</sup>
Insulin (μU/mL)	8.69±0.69 <sup>b</sup>	12.59±0.85 <sup>a</sup>	8.84±0.75 <sup>b</sup>
HOMA-IR	1.53±0.27 <sup>b</sup>	4.36±0.41 <sup>a</sup>	1.62±0.29 <sup>b</sup>

Data are expressed as mean ± SE (standard error),  $n=8$  per group. Values with different superscript letters in the same row are significantly different ( $P<0.05$ ) as assessed by **Tukey's test**. Control, mice receiving normal diet; MS (Metabolic syndrome), mice receiving HFD+WB diet; MS+Lf, mice receiving HFD+WB diet and *L. fermentum* CRL1446.

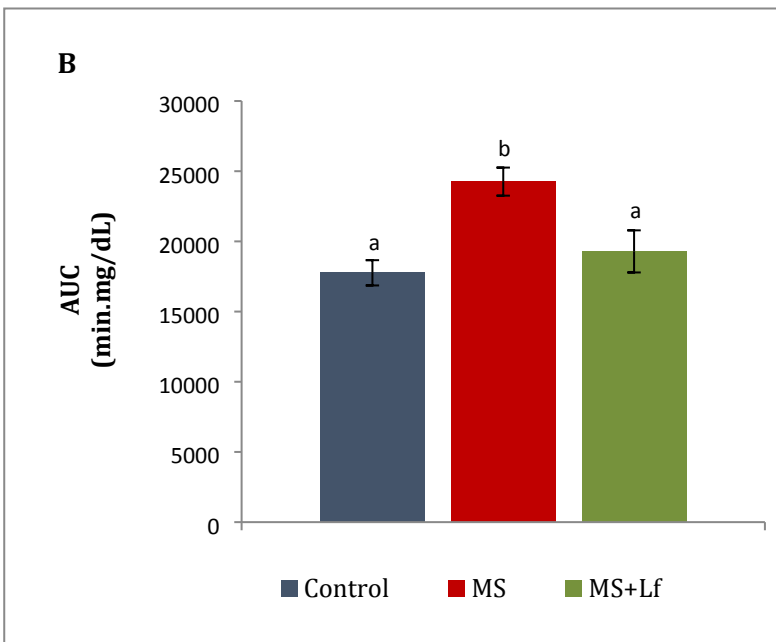
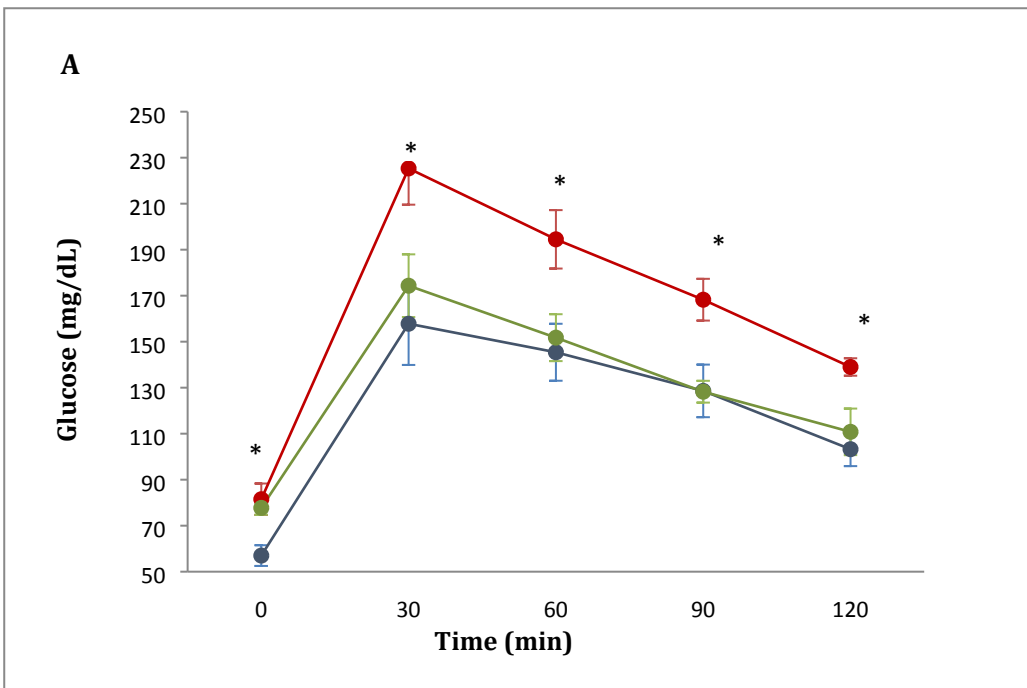


Figure 5

**Table 5: Intestinal feruloyl esterase, glutathione peroxidase and glutathione reductase activities and TBARS levels**

Enzymes	Groups		
	Control	MS	MS+Lf
IFE (U/g)	1255.60±50.27 <sup>a</sup>	733.00±82.90 <sup>c</sup>	1074.00±170.14 <sup>b</sup>
GPx (U/g)	34.15±0.97 <sup>b</sup>	30.50±0.73 <sup>c</sup>	41.84±3.12 <sup>a</sup>
GR (U/g)	53.91±1.30 <sup>b</sup>	45.90±1.94 <sup>c</sup>	75.72±2.74 <sup>a</sup>
TBARS (nmol/g)	32.42±6.60 <sup>b</sup>	47.94±4.54 <sup>a</sup>	32.32±2.71 <sup>b</sup>

IFE: intestinal feruloyl esterase, GPx: glutathione peroxidase, GR: glutathione reductase, TBARS: thiobarbituric acid reactive substances. Values are expressed as mean ± SE (standard error),  $n=8$  per group. Values with different superscript letters in the same row are significantly different ( $P<0.05$ ) as assessed by Tukey's test. Control, mice receiving normal diet; MS (Metabolic syndrome), mice receiving HFD+WB diet; MS+Lf, mice receiving HFD+WB diet and *L. fermentum* CRL1446.

**Table 6: Detection of FA-derived metabolites in colon contents of mice fed for 14 weeks by HPLC-MS.**

Compound	Rt (min)	Molecular ion [ M - H ] <sup>-</sup> (m/z)	Control group	MS group	MS+Lf group
FA	12.55	193.0	-	-	-
DHF	8.26	195.1	+	+	+
DHPPA	7.36	181.1	+	+	+
HPPA	8.56	165.1	++	+	+++
BA	13.2	121.0	-	-	-

The mass/charge ratios ( $m/z$ ) and retention times (RT) for each compound are shown. FA: ferulic acid; DHF: dihydroferulic acid; DHPPA: dihydroxyphenylpropionic acid, HPPA: hydroxyphenylpropionic acid and BA: benzoic acid. The metabolites detected are shown for the 3 groups of mice tested ( $n=3$  each). (-): metabolite not detected (+): metabolite detected. More signs (++ or +++) indicate greater area of the peaks in the spectrum.

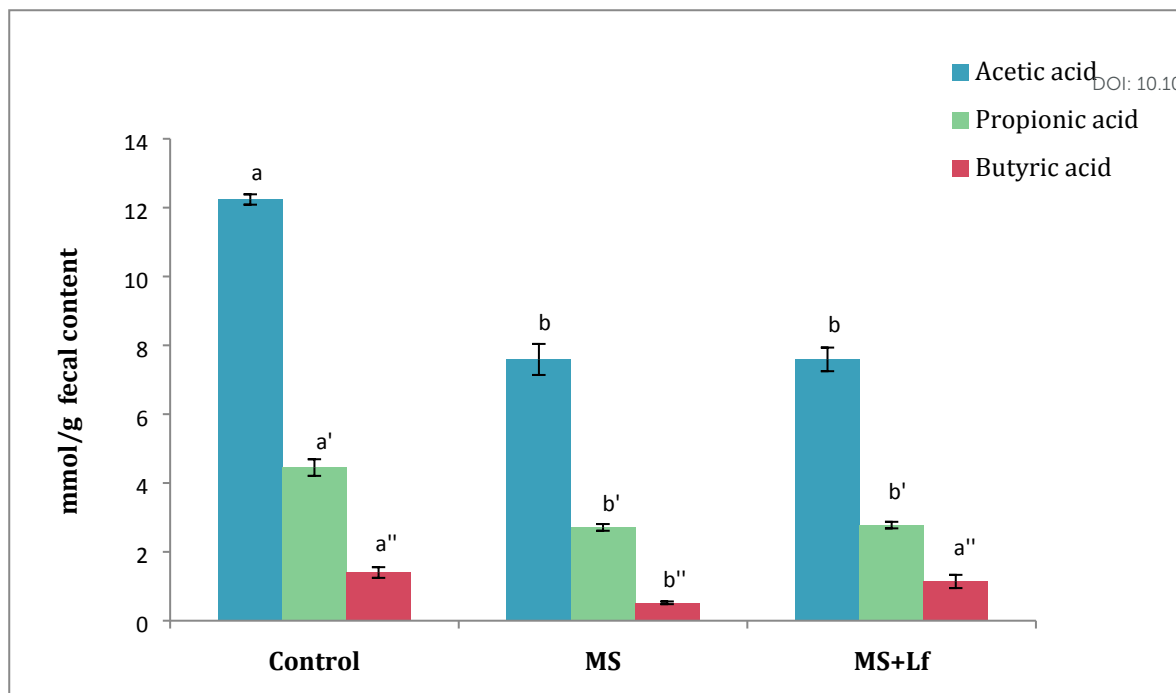


Figure 6



**Table 7: Alpha diversity metrics**

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Metric	Groups		
	Control	MS	MS+Lf
<b>Shannon</b>	5.38±0.46 <sup>a</sup>	6.29±0.12 <sup>b</sup>	6.28±0.32 <sup>b</sup>
<b>Chao 1</b>	297.98±23.82 <sup>a</sup>	267.06±4.04 <sup>a</sup>	330.36±0.74 <sup>b</sup>
<b>Observed OTUs (Species)</b>	274.10±28.50 <sup>a</sup>	255.65±0.75 <sup>a</sup>	313.75±7.95 <sup>b</sup>
<b>PD whole tree</b>	20.86±0.91 <sup>a</sup>	19.28±0.30 <sup>a</sup>	22.80±0.12 <sup>b</sup>

Chao1, Shannon's diversity and phylogenetic diversity (PD whole tree) of colon contents of mice fed for 14 weeks. Data are expressed as mean ± SE (standard error),  $n=3$  per group. Values with different superscripts, within the same row, are significantly different ( $P < 0.05$ ).

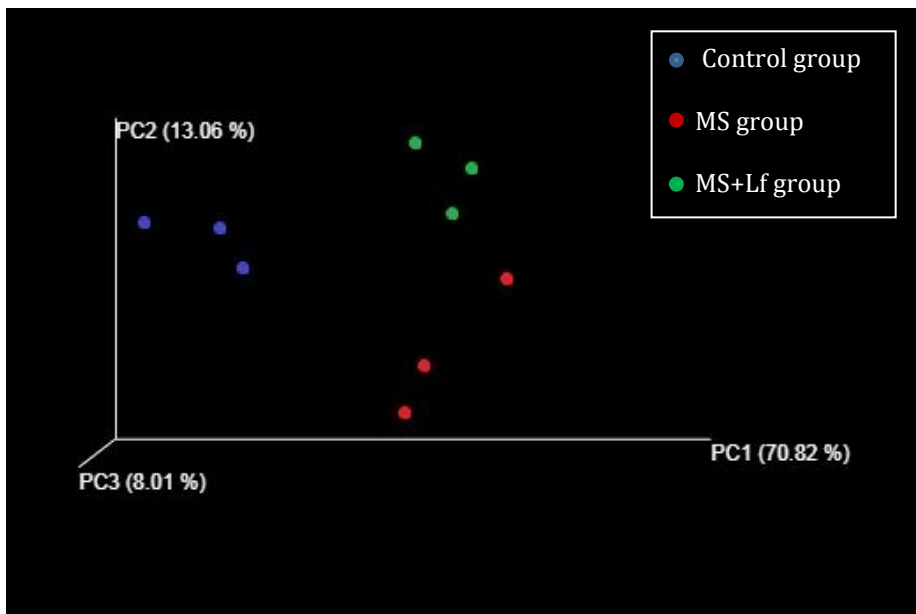
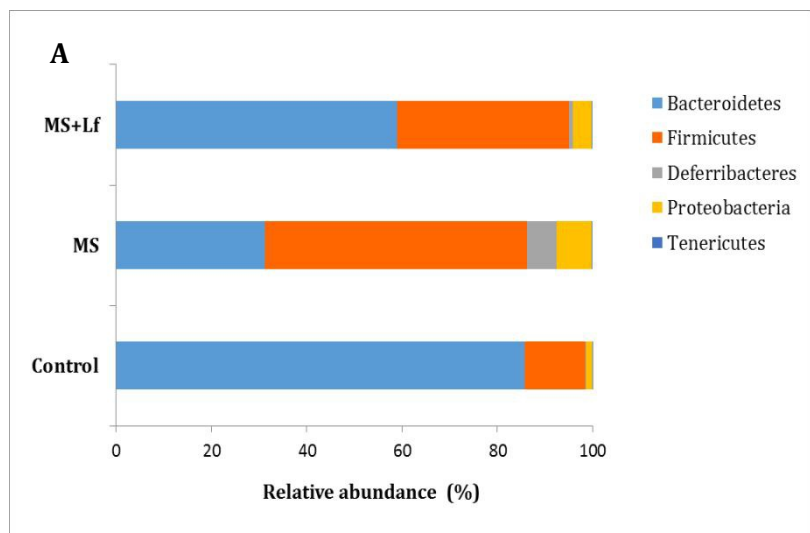
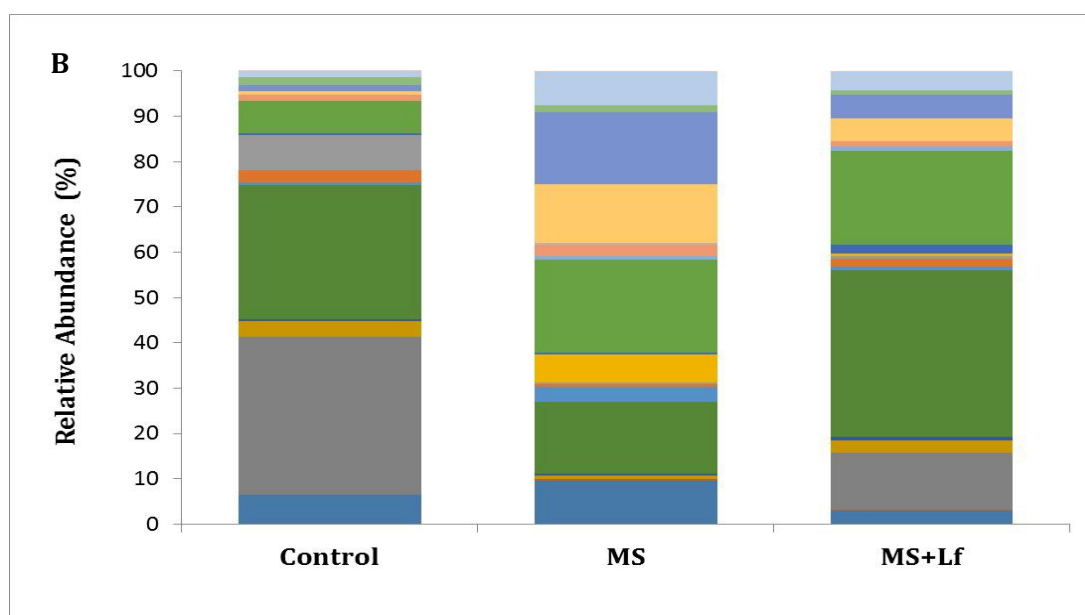


Figure 7

Figure 8



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- Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma
- Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio
- Clostridiales; Ruminococcaceae; Ruminococcus
- Clostridiales; Ruminococcaceae; Oscillospira
- Clostridiales; Ruminococcaceae - Other
- Clostridiales; Lachnospiraceae; Coprococcus
- Clostridiales; Lachnospiraceae - Other
- Clostridiales; Dehalobacteriaceae; Dehalobacterium
- Clostridiales - Other
- Lactobacillales; Lactobacillaceae; Lactobacillus
- Deferribacterales; Deferribacteraceae; Mucispirillum
- Bacteroidales; Paraprevotellaceae; Prevotella
- Bacteroidales; Paraprevotellaceae; Paraprevotella
- Bacteroidales; Odoribacteraceae; Odoribacter
- Bacteroidales - Other
- Bacteroidales; Rikenellaceae; Rikenella
- Bacteroidales; Rikenellaceae - Other
- Bacteroidales; Prevotellaceae; Prevotella
- Bacteroidales; Porphyromonadaceae; Parabacteroides
- Bacteroidales; Bacteroidaceae; Bacteroides

### Graphic abstract

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