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International Dairy Journal

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--Manuscript Draft--

Manuscript Number:	INDA-D-20-00321R3
Article Type:	Research Article
Keywords:	obesity; Yoghurt; cholesterol; Obese mice model; Pro-inflammatory cytokines
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This is the peer−reviewed accepted manuscript of the article, which has been published in final form in volume 114, March 2021, number 104914, DOI https://doi.org/10.1016/j.idairyj.2020.104914

Sex-dependant effects of a yoghurt enriched with proteins in a mouse model of diet-induced obesity

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ABSTRACT

This research aimed at assessing the effects of a yoghurt enriched with proteins on pro-inflammatory cytokines and lipids profile using a mouse model of diet-induced obesity in males and females C57BL/6 mice. The results obtained showed a clear sex-dependent behavior of the mice. Female gained less weight gain when compared to male mice independently to the diet. Considering the effect of the diet, when the high fat diet was implemented with yoghurt a reduction of cholesterol and triglycerides, in liver and blood serum of males, and triglycerides in the liver of female mice was observed. For male mice fed with yoghurt, a significant reduction of the levels pro-inflammatory cytokines measured was detected. However, in female mice, the anti-inflammatory effect due to yoghurt consumption was observed to a minor extent. The principal component analysis, obtained considering all the data, confirmed that gender or diet was able to group individual animals. A higher variability of data was observed in females compared to male mice, being this probably the reason why less significant differences were observed in the former.

Keywords: Obesity; Yoghurt; Cholesterol; Obese mice model; Pro-inflammatory cytokines

1. Introduction

Overweight and obesity have been constantly increasing in the last decades. In fact, the incidence of obesity has tripled since 1975 (WHO, 2019). In 2016, over 1.6 billion of adults were overweight and 650 million were obese resulting in 13% of the world population being obese (WHO, 2019). The energy imbalance between calories consumed and spent is reported to be the main reason of overweight and obesity (Romieu et al., 2017; WHO, 2019). The increase in the incidence of obesity

is generally linked to a greater consumption of energy-dense foods, high in fats and sugars, associated to a reduction in physical activity and fiber intake (Hruby & Hu, 2015), but the early role of intestinal microbiota establishment is also being considered (Dao & Clément, 2018). Obesity can be associated to different causing factors including genetic, environmental (C-section, reduced breast-feeding, antibiotics consumption), or neural hormonal functions among others (Nguyen & El-Serag, 2010; Williams, Mesidor, Winters, Dubbert, & Wyatt, 2015). The consequences of obesity are linked to multiple health problems such as cardiovascular diseases, diabetes, musculoskeletal disorders (especially osteoarthritis) and some cancers (including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon) (Must & McKeown, 2000; WHO, 2019).

Several studies report that the consumption of fermented dairy products may exert a beneficial effect on risk factors of metabolic disorder, such as dyslipidaemia, insulin resistance and high blood pressure, that, if not properly addressed, may dramatically increase the risk of diabetes and cardiovascular diseases (Astrup, 2014). Some epidemiological trials report that yoghurt consumption is contrariwise associated to the incidence of obesity in humans, in particular when associated to the consumption of fruit and vegetables (Rautiainen et al., 2016; Sayon-Orea, Martínez-González, Ruiz-Canela, & Bes-Rastrollo, 2017). Dairy foods make available important nutrients, including proteins and calcium. The consumption of dairy products in observational studies, and to some extent in randomized controlled trials, is associated with reduced risk of body fat gain, obesity and cardiovascular diseases. Yoghurt, because of its particular manufacturing process that includes fermentation, represents a unique dairy product. In fact, in yoghurt many nutrients, including protein, riboflavin, vitamin B-6, vitamin B-12, calcium, potassium, zinc, and magnesium, are more concentrated than in milk, including enhanced bioavailability of calcium (Jacques & Wang, 2014). Several literature data suggest that a regular consumption of yoghurt may

exert positive health effects through the reduction of the incidence of colorectal cancer, the levels of total cholesterol, low density lipoprotein- cholesterol, and triglycerides (Astrup, 2014; Ejtahed et al., 2011; Fernandez & Marette, 2017; Kang et al., 2015; Rodríguez-Figueroa, González-Córdova, Astiazaran-García, Hernández-Mendoza, & Vallejo-Cordoba, 2013). Although the relationship between yogurt and reduction of obesity has been extensively studied, still limited findings support this hypothesis and further trials are needed. In particular, limited information is available on the effect of gender on the efficacy of yoghurt to modulate obesity.

Mice can be used as a model for diet-induced obesity, in order to study mechanisms and find alternatives for obesity prevention or treatment (Della Vedova et al., 2016; Wang & Liao, 2012; Zou et al., 2018). The mouse strain C57BL/6 is reported to be highly susceptible to diet-induced obesity (Wang & Liao, 2012; Yang, Smith, Keating, Allison, & Nagy, 2014), and is the strain the most used one in this field. This research aimed at assessing the effects of a yoghurt enriched with proteins on pro-inflammatory cytokines and lipids profile using a mouse model of diet-induced obesity in males and females C57BL/6 mice.

2. Material and methods

2.1 Yogurt preparation

Standardized milk (composition (w/v): 3%, proteins, 4.8% carbohydrates,1.5% fat,) and skim milk powder (composition (w/w): 33% proteins, 53% carbohydrates, 1.1% fat, 7% ashes) was kindly provided by Milkaut S.A. (Santa Fe, Argentina). Whey Powder Concentrate WPC40 (composition (w/w): 40% proteins, 42% carbohydrates, 3% fat, 6% ashes) was produced and provided by García Hnos. Agroindustrial S.R.L. (Santa Fe, Argentina). A freeze-dried commercial direct vat set (DVS) starter culture composed by *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (named SLB95) was kindly supplied by Diagramma S.A. (Santa Fe, Argentina).

Milk base (500 mL) for yoghurt manufacture was formulated mixing standardized milk, skim milk powder (3.0 % w/v) and WPC40 (2.0 % w/v), which was kept overnight at 5 °C for proper hydration of powder ingredients. The milk base was heat-treated at 85 °C for 20 min and cooled down to 42 °C. The starter culture was then inoculated to the milk base, according to the manufacturer's instructions. The fermentation process was conducted at 42 ± 1 ºC in a water bath until pH 4.75 ± 0.05. Yoghurts were immediately cooled down to room temperature and then stored at 5 ºC. Yoghurt was produced every 2 weeks for animal feeding. Gross composition (mean ± standard deviation) of yoghurts after 7 days of storage at 4 °C was: pH, 4.56 ± 0.10; total solids, 14.40 ± 0.45%, protein, 4.98 ± 0.25%, and fat, 1.60 ± 0.15%.

2.2 In vivo trial

2.2.1 Animals

Eighteen 5-6 old-week male C57BL/6 mice weighing 16.5 to 23.5 g and eighteen 5-6 old-week female C57BL/6 mice weighing 14.0 to 19.5 g were obtained from the random-bred colony of the Veterinary Sciences Institute of Litoral (Instituto de Ciencias Veterinarias del Litoral, ICiVet-Litoral), from the Faculty of Veterinary, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina).

Animals were transported to the INLAIN animal facility and allowed to stand for a week before starting the experiments. They were housed individually in plastic cages and kept in a controlled environment (21±2 °C, 55 ± 2% humidity, with a 12 h light/dark cycle and renovation of 20 volumes of air every h). Mice were maintained and treated according to the guidelines of the National Institutes of Health (NIH, USA). The animal assay was approved by the Safety and Bioethical Committee of the CCT-CONICET, N° 22920160100023CO, Santa Fe.

2.2.2 Composition and preparation of the diet

The composition of the control diet was 14.2% (w/w) protein, 73.1% (w/w) carbohydrates, 4.0% (w/w) fat, 5.0% (w/w) raw fiber, 3.5% (w/w) minerals and vitamins. The High Fat Diet (HFD) was composed as follow: 26.2% (w/w) protein, 26.3% (w/w) carbohydrates, 34.9% (w/w) fat, 6.4% (w/w) raw fiber, 5.8% (w/w) minerals and vitamins. The formulation of both control and HFD is reported in Table 1. In the group that received HFD and yogurt (HFD+Y), 6.5% of the total calories of the HFD diet were replaced by yogurt. HFD+Y was prepared daily by thoroughly mixing 0.65 parts of the HFD diet with 0.35 parts of yoghurt.

2.2.3 Experimental design

Animals (male and female) were randomly divided into 3 groups (6 males and 6 females): Control (C): conventional diet 9.4% kcal from fat, High Fat Diet (HFD): hypercaloric diet 60% kcal from fat, HFD + yogurt (HFD+Y): 6.5% of the total calories replaced by yogurt. Total calories of each group were the following: C: 2.80 kcal g^{-1}, HFD: 4.96 kcal g^{-1}, HFD+Y: 3.36 kcal g^{-1}. Animals received food and sterile tap water *ad libitum*. The animals were fed the corresponding diet for 10 consecutive weeks. During the feeding period, animals were weighed weekly, while food intake was measured every two days.

2.2.4 Sacrifice and tissue sampling

The day before sacrifice, animals were fasted for 15 h. On the day of sacrifice, animals were anaesthetised intraperitoneally (0.2 mL per mouse) according to Burns et al. (2015), with a rodent cocktail (9 parts of ketamine (100 mg mL^{-1}) + 9 parts of xylazine (20 mg mL^{-1}) + 3 parts of acepromazine (10 mg mL^{-1}) + 79 parts of sterile saline solution). Blood was collected by cardiac puncture and kept at room temperature for 30 min to coagulate. Serum was recovered after

centrifugation (2000 ×*g*, 15 min, room temperature) and stored at -70 °C until analysis. Liver was removed and immediately frozen (-70 °C). Small and large intestines were removed, immediately placed on an ice bath, flushed twice with 5 mL of cold PBS buffer containing (0.1% v/v) of a protease inhibitor cocktail (P8340, Sigma Aldrich, St. Louis, MO, USA), and kept frozen (-70 °C) until use.

2.2.5 Cholesterol and triglycerides determination in blood serum and liver.

Liver portions were cut into small pieces and 0.25 g portions were added to a 5 mL mixture of chloroform:methanol (2:1). The suspension was homogenized (15000 rpm, 1 min, room temperature, Ultra Turrax T8, Ika Labortechnik, Staufen, Germany), and centrifuged (2000 ×*g*, 30 min, room temperature). The supernatant was collected and stored at -70 °C until analysis.

Liver and serum cholesterol were determined using the Colestat enzymatic kit (Wierner Lab., Rosario, Argentina) following the procedure indicated by the manufacturer. Liver and serum triglycerides were determined through the TG COLOR GPO/PAP AA enzymatic kit (Wierner Lab., Rosario, Argentina), according to the manufacturer's instructions.

2.2.6 Cytokines determination

Intestine samples were prepared as reported by Burns et al. (2015). Portions of 100 mg of the small or large intestine were placed in 1 mL of PBS solution containing 1% (v/v) antiprotease cocktail P8340 (Sigma Aldrich, St. Louis, MO, USA), 10 mmol L^{-1} EDTA (Sigma Aldrich, St. Louis, MO, USA) and 0.05% (v/v) Tween 20 (Sigma Aldrich, St. Louis, MO, USA). Suspensions were homogenised (Ultra Turrax T8, Ika Labortechnik, Staufen, Germany). Homogenates were centrifuged (10,000 *g*, 10 min, 4 °C) and the supernatant was collected and maintained at -70 °C until cytokine quantification. IL-10, IL-6, IFN-γ and TNFα concentrations were measured by

commercial ELISA kits (BD Biosciences Pharmingen, San Diego, CA, USA), according to the procedures supplied by the manufacturer.

2.3 Statistical analysis

The energy intake and bodyweight were expressed as mean ± standard deviation of each. The data were analysed using the software Statistica (version 8.0; StatSoft, Tulsa, Oklahoma, USA) and subjected to the analysis of variance (ANOVA) and the test of mean comparison, according to Fisher's least significant difference (LSD). Level of significance (p) was 0.05. To analyse cholesterol, triglycerides and cytokines the log transformation of the values was used, a linear random effect model was used to determine the effect of the diet on these parameters and to test the different hypothesis, depending on the expected behaviour of the parameter considered. Statistical treatments and Principal Component Analysis (PCA) were performed using the software R (R Core Team, 2014).

3. Results and Discussion

3.1 Weight and bodyweight gain

In studies where a functional food like yoghurt (Y) is offered to mice during a long term feeding period together with a high-fat diet (HFD), two feeding strategies are possible: to replace part of the HFD, in terms of calories, with the Y and offer the animals the mix *ad libitum*, or to offer the HFD and the Y separately, *ad libitum* too. The former strategy has the limitation that, unavoidable, the overall original HFD composition will change due to the incorporation of the Y. The latter strategy may have more limitations: animals may prefer to eat only the Y, animals may not eat the Y at all, or animals may eat both, the HFD and the Y, but in different proportions along the experiment that uses to take several months, introducing uncontrolled bias to the experiment. In

case the Y is administered independently from the HFD by gavage (controlled way of administration), the daily stress of the oral intubation may lead to bias in results too. For example: during some periods animals may eat less because their throats are injured or yet anticipated death may occur due to irreversible damages on their throats. Both options have their own limitations. However, we believe a much more-controlled study is achieved by choosing the first strategy, as was the case of this study.

During the 10 weeks of the feeding trial, food intake was measured and the energy (kcals) consumed by each group was calculated weekly, considering gender as well (Figure 1). The kcals ingested by the different groups were influenced by the type of diet and gender. The effect of the diet was more evident in male than in female mice. In fact, from week 6 onwards, male mice that received HFD consumed a significantly higher amount of energy ($p < 0.05$) in comparison to the HFD+Y group, while the control group showed a significantly lower energy intake compared to the HFD group only at week 10 of feeding. No significant differences, in males, between HFD+Y group and control group were detected during the whole feeding period. Regarding females, from week 4 onwards, no significant differences were detected among the three groups. In the first 3 weeks of feeding, control female mice ingested a significant higher amount of energy compared to the other groups.

Food efficiency ratio (FER) after 10 weeks of feeding is shown in Figure 2. For each diet, males showed a significantly higher FER ($p < 0.05$) than female mice. Considering gender, the HFD groups showed significantly higher FER ($p < 0.05$) compared to HFD+Y and control groups. No significant differences were detected between HFD+Y and control group for male mice while control group had a significant lower FER ($p < 0.05$) than HFD+Y in female mice.

Bodyweight gain was influenced by mice gender. In general, female groups showed a significantly lower bodyweight gain ($p < 0.05$) compare to male mice, starting from week 5 of feeding (data not

shown). At the end of the feeding period, the biggest differences in bodyweight gain between females (ranging between 8.9 and 10.7 g) and males (ranging between 14.0 and 16.8 g) were achieved, regardless the type of diet (Figure 2). Considering the influence of diet, even if a different trend in bodyweight gain was observed, no significant differences were observed in males or in females. Considering both males and females, the HFD groups showed the biggest increase in bodyweight gaining after 10 weeks of feeding, while the HFD+Y group was characterized by a lower value, however not significant, may be due to data dispersion that led to wide standard deviations that resulted in no significant differences among the three treatments, independently of the gender. The trend observed in bodyweight gaining was that the incorporation of yoghurt into the HFD pointed to a reduction in weight gain in both male and female mice, at the end of the feeding period.

Results obtained so far showed that males were more likely to gain weight than female mice, regardless the feeding group. This result is in agreement with Hwang et al. (2010) who reported that male mice were more susceptible to HFD-induced weight gain in terms of onset or magnitude, compared to female mice. Yang et al. (2014) reported significant differences in bodyweight gaining between male and female mice, feed HFD and low fat diet, earlier in males than in females, indicating that males may respond quicker to HFD than females. Similar results were obtained with studies conducted on rats where less incidence of obesity in female was mainly attributed to the effect of estrogen and estrogen receptor α (Gao et al., 2007). In addition, Ingvorsen, Karp, & Lelliott (2017) reported that sex has a significant impact on the onset of obesity in HFD C57BL/6N mice. They observed a sexual dimorphic effect as a significant modifier of the impact of HFD with males affected at a higher degree than females. In addition, the food intake observed in male and female groups, with few exceptions, resulted not significantly different throughout the whole feeding period. However, observing the bodyweight gain, males showed a significantly higher

241 weight gain than females starting from week 5 of feeding. This phenomenon could be a result of
242 the less-energy expenditure of male, as compared to the females on HFD with similar energy intake
243 correlated to the ovarian hormone estradiol, that can increase energy expenditure by regulating
244 physical activity (Ding et al., 2017). Other authors reported that differences in gross locomotor
245 activity in males and females may induce differences due to gender in the response to HFD (Benz
246 et al., 2012; Yang et al., 2014).
247 No significant differences in bodyweight gaining among the three diet groups were observed in
248 males or females. Literature data on mouse models concerning the supplementation of the diet with
249 conventional yogurt, added or not with probiotics or functional compounds are controversial
250 (Balcells et al., 2018; Chen et al., 2016; Park, Seong, & Lim, 2016). Balcells et al. (2018), in a trial
251 based on the administration of yoghurt or probiotic yogurt to mice in a model of obesity, observed
252 that after 2 months of probiotic yoghurt administration, smaller body weight was observed in the
253 obese group than in the control one, while animals that received conventional yoghurt did not show
254 differences respect to obese control mice. Park et al. (2016), studying the effects of milk fermented
255 by *Lactobacillus plantarum* Q180 on metabolic parameters of Sprague-Dawley rats fed HFD,
256 observed no significant differences between groups during 8 weeks of feeding. The variability of
257 the bodyweight gaining observed can be due to the high heterogeneity within each group. In fact,
258 the presence of mice of different age can also strongly affect the susceptibility to obesity that is
259 influenced by intrinsic and environmental factors (Schwartz et al., 2017). In addition, the mode of
260 administration of yoghurt can certainly influence its effect. In our study, yogurt was mixed with
261 the diet, avoiding causing any stress to animals due to oral administration by gavage, for example,
262 that represents one of the most used administration routes.
263
264 *3.2 Cholesterol and triglycerides*

At the end of the 10 weeks feeding period, animals were sacrificed, and blood and liver samples were taken for cholesterol and triglycerides analysis. In addition, the concentrations of pro-inflammatory (IL-6, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines were assessed in homogenates of the small and large intestines.

The log transformation of the values obtained was used, and a linear random effect model was used. The aim was to determine the impact of the diet and to test the hypothesis that HFD+Y would be able to low down triglycerides and cholesterol levels to control values, i.e., to test the experimental hypothesis that cholesterol and triglycerides of HFD+Y have no differences when compared to control, and that their levels are smaller than those found in animals fed with the HFD diet. Data obtained support the hypothesis that for males, levels of cholesterol and triglycerides, in both liver and blood serum, were the same for animals in the control and HFD+Y groups, and values in the HFD+Y group were lower than in the HFD group (Table 2). For female mice, the data support the hypothesis made only for triglycerides in the liver. Even if the hypothesis would be true, as a trend can be observed into that direction, differences were not significant may be due to the variability of data in females, i.e. the magnitude of the standard deviation compared to the mean.

It is well established that, in obesity, levels of triglycerides in blood serum is raised, due to the so-called "metabolic syndrome" (Cornier et al., 2008; Han & Lean, 2016). In addition, it was also reported that a regular consumption of dairy products such as yogurt or kefir, supplemented or not with probiotics, can reduce serum levels of cholesterol, LDL, HDL and triglycerides (Balcells et al., 2018; Kim, Jeong, et al., 2017; Kim, Kim, et al., 2017; Kobyliak et al., 2016). In this study, yoghurt administration induced a decrease in serum and liver cholesterol and triglycerides to values similar to those of the control group in a HFD given to male mice. In females, the same effect was observed only for serum triglycerides, but, as argued above, this can be due to the high variability

of results for females in all feeding groups. Female mice are markedly under-investigated in the biological and behavioural sciences due to the presumption that cyclic hormonal changes across the ovulatory cycle introduce excessive variability to the measures under consideration, compared to males (Smarr, Grant, Zucker, Prendergast, & Kriegsfeld, 2017).

3.3 Cytokines analysis

For cytokine analysis, the log transformation of data and a standard linear model were used too. After fitting the linear models, the desired contrasts were performed, in order to test the hypothesis that, pro-inflammatory cytokines (IL-6, IFN-γ and TNFα) in HFD+Y group < HFD group and, in the control group < HFD group. For IL-10, the hypothesis was that HFD+Y > HFD and HFD+Y > control. Boxplots showing results for all cytokines are depicted in Figures 3 and 4.

IFN-γ, and under certain circumstances IL-6, are cytokines with pro-inflammatory activity, and high levels may indicate a state of inflammation (Luo & Zheng, 2016). In special, IFN-γ can promote inflammation in fat tissue (Rocha et al., 2008). TNF-α is able to strongly promote and stimulate the immune system, playing a key role as an inflammatory mediator too. Usually, a high concentration of TNF-α correlates to several diseases and tissue damage (Cuffia et al., 2019; Lollo et al., 2013).

For male animals, and except for TNF-α in the large intestine, there was a significant reduction of the levels of the three pro-inflammatory cytokines measured in the HFD+Y group, compared to the HFD group. In female mice, again a high variability of values was observed, and significant differences were detected only for IFN-γ and IL-6 in the large intestine, again when HFD+Y was compared to the HFD. In the groups that received yoghurt, regardless the gender, lower levels of inflammation than in the HFD group were observed (Figure 3). A prevention of the increase of IL-

6 in plasma, associated to kefir consumption in male C57BL/6 mice, was reported by Kim, Jeong, et al. (2017). Other authors have reported a reduction in the patterns of pro-inflammatory cytokines, such as IL-6 and IFN-γ, following the regular consumption of probiotic microorganisms in murine models (Burns et al., 2017; Cuffia et al., 2019).

The main biological function of IL-10 is to control the inflammatory response (Febbraio, 2014). It may control the production of inflammatory mediators such as IL-1, IFN-γ, IL-4, IL-5 and TNF-α (Ropelle et al., 2010). In this study, the levels IL-10 in the small intestine of female and male mice were significantly reduced by the HFD, whereas HFD+Y restored this parameter to levels comparable to control mice, in the small and large intestine of all animals (Figure 4). In fact, the question whether the effects observed were due to the reduction in fat in the HFD-Y or due to the yoghurt addition itself to the HFD-Y is not answered by the experimental design of this study. The mechanism of action by which the reduction of pro-inflammatory cytokines occurred remains unknown. It may be due to the anti-inflammatory properties of yoghurt, to the reduction in the fat content of the HFD-Y, or by a mixed effect. The fact that anti-inflammatory properties of yogurt and their living lactic acid bacteria were demonstrated in the past, led us hypothesize that the effect was, may be partially, due to yoghurt, and may be not to, just, a reduction in fat.

3.4 Principal Components Analysis

In order to better understand the effects of the different diets on the parameters measured, different principal component analyses (PCA) were performed. Figure S1 displays the distribution of individuals on the factorial plane defined by PC1 and PC2, considering mice gender, and the directions pointed by the biological parameters measured. There was a remarkable separation of samples based on gender, regardless of the diet. The separation occurred along the main component

(PC1), which explains 77% of the variability of the data. Females were characterized by the highest concentrations of IL-10, while the analytical parameters that determined the clusterization of males were cholesterol, triglycerides and IL-6 in the large intestine, in opposite direction compared to females. These data distributions confirm the strong effect of gender on results, independently of the diet. Alex et al. (2009), reported that some cytokines such as IL-6 and IL-12 stratified gender-associated disease activity in chronic colitis in C57BL/6 murine models of DSS and TNBS-induced colitis.

When results were displayed in the factorial plane considering the different diets administered (Figure S2), individuals distributed in a way that mice from the control group overlapped with animals from the HFD+Y group, regardless of gender, suggesting a biological proximity among them, and at the same time separated also from individuals of the HFD group, that were located in the upper-left corner of the plane. Again, 77% of the variability of the data was explained by the first component of the PCA. When biological parameters were considered, the anti-inflammatory cytokine IL-10 pointed towards the location of the control and HFD+Y groups and in opposite direction to cholesterol, triglycerides and pro-inflammatory cytokines, that pointed to the place where individuals that received the HFD were located. The same distribution was observed for male mice when the PCA was conducted considering gender and treatment at the same time (Figure S3, left). However, for female mice (Figure S3, right), the clusterization was less clear. Finally, we would like to acknowledge some limitations of this study. When a complex food matrix (HFD) is replaced by another complex food matrix (yoghurt), the quality of macronutrients (proteins, carbohydrates, and lipids), for instance, may be different, even if the calories provided are the same. However, this is an inherent limitation that happens in studies of this kind, as for example in Lasket et al., 2019. Another fact that may look as a limitation is to replace part of the HFD by a food high

in water, like yoghurt. In this case, the animal will eat more, until energy requirement is satisfied, as the added food matrix contained mostly water.

4. Conclusions

In this work, the effect of a yoghurt enriched with proteins in a HFD was evaluated in mice, considering gender. Female mice, regardless of the diet, gained less weight when compared to male mice. The HDF+Y was able to reduce cholesterol and triglycerides, in liver and blood serum of males, but only triglycerides in the liver of female mice. For male animals, there was a significant reduction of the levels of the three pro-inflammatory cytokines measured when animals were fed the HFD+Y group. However, in female mice, the anti-inflammatory effect was observed but to a less extension. The anti-inflammatory effect was confirmed by the increased IL-10 levels observed. The PCA confirmed that gender or diet was able to group individual animals. IL-10 pointed to control and HFD+Y individuals, that also overlapped among them, whereas cholesterol, triglycerides and pro-inflammatory cytokines pointed towards HFD individuals. Higher variability of data was observed in females compared to male mice, being this probably the reason why less significant differences were observed in the former.

Acknowledgements

This research was funded by the project PUE-CONICET "Desarrollo de una bebida láctea fermentada multifuncional para contribuir a las estrategias contra la globesidad", N° 22920160100023, Res. D4119/2016. Lorenzo Siroli received a 12-months posdoctoral grant by the Universidad Nacional del Litoral (2018).

Author contributions

LS: Investigation (animal feeding, tissue processing, cytokine analysis), PB: Investigation (animal sacrifice and tissue processing), FB: Investigation (yoghurt preparation), MP: Investigation (cytokine analysis, cholesterol and triglycerides), SD: Conceptualization, LF: Formal statistical analysis, MED: Conceptualization, JR: Funding acquisition, CP: Methodology and yoghurt preparation, GV: Conceptualization and writing of the original draft.

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Figure caption

Figure 1 – Consumption of energy (accumulated kcals) along 10 weeks of feeding. Columns with different top letter, for the same feeding period, are significantly different ($p < 0.05$).

Figure 2 - Food efficiency ratio (FER) of mice fed for 10 weeks. FER = Weight gain (g)/Food intake (g) × 100 and Body weight gain (g) at the end of the 10 weeks feeding period. Columns displaying different top letter are significantly different ($p < 0.05$).

Figure 3 - Boxplots showing the concentration of TNF-α, IFN-γ and IL-6 in homogenates of the small (SI) and large (LI) intestine of female and male mice, fed with Control (■), High Fat Diet (HFD, ■) or High Fat Diet plus Yoghurt (HFD+Y, ■) diet. p-values (Dunnet test) are shown between groups where significant differences were observed.

Figure 4 - Boxplots showing the concentration of IL-10 in homogenates of the small (SI) and large (LI) intestine of female and male mice, fed with Control (■), High Fat Diet (HFD, ■) or High Fat Diet plus Yoghurt (HFD+Y, ■) diet. p-values (Dunnet test) are shown between groups where significant differences were observed.

Table 1 - Composition of the control and the high-fat diet.

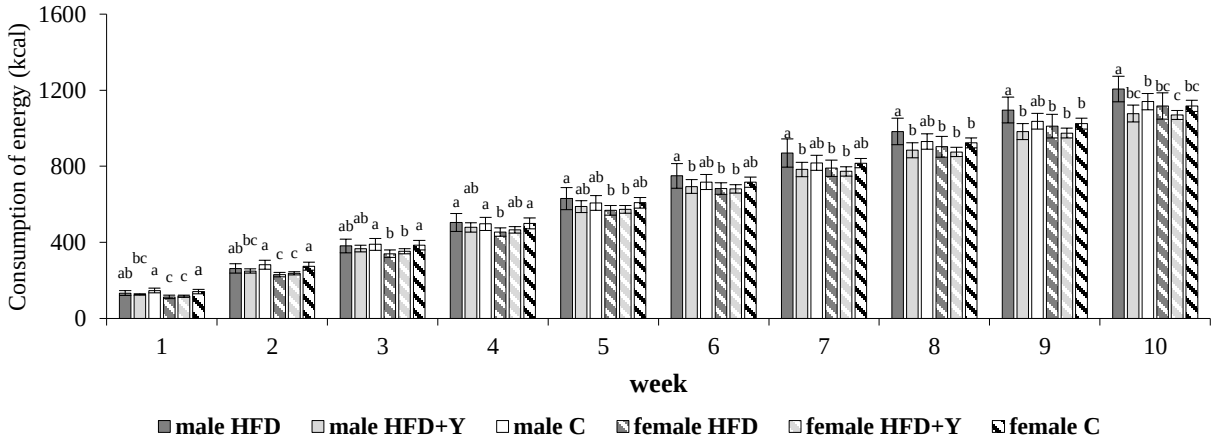
Ingredient	Diet composition (g kg^{-1})	
	Control	High-fat
Casein	140.0	258.5
L-Cystine	1.8	3.9
Corn starch	495.7	-
Maltodextrin	125.0	161.5
Sucrose	100.0	88.9
Cellulose	50.0	64.6
Soybean oil	40.0	32.3
Lard	-	316.6
DiCalcium Phosphate	-	16.8
Calcium Carbonate	-	7.1
Potassium Citrate	-	21.3
Choline bitartrate	2.5	2.6
Mineral mix S10022M	35.0	-
Mineral mix S10026	-	12.9
Vitamin mix V10037	10.0	-
Vitamin mix V10001	-	12.9
Energy (kcal/kg)	3810.0	5191.3

Table 2 - Total cholesterol and triglycerides in blood serum and liver. For each gender, values in columns with different superscript are significant different ($p < 0.05$, Dunnet test, compared to control).

		Triglycerides (mg dL^{-1} ± SD)		Cholesterol (mg dL^{-1} ± SD)	
Gender	Group	Liver	Serum	Liver	Serum
Male	HFD	$332±36^{b}$	$106±27^{b}$	$60±9^{b}$	$75±5^{b}$
	HFD+Y	$274±35^{a}$	$77±6^{a}$	$39±8^{a}$	$54±7^{a}$
	C	$266±40^{a}$	$83±6^{a}$	$46±4^{a}$	$60±8^{a}$
Female	HFD	$193±41^{b}$	$74±26^{a}$	$35±11^{a}$	$59±9^{b}$
	HFD+Y	$160±30^{a}$	$49±13^{a}$	$35±10^{a}$	$42±8^{b}$
	C	$146±19^{a}$	$60±19^{a}$	$33±8^{a}$	$33±8^{a}$

Figure

1



2

3 **Figure 1**

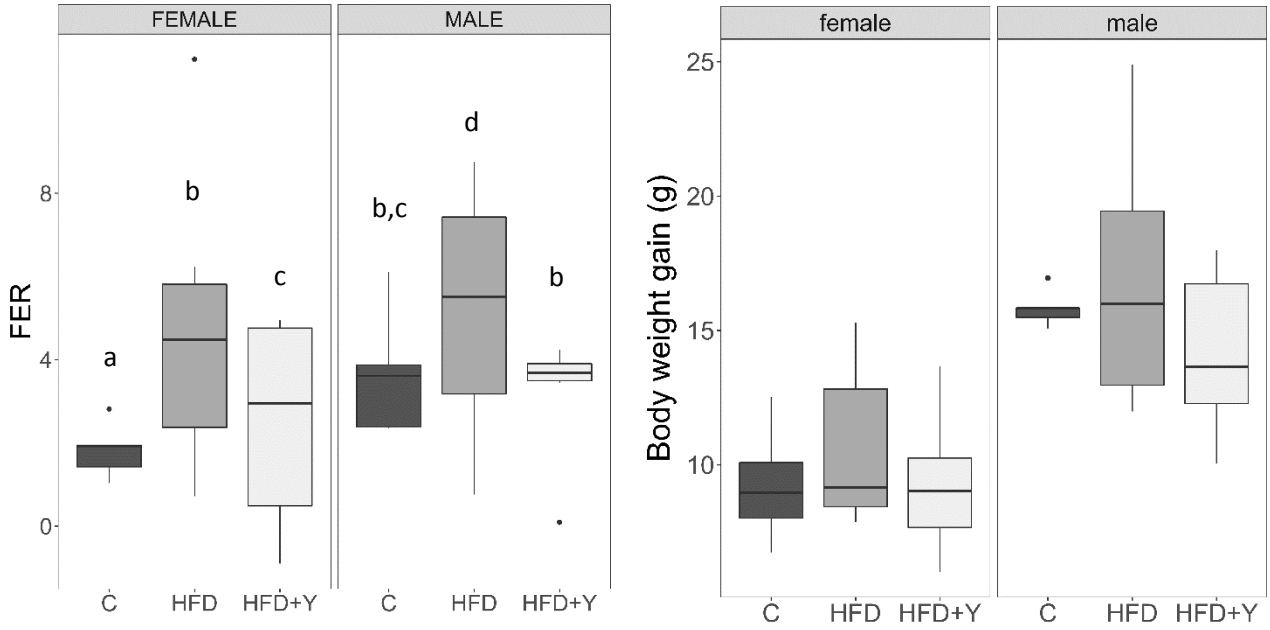

FEMALE
MALE
FER
8
4
0
a
b
c
b,c
d
b
C
HFD
HFD+Y
female
male
Body weight gain (g)
25
20
15
10

Figure 2

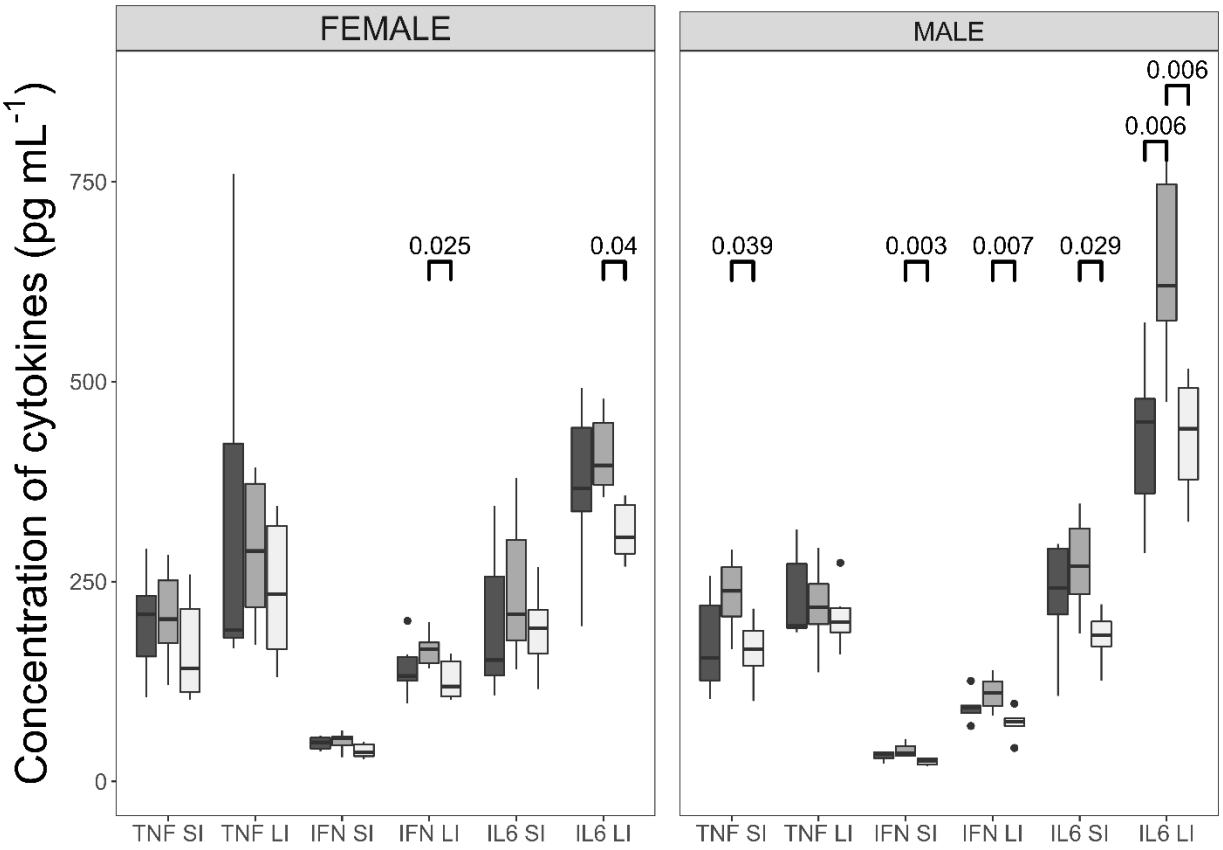

FEMALE
MALE
Concentration of cytokines (pg mL-1)
750
500
250
0
0.025
0.04
0.039
0.003
0.007
0.029
0.006
0.006
TNF SI
TNF LI
IFN SI
IFN LI
IL6 SI
IL6 LI

Figure 3

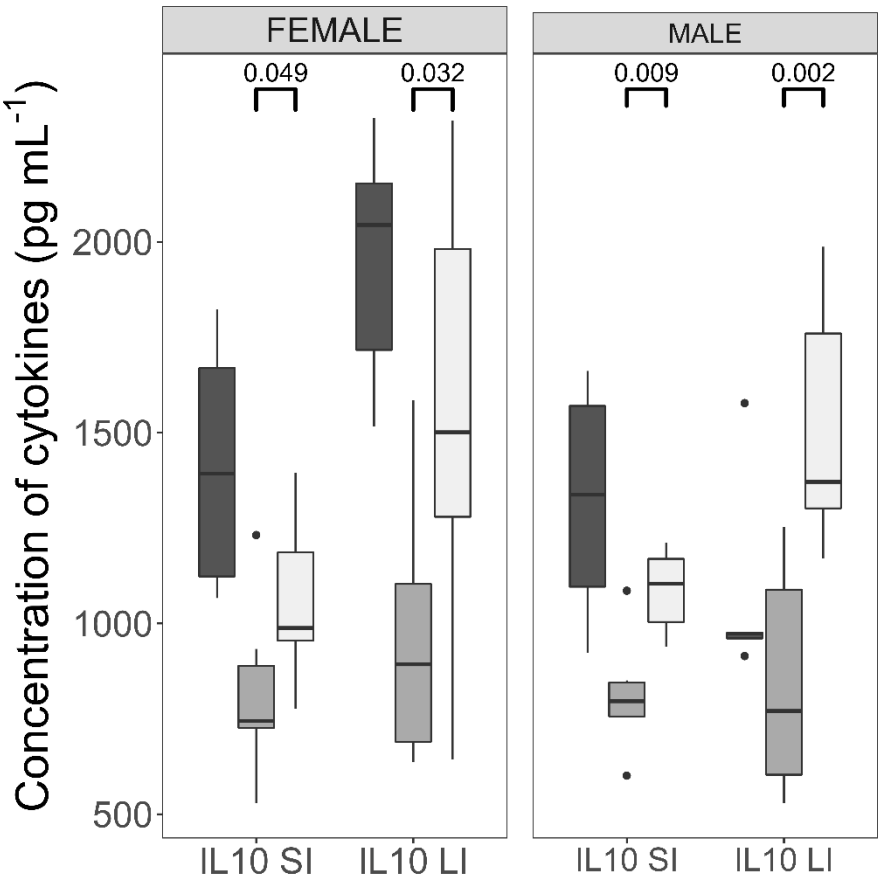

FEMALE
MALE
0.049
0.032
0.009
0.002
Concentration of cytokines (pg mL-1)
2000
1500
1000
500
IL10 SI
IL10 LI
IL10 SI
IL10 LI

Figure 4

Figure

Supplementary material

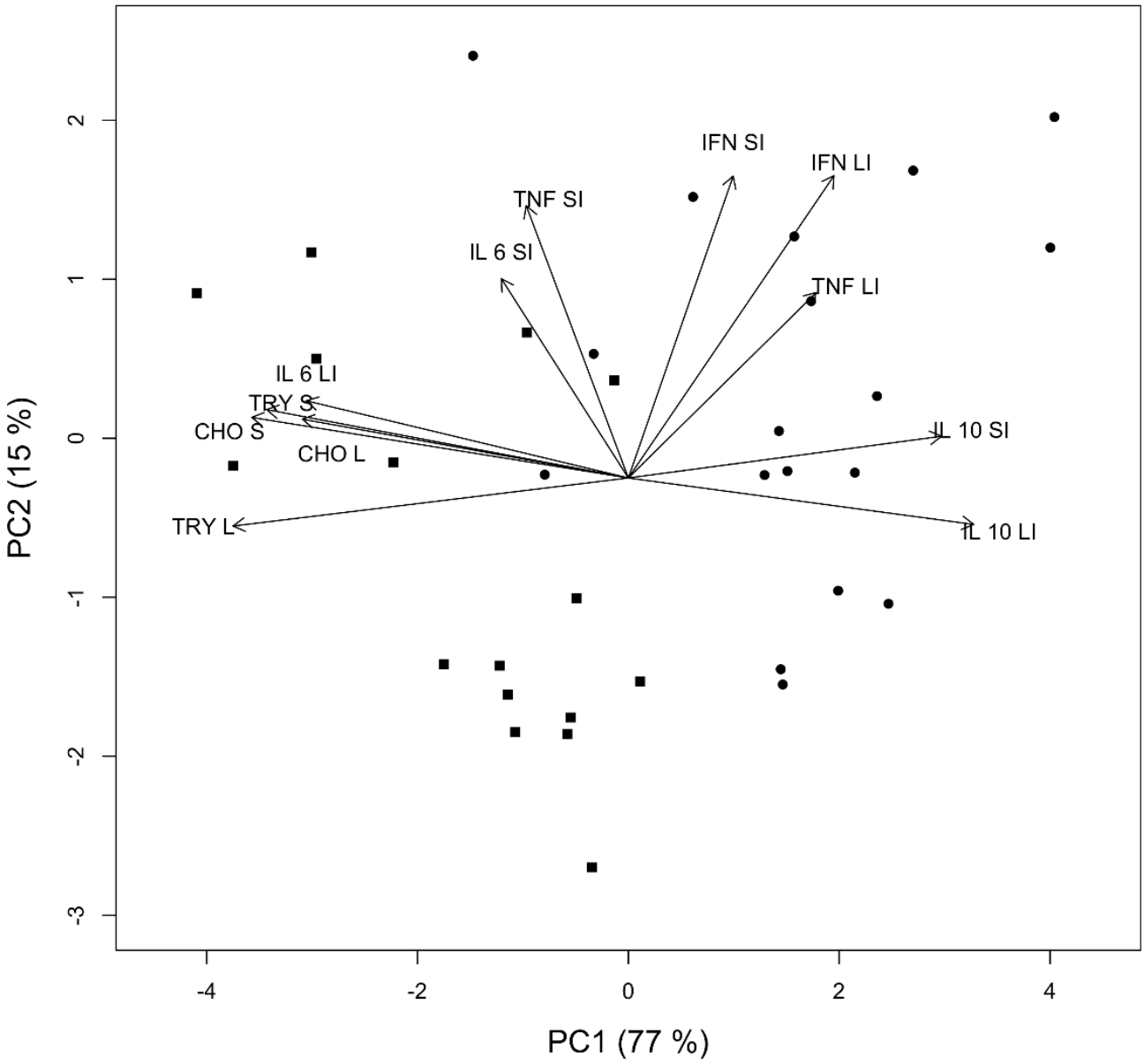


Figure S1 - Projection of the variables measured in individual female (●) and male (■) mice. TRY: triglycerides, CHO: cholesterol, L: liver, S: blood serum, SI: small intestine, LI: large intestine.

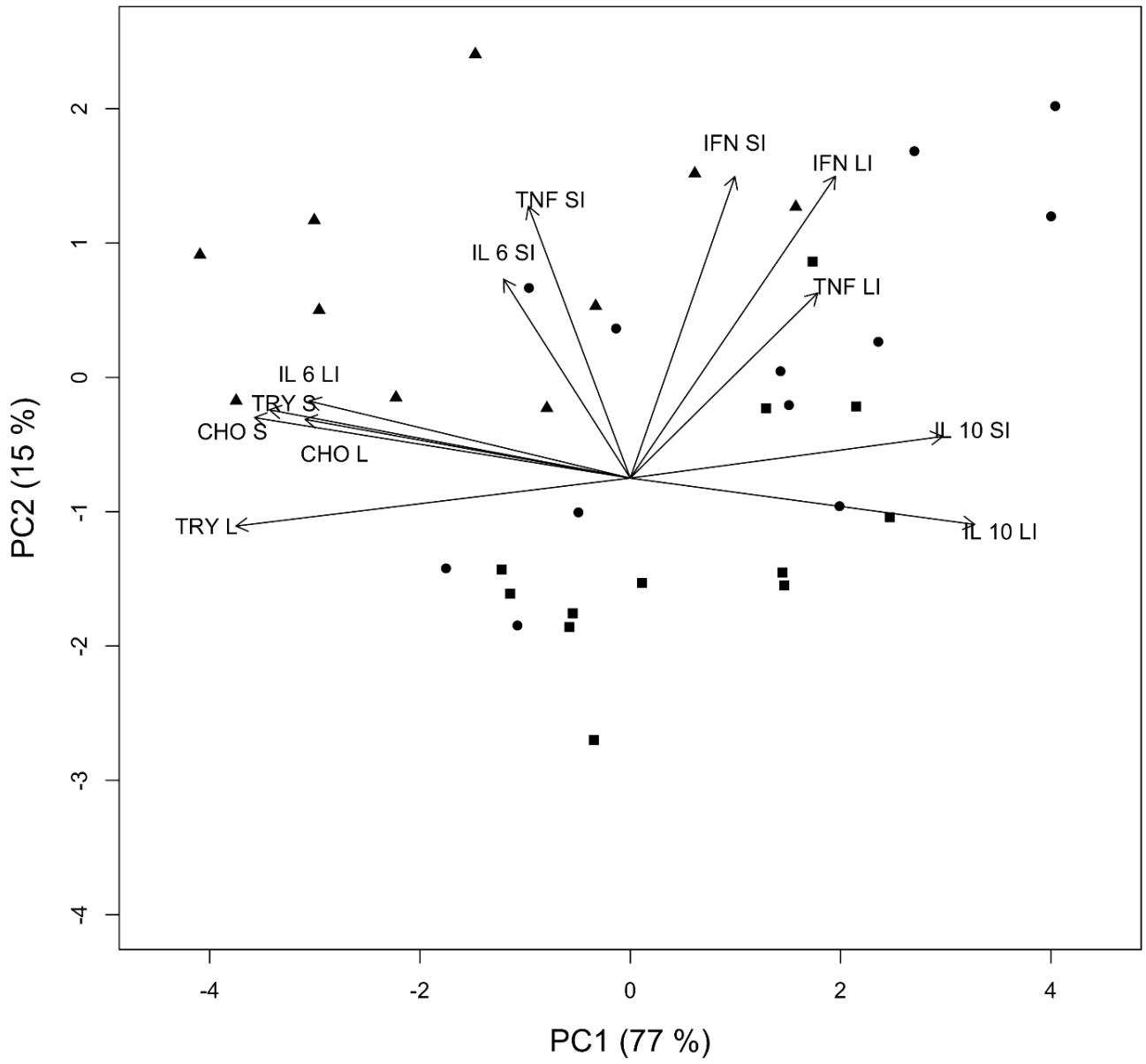


Figure S2 - Projection of the variables measured in individual mice fed with control (●), HFD (▲) or HFD+Y (■) diet. TRY: triglycerides, CHO: cholesterol, L: liver, S: blood serum, SI: small intestine, LI: large intestine.

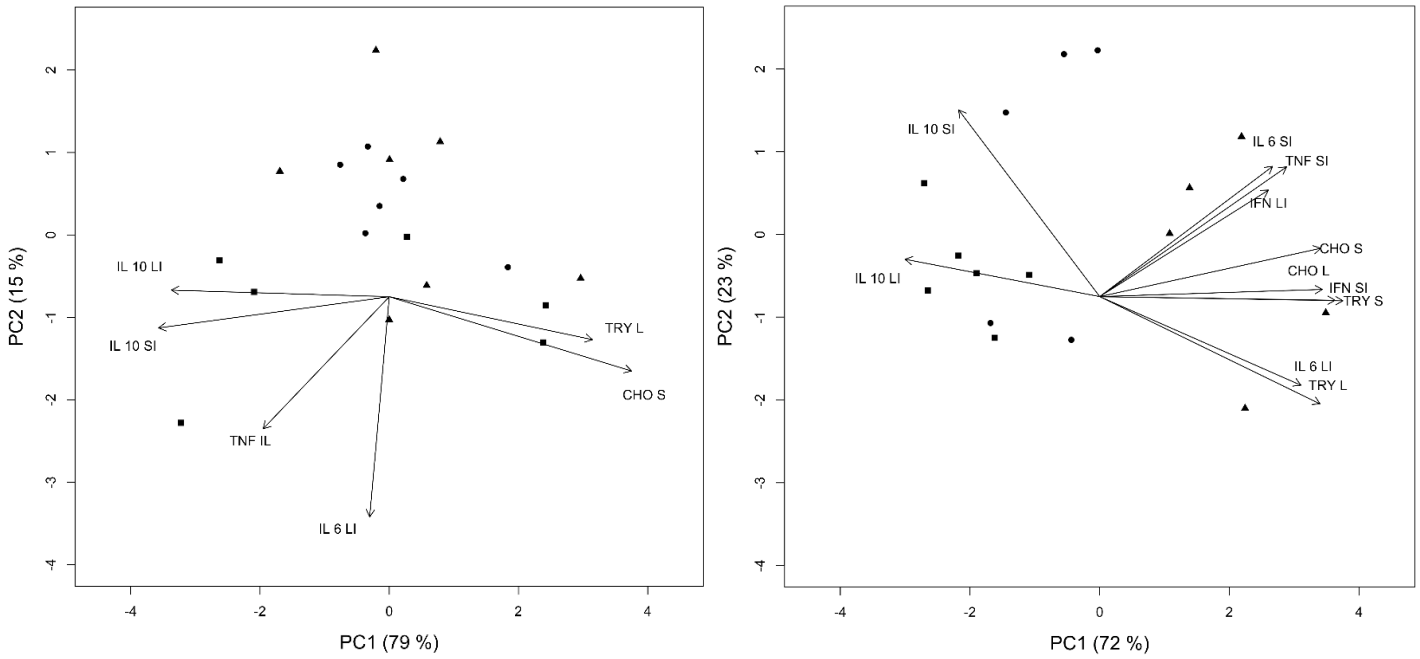


Figure S3 - Projection of the variables measured in individual female (left) or male (right) mice fed with control (●), HFD (▲) or HFD+Y (■) diet. TRY: triglycerides, CHO: cholesterol, L: liver, S: blood serum, SI: small intestine, LI: large intestine.