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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Siroli, L., Burns, P., Borgo, F., Puntillo, M., Drago, S., Forzani, L., et al. (2021). Sex-dependent effects of a yoghurt enriched with proteins in a mouse model of diet-induced obesity. *INTERNATIONAL DAIRY JOURNAL*, 114, 1-8 [10.1016/j.idairyj.2020.104914].

Availability:

This version is available at: <https://hdl.handle.net/11585/783360> since: 2022-11-14

Published:

DOI: <http://doi.org/10.1016/j.idairyj.2020.104914>

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(Article begins on next page)

International Dairy Journal

Sex-dependant effects of a yoghurt enriched with proteins in a mouse model of diet-induced obesity --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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Abstract:	<p>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.</p>

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>

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27

28 **ABSTRACT**

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

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

42

43 **1. Introduction**

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

49 is generally linked to a greater consumption of energy-dense foods, high in fats and sugars,
50 associated to a reduction in physical activity and fiber intake (Hruby & Hu, 2015), but the early
51 role of intestinal microbiota establishment is also being considered (Dao & Clément, 2018).
52 Obesity can be associated to different causing factors including genetic, environmental (C-section,
53 reduced breast-feeding, antibiotics consumption), or neural hormonal functions among others
54 (Nguyen & El-Serag, 2010; Williams, Mesidor, Winters, Dubbert, & Wyatt, 2015). The
55 consequences of obesity are linked to multiple health problems such as cardiovascular diseases,
56 diabetes, musculoskeletal disorders (especially osteoarthritis) and some cancers (including
57 endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon) (Must & McKeown,
58 2000; WHO, 2019).

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

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

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

87

88 **2. Material and methods**

89 *2.1 Yogurt preparation*

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

97 Milk base (500 mL) for yoghurt manufacture was formulated mixing standardized milk, skim milk
98 powder (3.0 % w/v) and WPC40 (2.0 % w/v), which was kept overnight at 5 °C for proper hydration
99 of powder ingredients. The milk base was heat-treated at 85 °C for 20 min and cooled down to 42
100 °C. The starter culture was then inoculated to the milk base, according to the manufacturer's
101 instructions. The fermentation process was conducted at 42 ± 1 °C in a water bath until pH $4.75 \pm$
102 0.05 . Yoghurts were immediately cooled down to room temperature and then stored at 5 °C.
103 Yoghurt was produced every 2 weeks for animal feeding. Gross composition (mean \pm standard
104 deviation) of yoghurts after 7 days of storage at 4 °C was: pH, 4.56 ± 0.10 ; total solids, $14.40 \pm$
105 0.45% , protein, $4.98 \pm 0.25\%$, and fat, $1.60 \pm 0.15\%$.

106

107 *2.2 In vivo trial*

108 *2.2.1 Animals*

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

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

120

121 2.2.2 *Composition and preparation of the diet*

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

129

130 2.2.3 *Experimental design*

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

138

139 2.2.4 *Sacrifice and tissue sampling*

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

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

150

151 *2.2.5 Cholesterol and triglycerides determination in blood serum and liver.*

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

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

160

161 *2.2.6 Cytokines determination*

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

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

171

172 *2.3 Statistical analysis*

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

182

183 **3. Results and Discussion**

184 *3.1 Weight and bodyweight gain*

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

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

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

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

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

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

228 Results obtained so far showed that males were more likely to gain weight than female mice,
229 regardless the feeding group. This result is in agreement with Hwang et al. (2010) who reported
230 that male mice were more susceptible to HFD-induced weight gain in terms of onset or magnitude,
231 compared to female mice. Yang et al. (2014) reported significant differences in bodyweight gaining
232 between male and female mice, feed HFD and low fat diet, earlier in males than in females,
233 indicating that males may respond quicker to HFD than females. Similar results were obtained with
234 studies conducted on rats where less incidence of obesity in female was mainly attributed to the
235 effect of estrogen and estrogen receptor α (Gao et al., 2007). In addition, Ingvorsen, Karp, &
236 Lelliott (2017) reported that sex has a significant impact on the onset of obesity in HFD C57BL/6N
237 mice. They observed a sexual dimorphic effect as a significant modifier of the impact of HFD with
238 males affected at a higher degree than females. In addition, the food intake observed in male and
239 female groups, with few exceptions, resulted not significantly different throughout the whole
240 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*

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

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

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

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

293

294 *3.3 Cytokines analysis*

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

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

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

312 6 in plasma, associated to kefir consumption in male C57BL/6 mice, was reported by Kim, Jeong,
313 et al. (2017). Other authors have reported a reduction in the patterns of pro-inflammatory cytokines,
314 such as IL-6 and IFN- γ , following the regular consumption of probiotic microorganisms in murine
315 models (Burns et al., 2017; Cuffia et al., 2019).

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

328

329 *3.4 Principal Components Analysis*

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

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

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

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

360

361 **4. Conclusions**

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

374

375 **Acknowledgements**

376 This research was funded by the project PUE-CONICET “Desarrollo de una bebida láctea
377 fermentada multifuncional para contribuir a las estrategias contra la globesidad”, N°
378 22920160100023, Res. D4119/2016. Lorenzo Siroli received a 12-months postdoctoral grant by the
379 Universidad Nacional del Litoral (2018).

380

381 **Author contributions**

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

387

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557 **Figure caption**

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

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

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

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

571

1 **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

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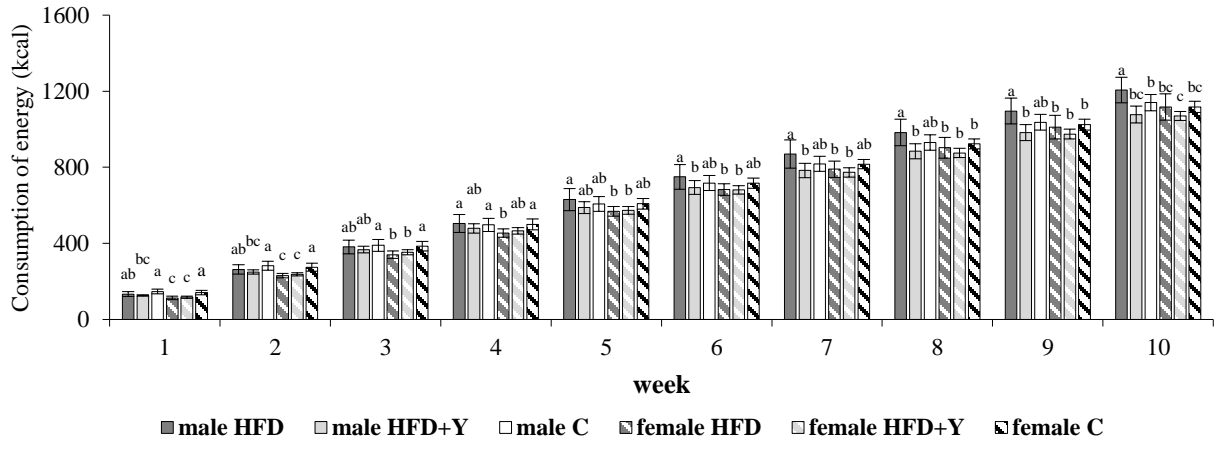
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4 **Table 2** - Total cholesterol and triglycerides in blood serum and liver. For each gender, values in
 5 columns with different superscript are significant different ($p < 0.05$, Dunnet test, compared to
 6 control).

Gender	Group	Triglycerides (mg dL ⁻¹ ± SD)		Cholesterol (mg dL ⁻¹ ± SD)	
		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

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3 **Figure 1**

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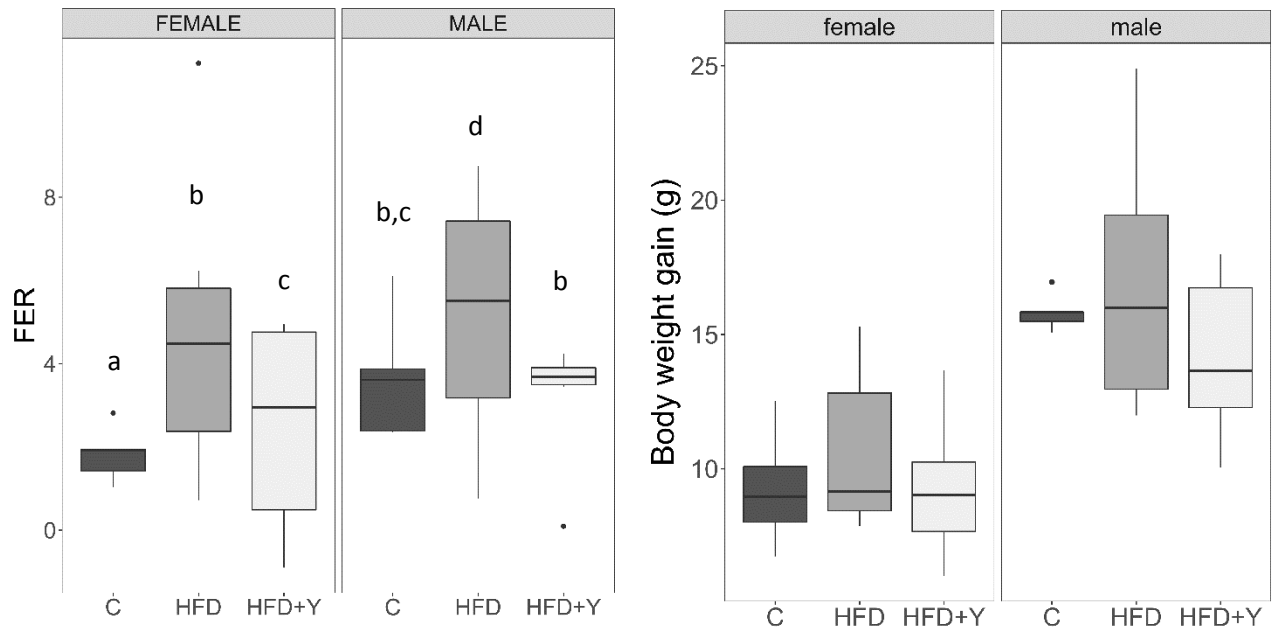
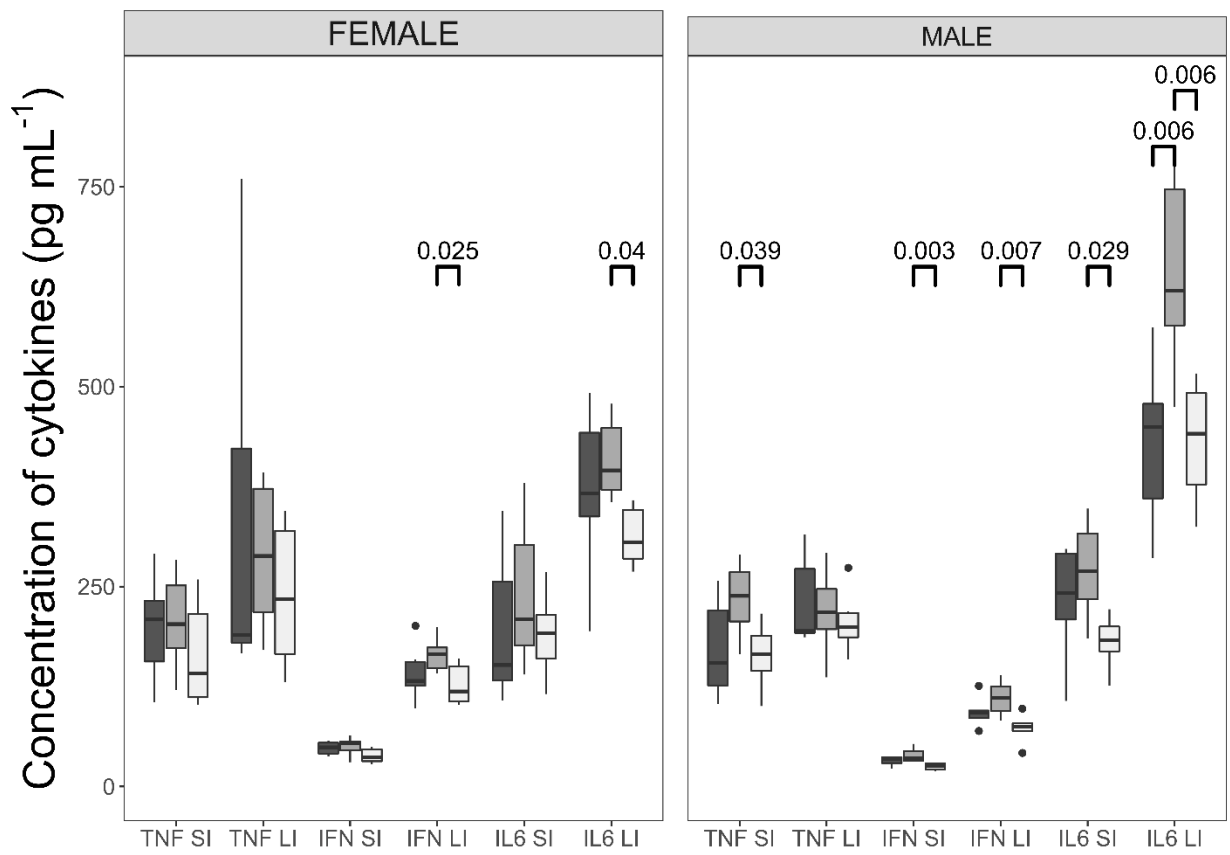


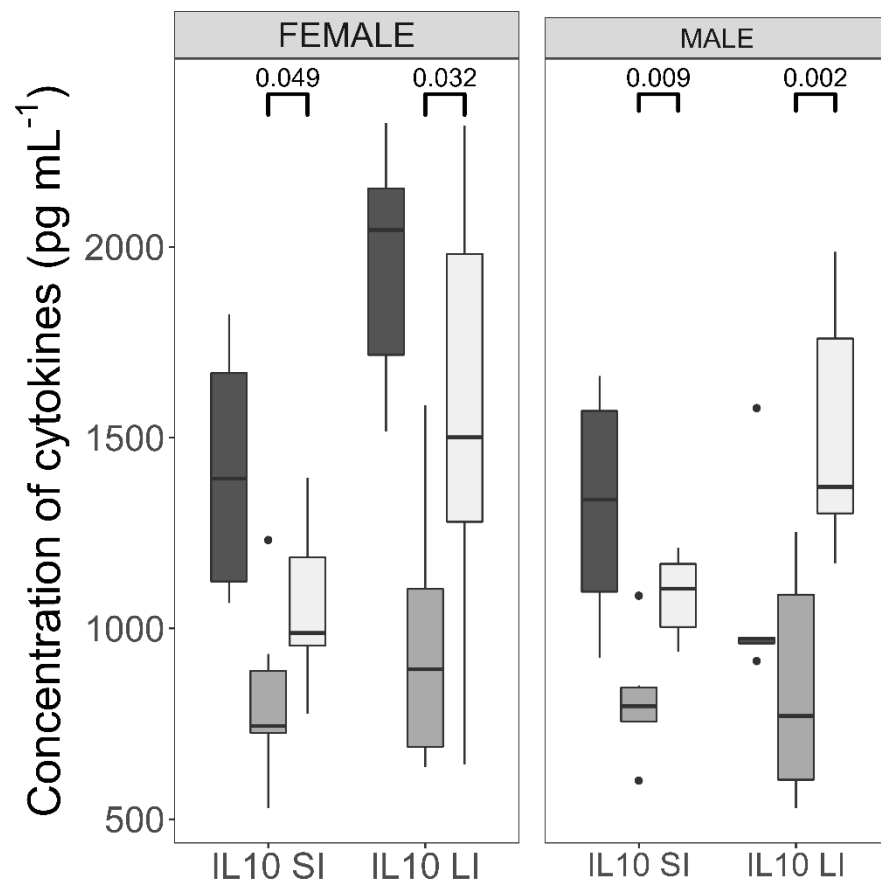
Figure 2



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19 **Figure 3**

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23 **Figure 4**

1 **Supplementary material**

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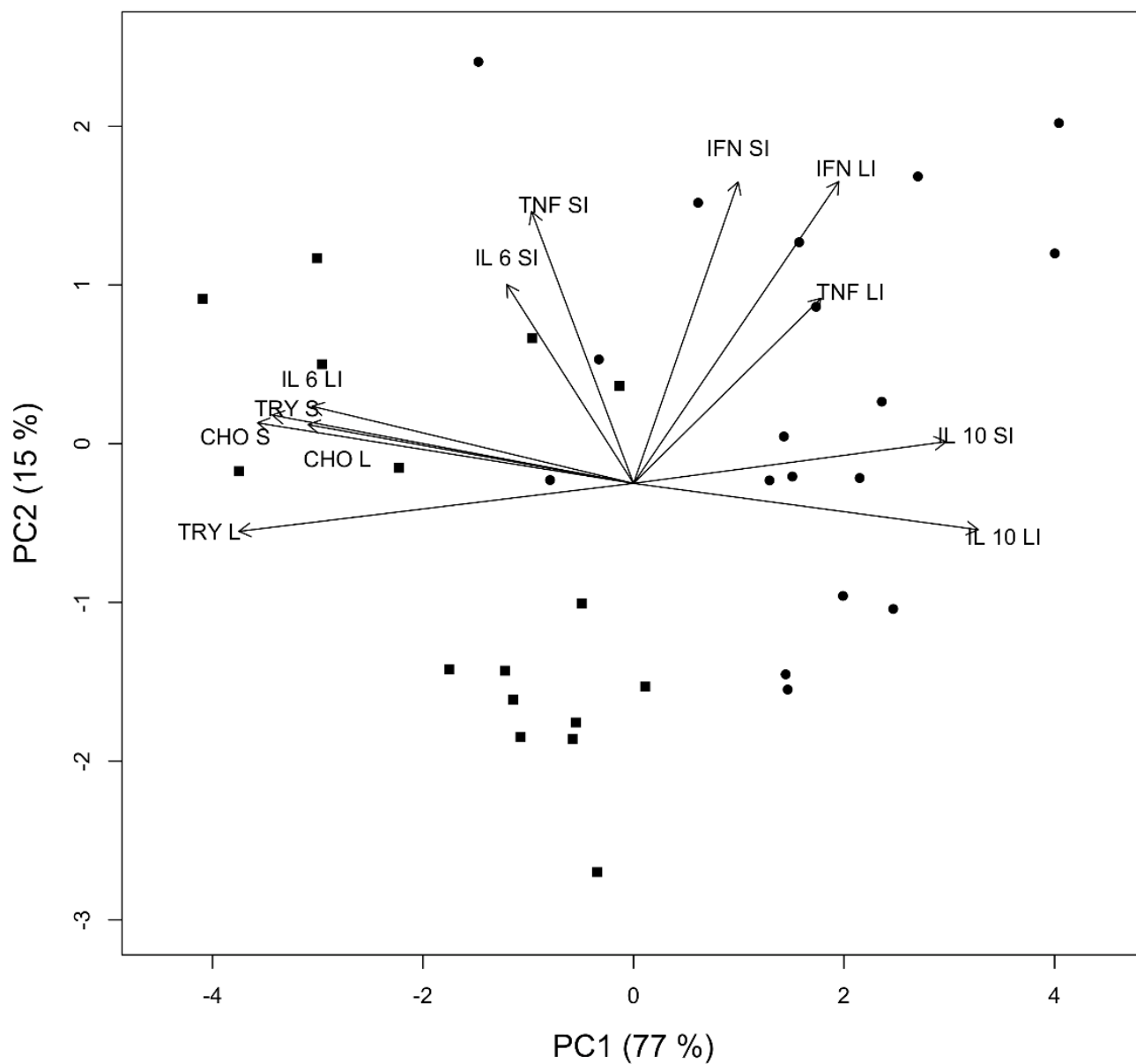
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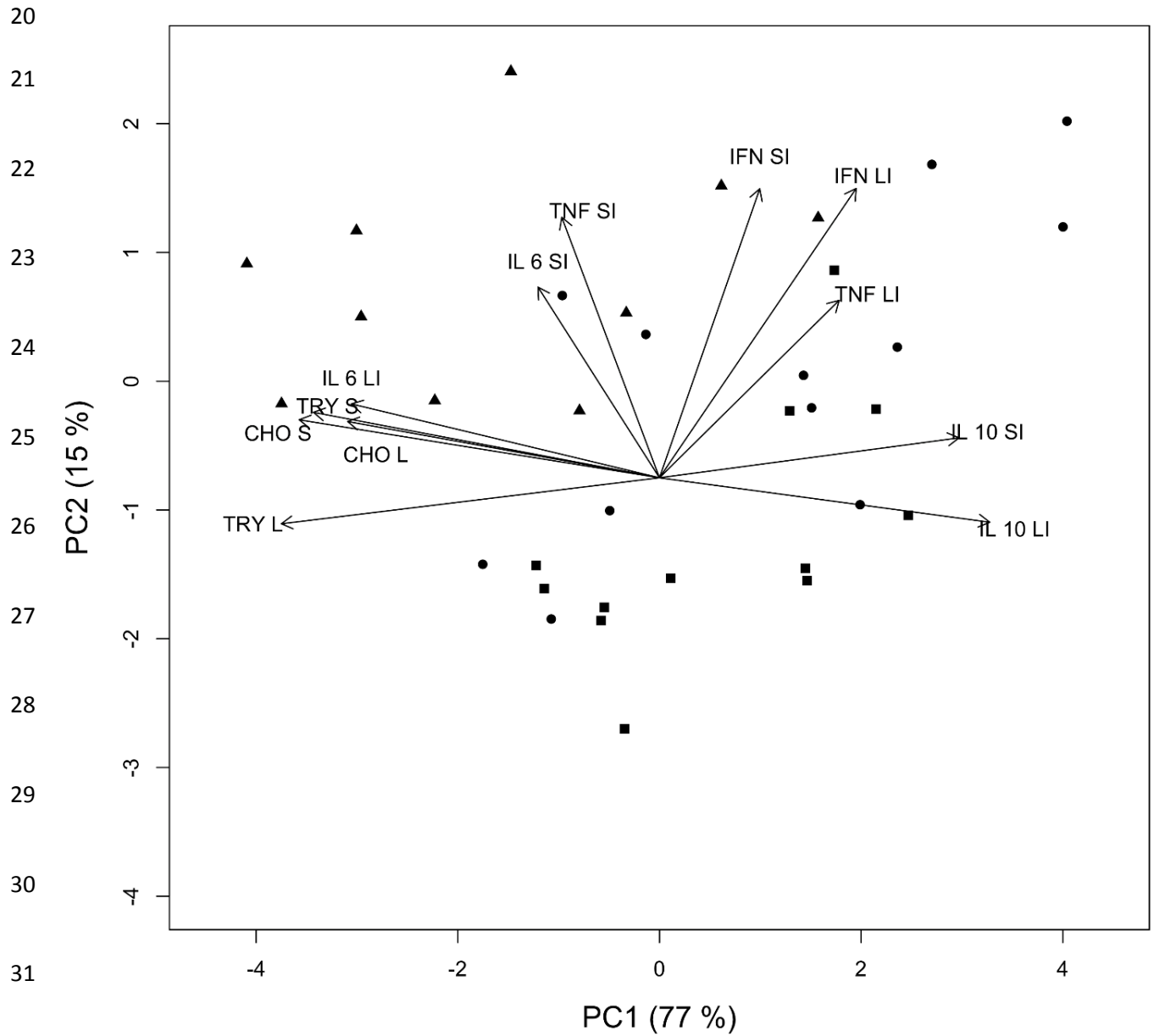


16 **Figure S1** - Projection of the variables measured in individual female (●) and male (■) mice. TRY:

17 triglycerides, CHO: cholesterol, L: liver, S: blood serum, SI: small intestine, LI: large intestine.

18

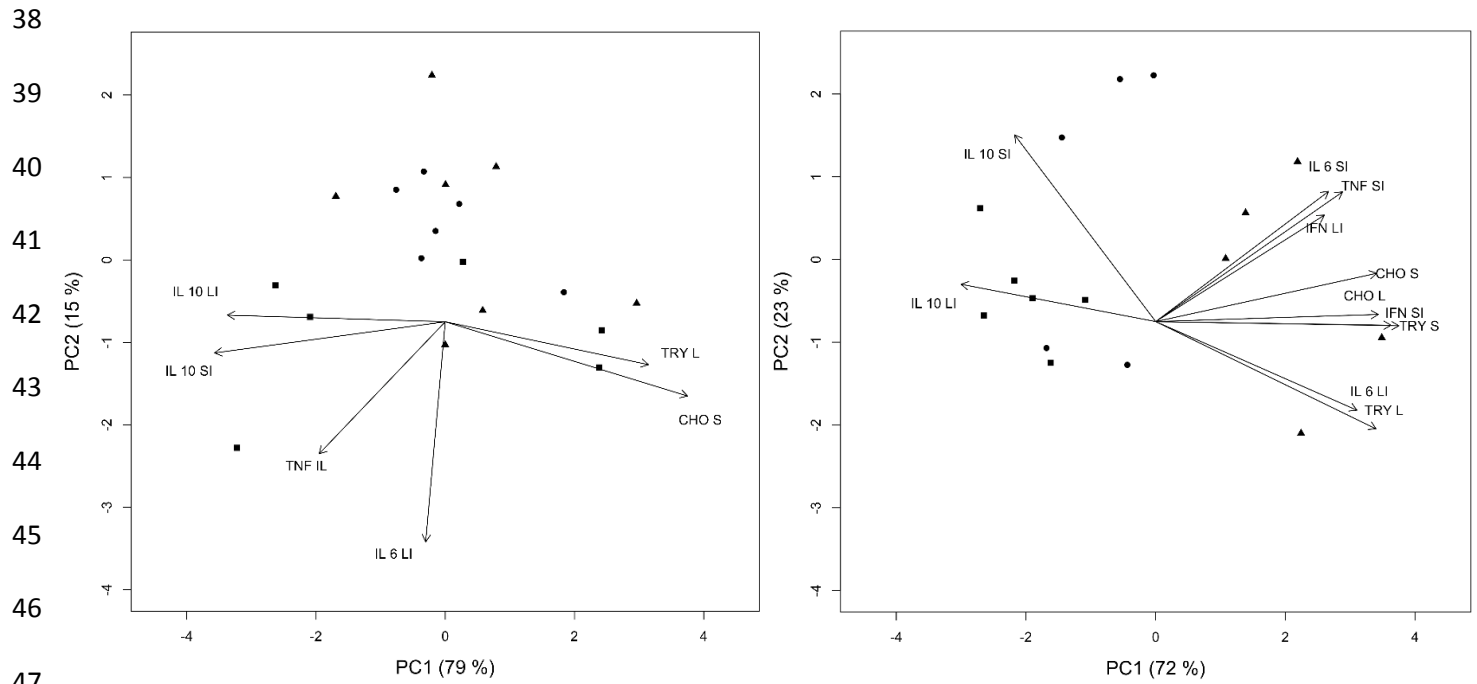
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33 **Figure S2** - Projection of the variables measured in individual mice fed with control (●), HFD (▲)
 34 or HFD+Y (■) diet. TRY: triglycerides, CHO: cholesterol, L: liver, S: blood serum, SI: small
 35 intestine, LI: large intestine.

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