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1 **Chicory root flour - a functional food with potential multiple health benefits evaluated**  
2 **in a mice model**

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22 **Abstract**

23 **Industrial chicory roots** (*Cichorium intybus* var. *sativum*) have been the subject of many  
24 different studies investigating their supposed nutritional and medicinal benefits. Although an  
25 impressive number of clinical evidences has supported the health effects of this plant, the  
26 molecular mechanisms remain poorly understood. In this work, we looked at chicory flour  
27 that is proposed as an ingredient for pastries **and a water extract of chicory root flour was**  
28 **tested**. We experimented on the murine model and highlighted new mechanisms by which the  
29 chicory roots brings health benefits. For this purpose, a nutrigenomic analysis was associated  
30 with a metabolic hormone dosage and a metagenomic analysis. New **potential** mechanisms by  
31 which the chicory acts on cancer prevention on antibacterial and antiviral defense, on  
32 hypoglycemic and hypolipidemic effect as well as on the antioxidant effect were identified.  
33 Also, the prebiotic effect of the chicory **root flour** was discussed by comparison with inulin. In  
34 general, almost all effects were found to be more pronounced in male mice than in female  
35 mice.

36

37 **Keywords:** *Cichorium intybus*, **chicory root flour**, cancer protection, antimicrobial protection,  
38 metabolic regulation, prebiotics

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## 45 1. INTRODUCTION

46 Chicory (*Cichorium intybus* L.) is mostly known for its multiple uses in the food industry and  
47 herbal medicine. This plant has long been consumed as a vegetable by humans. Leaves and  
48 root have been used for thousands of years for nutritional purposes (Puhlmann and de Vos,  
49 2020) and since the end of the 16th century and the reign of Napoleon, roasted roots have  
50 been used as a substitute to coffee beans (Lucchin *et al.*, 2008).

51 Chicory roots are rich in fiber and bioactive compounds. Inulin is the main storage  
52 carbohydrate consisting of fructose molecules whose linkage cannot be broken down by  
53 human intestinal enzymes and was classified as dietary fiber. Nowadays, chicory inulin is  
54 used as food ingredient for its gelling, texturing and stabilizing properties (Arkel *et al.*, 2012),  
55 acting as a fat (Keenan *et al.*, 2014) or sugar substitute (Gonzalez Thomas *et al.*, 2009).  
56 Chicory inulin was also defined as a prebiotic because this substrate is selectively used by the  
57 gut microorganisms and confers a health benefit (Gibson *et al.*, 2017). This benefit includes  
58 the stimulatory effect mainly on bifidobacteria and the increase in short-chain fatty acids  
59 (SCFA) production (Le Bastard *et al.*, 2020).

60 Chicory was already cultivated as a medicinal plant by the ancient Egyptians and is still used  
61 in traditional dishes in various parts of the world (Puhlmann and de Vos, 2020). Liv-52 and  
62 Jigrine, two traditional Indian formulations containing parts of this plant are largely used for  
63 their hepatoprotective effects (Huseini *et al.*, 2005). In addition to being used as a tonic in the  
64 form of syrup, chicory roots are conventionally taken to relieve different digestive disorders  
65 (Judzentiene and Budiene, 2008). The whole plant is also known for its hypoglycemic  
66 (Roberfroid *et al.*, 1998) and hypolipidemic properties, hypothetically due to the chlorogenic  
67 acids (CGA) content of chicory (Jackson *et al.*, 2017). Extracts of chicory roots have also

68 been stated to have antimicrobial (Liu *et al.*, 2013), immunomodulatory (Amirghofran *et al.*,  
69 2000), anti-inflammatory (Lee *et al.*, 2015), and anti-cancer properties (Behboodi *et al.*,  
70 2019). Historically used in Afghanistan as a remedy for malarial fevers, chicory has proven to  
71 be a promising antimalarial agent, due to its bitter compounds such as lactucin, lactucopicrin,  
72 8-deoxylactucin and their derivatives (Bischoff *et al.*, 2004). Due to its content in  
73 sesquiterpene lactones (STL), chicory presents also anthelmintic activity in animals (Jensen *et*  
74 *al.*, 2011).

75 In recent years, functional foods have developed as part of health and wellness programs  
76 because they are highly nutritious and associated with several powerful health benefits at the  
77 same time. Inulin was adopted as a functional ingredient and various studies evaluated its  
78 efficacy as prebiotic, for promoting good digestive health, for influencing lipid metabolism  
79 and for its beneficial roles in optimum plasmatic levels of glucose and insulin maintaining  
80 (Ahmed and Rashid, 2017).

81 As chicory roots contain inulin but also many other bioactive compounds, we considered the  
82 whole root as a potential functional food and investigated the molecular mechanisms of its  
83 effects on health. To distinguish the effect of the complete food matrix from that of inulin, a  
84 murine model was used and a force-feeding was performed with chicory root flour or inulin.  
85 The distinct effects in males and females were also examined. We analyzed changes in gene  
86 expression profiles, in plasmatic metabolism marker levels and also in gut microbiota  
87 composition, in order to correlate the different observations with respective effects on health.

88

## 89 **2. MATERIALS AND METHODS**

### 90 **2.1. Chicory flour production and composition**

91 Two industrial chicory genotypes (*Cichorium intybus* var. *sativum*) were used to produce  
92 fresh chicory roots. These genotypes named G12 and G35 were selected by Florimond-  
93 Desprez Veuve et Fils SAS (Capelle-en-Pévèle, France). Roots were processed by Leroux  
94 SAS (Orchies, France) to provide corresponding flour. Samples were freeze-dried (48 h) and  
95 ground (ball mill, Retsch, Eragny sur Oise, France) for 30 min at room temperature using  
96 50 mL wells and 25 mm balls to obtain a fine powder. Flour composition of both G12 and  
97 G35 chicory genotypes was identical with root composition and it is indicated in Table 1 as  
98 previously quantified (Fouré et al., 2018). Mice were fed with an aqueous decoction of the  
99 dried powders from the two selected genotypes (G12 and G35). Hundred milliliters of warm  
100 water (85°C) were added to a 150 mL glass tube containing 3 grams of powder. The mixture  
101 was incubated for 20 min at 80°C under weak agitation. The solution was filtered through a  
102 coffee filter, filtrates were centrifuged at 30 000 g for 10 min at 4°C and the supernatants  
103 were store at – 80°C until feeding. The concentration of the decoctions was normalized to 20  
104 ± 0.5 mg/ml and their composition was assessed for CGA and STL levels by LC-UV analyses  
105 performed according to Willeman et al. (2014). The composition of both decoctions was  
106 considered stable for these compounds, which are fully or partially soluble in water (Table 2).  
107 Nevertheless, nonpolar and hydrophobic compounds will not be present in the resulting  
108 aqueous extract, and will remain in the sediment not diluted in water. Inulin water solution  
109 was prepared with 2 g inulin (Inulin from chicory, Sigma-Aldrich) for 150 ml warm water  
110 (85°C), incubated for 20 min at 80°C under weak agitation and filtered as flour decoctions.

## 111 2.2. Animal experiments and ethics statements

112 Male and female BALB/c BYj mice were used for experiments in agreement with Directive  
113 2010/63/ EEC for the protection of animals used for fine scientists and in accordance with  
114 Law 2012-10 (2012) and 2013-118 (2013). The protocol was approved by the Ethics  
115 Committee for the experiment on animals. The mice were randomly divided into groups (n =

116 6/group) and housed in a controlled environment (with a temperature of 22 °C, a 12 h/12 h  
117 light/dark cycle and *ad libitum* access to standardized food and water). Mice were fed with an  
118 aqueous decoction of roots flour from two selected chicory genotypes (G12 and G35) and  
119 with an inulin solution (I2255 Sigma Aldrich, MO, USA). The mice gavages consisted in a  
120 daily force-feeding of 500 µL of stemming solution decoctions of chicory flour or inulin  
121 water solution besides the standard chow (Diet A04C-10, Scientific Animal Food and  
122 Engineering, Augy, France). For the chicory flour, the decoction corresponded to 10 mg root  
123 powder/mouse/day that was considered to be the human equivalent weight/body mass for a  
124 moderated alimentary dose (Fouré *et al.*, 2018). The administered dose of inulin was  
125 calculated to correspond to the concentration of inulin in the G35 aqueous extract of roots for  
126 the daily consumption, and was estimated at 6.9 mg/mouse/day. Controls underwent an  
127 equivalent force-feeding with water. As experiments were conducted sequentially for two  
128 different periods of time, two control sets were used: first control group (Ctr1) in parallel with  
129 G12 and G13 diet and a second control group (Ctr2) in parallel with the inulin diet. Six mice  
130 for each gender per condition were used for 30 days followed by a week of resilience.  
131 Individual body weight was regularly registered. Three mice for each condition were  
132 sacrificed before the beginning of the treatment (D0), after 30 days of treatment (D30) and  
133 after washout period (D36) and the central core of the liver left lobe was cut into cubes and  
134 frozen as individual samples at -80°C for transcriptomics. The feces were individually  
135 harvested before the beginning of the treatment (D0) after 30 days of treatment (D30) and  
136 after washout period (D36), and stored at -80°C for microbiota analyses. For hormonal assays  
137 100 µL of blood from the caudal vein was sampled from each animal 30 min after gavage at  
138 D30. The blood from each mouse was collected in tubes containing 20 µL EDTA 10%  
139 anticoagulant solution and 1 µL dipeptidyl peptidase-4 inhibitor (DPP4-010, Marck, Milipore,

140 Darmstadt, Germany), then centrifuged at 14,000 rpm for 10 min, the plasma containing  
141 supernatants collected and stored at -80°C.

### 142 **2.3. RNA isolation and microarrays analysis**

143 Total RNA was extracted from hepatic tissues using RNeasy spin columns (Macherey-Nagel).  
144 RNA quality was observed with Nanodrop and absorbance ratios A260/280 and A260/230  
145 were found between 2.0 and 2.2. RNA quality was also examined with Bioanalyzer 2100  
146 (Agilent, Les Ulis, France) and a minimal RNA integrity number (RIN) of 0.8 was required  
147 for all samples.

148 For the microarray analysis, groups of mice (n=3) for both genders were used: i) a group that  
149 received a decoction of chicory flour G12 for 30 days as previously described, ii) a group that  
150 received a decoction of chicory flour G35 for 30 days, iii) a group that received a solution of  
151 inulin for 30 days, and iv) two control groups with standard drinking water as mentioned  
152 before.

153 Agilent *Mus musculus* Sure Print GE 4x44 v2 microarrays with oligonucleotide 45,220 probes  
154 were used to study the gene expression profile. RNA amplification, staining, hybridization  
155 and washing were conducted according to the manufacturer's specifications. Slides were  
156 scanned at 5 µm/pixel resolution using the GenePix 4000B scanner (Molecular Devices  
157 Corporation, Sunnyvale, CA, USA). Images were used for grid alignment and expression data  
158 digitization with GenePix Pro 6.0 software. Expression data were normalized by Quantile  
159 algorithm. The 3 control samples were filtered for p value <0.05 and the average was  
160 calculated for each gene. A fold change (FC) value was calculated between individual treated  
161 samples and the mean of corresponding controls. Differentially expressed genes (DEGs) were  
162 selected for a threshold > 2.0 or < 0.5. Functional annotation of DEGs was based on NCBI  
163 GenBank and related-genes physiological processes were assigned with NCBI, AmiGO 2



164 Gene Ontology and UniProt. KEGG pathway analysis was also used to identify relevant  
165 biological pathways of selected genes. All microarray data have been submitted to the NCBI  
166 GEO: archive for functional genomics data with the accession number GSE150218.

#### 167 **2.4. *In vivo* hormone detection**

168 Plasma concentrations of Glucagon-like peptide-1 (GLP-1), gut hormone peptide YY (PYY)  
169 and leptin were assessed using antibody-immobilized beads specific to each hormone in a  
170 Milliplex® Map Kit (Millipore Corporation, Billerica, MA, USA) according to the  
171 manufacturer's instructions. Kit sensitivity was 19 pg/mL for leptin, 23 pg/mL for GLP-1, and  
172 5 pg/mL for PYY. The quantification was carried out using the Luminex® 100/200 (Luminex  
173 Corporation, Austin, USA) system and the Luminex xPONENT® for LX100/200 software.

#### 174 **2.5. Microbiota analysis**

175 DNA extraction was carried out on 200 mg of mouse feces according to QIAamp stool DNA  
176 Kit (Qiagen, France). 16S targeted metagenomic analysis, and bioinformatics analyses were  
177 achieved according to optimized and standardized protocol developed by the company  
178 Genoscreen, Lille, France as described previously (Foligné *et al.*, 2019). Microbial diversity  
179 and taxonomic composition of samples were determined for each sample using the so-called  
180 metagenomic targeted methodology. Briefly, a fragment of the 16S rRNA gene framing the  
181 V3 and V4 hypervariable regions was amplified from 5 ng of gDNA of the samples according  
182 to an optimized and standardized amplicon library preparation protocol Metabiote®  
183 (Genoscreen, France). The final amplification products (each containing a nucleotide index to  
184 differentiate the samples as well as the adapters necessary for carrying out the sequencing)  
185 were purified on beads and then finally mixed in equal concentrations. The sequencing was  
186 performed with the 2\* 250 bp chemistry on the Illumina MiSeq platform (Illumina, San  
187 Diego) at Genoscreen. The resulting raw sequences were subjected to a cleaning process  
188 comprising i) sorting the sequences according to the indexes and according to the 16S primers

189 ii) the "trimming" of the sequences by truncating the bases from the 3 'end with a Phred  
190 threshold of 30 (iii) assembly of the two paired sequences according to a minimum overlap of  
191 30 bases and at least 97% identity over the overlapping area.

192 The computer analysis was carried out on a fully automated (Metabiote® OnLine) pipeline  
193 built around the QIIME v 1.9.1 software (Breton *et al.*, 2013). Following the steps of pre-  
194 processing, the full-length 16S rRNA gene sequences go through a step in which chimera  
195 sequences are detected and eliminated (in-house method based on the use of Usearch 6.1).  
196 Then, a clustering step is performed in order to group similar sequences with a nucleic  
197 identity defined threshold (97% identity for an affiliation at the genus level on the V3-V4  
198 regions of the 16S rRNA gene) with Uclust v1.2.22q (Cresci *et al.*, 2010) through an open-  
199 reference OTU picking process and complete-linkage method, finally creating groups of  
200 sequences or "Operational Taxonomic Units" (OTUs). The most abundant sequence of each  
201 OTUs is therefore considered as the reference sequence of its OTU and is then taxonomically  
202 compared to a reference database (Greengenes database, release 13\_8; [www.greengenes.gov](http://www.greengenes.gov))  
203 by the RDP classifier method v2.2 (Lahiri and Abraham, 2014). A final table listing all the  
204 created OTUs was generated comprising the number of sequences for each sample related to  
205 each OTU and their respective taxonomic affiliation.

206 All analyses were performed by comparing experimental groups to their respective controls.  
207 Normality was controlled with the Shapiro-Wilk test and homogeneity of variances with  
208 Bartlett's test. Statistical paired differences were assessed by ANOVA and Tukey's test using  
209 PRISM 7 (GraphPad Prism 6.0, California, USA) and differences were considered significant  
210 for a p value of <0.05. All the biosample raw reads have been deposited at the National  
211 Center for Biotechnology Information (NCBI) and are available under BioProject accession  
212 number PRJNA631462.

213

## 214 3. RESULTS

215 Two chicory genotypes G12 and G35 were used here for the different analyses. These two  
216 genotypes were selected from a previous study because they presented important differences  
217 in roots metabolite composition. The G12 genotype had significantly more citrate, fumarate,  
218 lactate, formate, acetate, and inositol than G35. Conversely, roots of the G35 genotype were  
219 richer in inulin, sucrose, and fructose as well as in 3-mono-*O*-caffeoylquinic acid and 11,13-  
220 dihydro-8-deoxylactucin (Fouré *et al.*, 2018). **Aqueous extracts of root flours from chicory**  
221 G12 and G35 were used to feed mice in order to detect the different health effects and to  
222 compare with **an inulin water solution**. For this purpose, many investigations were performed:  
223 a whole transcriptomic analysis using Agilent DNA microarrays, a hormonal level  
224 measurement using Luminex technology and a fecal microbiota metabarcoding analysis by  
225 Illumina 16S rDNA sequencing.

### 226 3.1. Chicory diet-driven profiles of gene expression

227 Diet-induced gene expression profiles were analyzed in the liver of mice after 30 days of  
228 chicory flours or inulin ingestion. A total number of 28 profiles were found differentially  
229 expressed during the chicory diet, with an unbalanced pattern between male and female  
230 subjects for 39% of profiles (Figure 1, Supplementary file 1). DEGs modified by the chicory  
231 diet are involved in cell growth arrest and apoptosis, immunity system, glucose and lipid  
232 homeostasis, xenobiotic metabolism and antioxidant activity. **As a trend**, all effects except  
233 antibacterial ones were more pronounced in male than in female mice. Despite quantitative  
234 differences in flour composition, the G12 and G35 chicory flour modulated similarly the gene  
235 expression. An obvious difference between chicory and inulin effects was observed as only 4  
236 DEGs were found differentially expressed during inulin intake. These observations suggest  
237 that bioactive constituents of chicory roots other than inulin could be involved in the observed

238 effects, **alternatively the combined effect of inulin and these different compounds**. After 7  
239 days of resilience, all genes were found to be expressed at the same level as the controls,  
240 regardless of diet (**Supplementary file 1**).

### 241 **3.2. Plasma level of hormones**

242 Despite that no significant differences were observed in plasmatic hormone levels, several  
243 tendencies must be highlighted. Using the MILLIPLEX® MAP Kit a multiple-fold increase  
244 of GLP-1 and PYY was registered in plasma of male subjects after chicory uptake (Figure 2  
245 and Supplementary file 2). Inulin was found to induce an increase of PYY in male mice only.  
246 As for gene expression level, the hormonal level seems to be more impacted in male than in  
247 female mice. Leptin level was found to be decreased between 1.3 and 2.1 times in plasma of  
248 all mice fed with chicory flour either G12 or G35 but not during inulin diet.

### 249 **3.3. Microbial diversity**

250 16S-rRNA gene-targeted metagenomic analysis was performed in individual fecal samples of  
251 male and female mice (n=6) for each dietary requirement, allowing comparison of the overall  
252 bacterial richness and phylogenetic composition of the microbiota. After sequence processing,  
253 a total of 29 different operational taxonomic units (OTUs) were identified with an abundance  
254 >0.1% (Supplementary file 3). The average number of OTUs per individual was  $264 \pm 44$  for  
255 males and  $362 \pm 43$  for females and agrees with other studies in mice fecal microbiota  
256 (**Foligné et al., 2019**). The alpha diversity Shannon index indicated a stabilized diversity for  
257 all subject groups and no disruptive modifications were found during the chicory or inulin diet  
258 (Figure 3A). Beta diversity among different conditions as a measure of genera turnover was  
259 considered by Principal Coordinate Analysis for affected taxa during different diets. This  
260 revealed a common but also a specific effect of chicory and inulin diet (Figure 3B).

### 261 **3.4. Microbial characteristics**

262 The community composition representing the bacterial phyla and genus level rankings for  
263 major OTU present in the subjects are shown in Figures 4 and 5 respectively. The most  
264 abundant phyla across all young subjects were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*,  
265 *Proteobacteria*, TM7 and *Tenericutes* (Figure 4A). The ratio *Firmicutes/Bacteroidetes* (F/B)  
266 was found diminished after 30 days of G35 chicory diet only, due to a significant increase in  
267 *Bacteroidetes* abundance in male and decrease of *Firmicutes* abundance in female mice  
268 (Figure 4B and Supplementary file 4).

269 At the genus level, all changes observed in the relative abundance after 30 days of chicory or  
270 inulin treatment are represented in Figure 5. No qualitative changes were observed but  
271 quantitative modifications appeared in the abundance of several taxa after chicory or inulin  
272 diet (Figure 5A). These modifications were closely followed as fold change of standardized  
273 abundance ratio (condition vs control) (Figure 5B). Three taxa appeared to increase in  
274 abundance during both G12 and G35 treatments: *Butyricoccus*, *Anaerostipes* and *Dorea*.  
275 *Bacteroidales\_unclassified* was more abundant in G35 condition only, and *Oscillospira* in male  
276 mice only, during the administration of both chicory diets. Conversely, abundance of  
277 *Peptococcaceae*, *Coprococcus* or *Clostridium* was diminished when chicory was  
278 administered. *Lactobacillus* increased specifically when inulin alone was consumed.

279

#### 280 4. DISCUSSION

281 Many studies have already been done on the health effects of inulin (Le Bastard *et al.*, 2020)  
282 or roasted roots of chicory (Fouré *et al.*, 2018). In this study we were interested in another  
283 known foodstuff obtained from chicory roots: the flour. Known to provide nutritional and  
284 medicinal benefits for not only livestock (Nwafor *et al.*, 2017) but also for humans (Puhlmann  
285 and de Vos, 2020), chicory flour was proposed to complement other bread-making ingredients

286 in consumer-friendly products and in products adapted to coeliac patients (Makovicky *et al.*,  
287 2018). To understand the mechanisms by which the **aqueous extract of chicory root** flour  
288 impacts health, we used the murine model and analyzed changes registered in gene expression  
289 profiles, in metabolic hormones level and also in gut microbiota composition during chicory  
290 diet. Our results revealed several major molecular mechanisms by which the whole roots of  
291 chicory – as opposed to inulin alone – could trigger an anticancer and antimicrobial action,  
292 glucose and lipid regulatory influence as well as a xenobiotic and antioxidant effect. A  
293 prebiotic but in part distinct effect was detected for both flour and inulin diet. These results  
294 are discussed below in support of previously published clinical results.

#### 295 **4.1. Activity on gene expression related with cell growth and apoptosis**

296 Nine genes involved in cell growth arrest and apoptosis regulation were found differentially  
297 expressed during chicory intake (Figure 1) suggesting the **potential** capacity of **the aqueous**  
298 **extract of chicory roots** consumption to protect against cancer by increasing the expression of  
299 pro-apoptosis factors and avoiding the proliferation of cancerous cells.

300 Gene coding for the growth arrest and DNA damage-inducible protein GADD45 beta  
301 (*Gadd45B*) was found up-regulated in both male and female mice after the chicory diet,  
302 instead of *Gadd45G* that was found up-regulated in only male mice (Figure 1, lines 1-2).  
303 GADD45 proteins serve as tumor suppressors and their pro-apoptotic activities have  
304 positioned these proteins as essential players in oncogenesis (Tamura *et al.*, 2012). They  
305 belong to the universe of the p53 target genes that was described as the “guardian of the  
306 genome”, being involved in response to DNA damage, apoptosis and cell cycle regulation  
307 (Levine *et al.*, 2006). Another p53-related gene coding for the protein phosphatase 1  
308 regulatory subunit 15A (*Ppp1r15a* or *Gadd34*) was found up-regulated in male mice only,  
309 after chicory intake (Figure 1, line 3). This protein is involved in cell growth suppression and

310 apoptosis of carcinoma (Lum *et al.*, 2006). Still belonging to the p53 universe (Jackson *et al.*,  
311 2003), the *Cd82* (cluster of differentiation 82) was found up-regulated by the chicory diet in  
312 all mice (Figure 1, line 4). It acts as a metastasis suppressor (Tonoli and Barrett, 2005) and  
313 was described as a solid biomarker for malignant tumors (Zoller, 2009). Similarly, *Dmbt1*  
314 (Deleted in malignant brain tumors 1 protein) (Figure 1, line 5) that codes for a tumor  
315 suppressor promoting apoptosis (Zhang, 2019). Gene coding for the ribosome biogenesis  
316 factor NOP53 was also found up-regulated (Figure 1, line 6) and is known to be engaged in  
317 tumor repression (Cho *et al.*, 2016) and in the p53 stabilization in response to stress (Lee *et*  
318 *al.*, 2012). This was also the case of *Hspa8* (Figure 1, line 7) that codes for a heat shock  
319 protein contributing to apoptosis, autophagy, cell growth and differentiation (Mayer and  
320 Bukau, 2005). Its confirmed role in autophagy seems essential in cancer protection, cell  
321 senescence and aging (Xilouri and Stefanis, 2016).

322 Two genes promoting tumor growth were found down-regulated in all mice during chicory  
323 diet: the apoptosis-antagonizing transcription factor (*Aatf*) and the leukemia inhibitory factor  
324 receptor (*Lifr*) (Figure 1, lines 8-9). AATF was described to repress apoptosis (Höpker *et al.*,  
325 2012) and promote tumor progression (Welcker *et al.*, 2018) by targeting genes p53-related.  
326 *Lifr* was identified as a promoter of chemoresistance in various types of carcinoma (Lv *et al.*,  
327 2018).

328 Summing up these aspects, we observed here for the first time evidence **of the modulation by**  
329 **chicory root flour administration of gene expression linked with an anti-proliferative effect.**

330 This strengthens the findings of previous studies (Behboodi *et al.*, 2019; Saleem *et al.*, 2014;  
331 Harza *et al.*, 2002) and could provide an answer related to the mechanism of this valuable  
332 effect. Unexpectedly, inulin intake did not trigger similar effects. Only *Gadd45B* and *Lifr*  
333 were found down-regulated in mice (Figure 1 lines 1 and 9). It would appear that the

334 anticancer protective effect of the chicory is obviously not attributable to inulin only but to  
335 other water-soluble chicory compounds present in the aqueous extract.

#### 336 **4.2. Activity on expression of immune genes**

337 Chicory was already found responsible for various antimicrobial effects (Puhlmann and de  
338 Vos, 2020) and in our experiments we found that the expression of several genes related to  
339 immunity and displaying antibacterial and antiviral activity could be responsible for these  
340 effects. Thus, an important up-regulation of *Lipocalin 2 (Lcn2)* was detected in male and  
341 female mice after chicory food intake (Figure 1, line 10). This gene mediates an innate  
342 immune response to bacterial infection. It codes for a secreted protein stimulated by Toll-like  
343 receptors upon infection, which is able to sequester catecholate-like siderophores and  
344 therefore renders bacteria unable to expand (Flo *et al.*, 2004). This role seems essential for  
345 antibacterial defense as the lipocalin 2 deficiency was lethal in acute infections with  
346 *Escherichia coli* H9049 (Flo *et al.*, 2004). In addition to its antibacterial activity, LCN2 play  
347 an important role in inflammation reducing by regulating the recruitment of immune cells to  
348 the site of inflammation (Shashidharamurthy *et al.*, 2013). Genes coding for serum amyloid  
349 A1 and A2 (*Saa1* and *Saa2*) were also found to be strongly up-regulated after chicory extract  
350 administration (Figure 1, lines 11-12). SAA1 can bind to the outer membrane protein A  
351 present on the surface of Gram-negative bacteria, enabling an increasing immunopathological  
352 response against invasive bacteria (Shah *et al.*, 2006). Both SAA1 and SAA2 were found to  
353 play an important role in immunity and regulation of inflammation (Sun *et al.*, 2015).

354 The antiviral effect of the chicory was suggested by the up-regulation of genes coding for  
355 interferon regulatory factor 1 (*Irf1*) and interferon regulatory factor 2 binding protein 2  
356 (*Irf2bp2*) and was observed mainly in male mice (Figure 1, lines 13-14). IRF1 was described  
357 as crucial in innate immunity (Honda *et al.*, 2006) and essential for defense against viruses<sup>47</sup>.



358 The gene coding for interferon regulatory factor 2 binding protein 2 (*Irf2bp2*) was reported to  
359 play a major role in the transcriptional regulation of type I interferon genes in response to  
360 viral infection (Childs and Goodbourn, 2003) and also in anti-inflammatory responses (Chen  
361 *et al.*, 2015).

362 In female mice only, the up-regulation of the gene coding for signal peptide peptidase like 2A  
363 (*Sppl2a*) was observed after chicory food intake (Figure 1, line 15). This gene plays a critical  
364 function in the regulation of innate and adaptive immunity. It catalyzes the cleavage of tumor  
365 necrosis factor alpha (TNF $\alpha$ ) (Friedmann *et al.*, 2006) and equally is directly involved in B  
366 cell maturation and survival (Beisner *et al.*, 2013).

367 Any significant effect of inulin was registered otherwise slight down-regulation of *Lcn2* and  
368 *Saal* suggesting a light decrease of the antibacterial defense on mice after inulin food intake  
369 (Figure 1 lines 10-11).

#### 370 **4.3. Activity on gene expression related to glycemia and lipidemia regulation**

371 Many clinical evidences supported the anti-hyperglycemic (Jackson *et al.*, 2017) or  
372 hypolipidemic effect of the chicory (Keshk and Noeman, 2015), but molecular mechanisms of  
373 these effects continue to remain poorly understood. Our experiment provides a possible  
374 answer in this way, as several genes involved in glycogen biosynthesis and lipidemia  
375 regulation were deregulated after the chicory diet. Thus, protein phosphatase 1, regulatory  
376 subunit 3G (*Ppp1r3g*), insulin-like growth factor 1 (*Igf1*) and its regulatory binding protein  
377 *Igf1bp1* genes, were found up-regulated following sex-dependent pathway particularities and  
378 suggesting a protective effect of chicory against hyperglycemia and weight gain (Figure 1,  
379 lines 16-18). PPP1R3G plays a significant role in both postprandial glucose homeostasis and  
380 lipid metabolism. It stimulates hepatic glycogen accumulation and decreasing hepatic  
381 triglyceride level after a meal, and has the additional benefice of reducing fat mass without

382 changing metabolic rate (Zhang *et al.*, 2014). IGF1 induces similar metabolic effects to  
383 insulin (Monzavi and Cohen, 2002) and is involved in glycogen synthesis (Dean and Rose,  
384 2018). IGF1BP1 can either act as an autocrine and paracrine regulator of IGF1, being involved  
385 in glucose metabolism modulation (Wheatcroft and Keamery, 2009), insulin resistance  
386 suppression, fat mass reducing as well as leptin levels decreasing (Rajkumar *et al.*, 1999).  
387 Indeed, the up-regulation of *Igf1* and *Igf1bp* genes could be linked to the decreasing tendency  
388 observed in plasmatic leptin levels of G12 ( $p = 0.682$ ) and G35 ( $p = 0.132$ ) male groups and  
389 G12 female group ( $p = 0.059$ ) (Figure 2).

390 *Apoa4* coding for the apolipoprotein A-IV was found up-regulated in both male and female  
391 mice after G12 and G35 chicory diet (Figure 1, line 19) and was described as an anti-  
392 atherogenic factor (Culnan *et al.*, 2009) protecting against atherosclerosis and diabetes (Qu *et*  
393 *al.*, 2019). The *Lpin1* and *Lpin2* genes were found up-regulated in male mice only by the  
394 chicory diet (Figure 1, lines 20-21) and are involved in lipid homeostasis regulation (Dwyer *et*  
395 *al.*, 2012). Two other genes coding for apolipoprotein B (*Apob*) and apolipoprotein C-IV  
396 (*Apoc4*) were found down-regulated in only male mice (Figure 1, lines 22-23). These genes  
397 were described to be involved in atherosclerosis (Borén and Williams, 2016) and liver  
398 steatosis (Kim *et al.*, 2008) and their down-regulation suggests a protective effect against  
399 these chronic illnesses.

400 The level of different metabolic markers detected in mice plasma indicated no statistical  
401 differences but noticeable variations suggesting a beneficial metabolic effect of the chicory  
402 diet. Thus, GLP-1 which is known to regulate the appetite and food intake (Holst *et al.*, 2007),  
403 and PYY which is a short-term food intake marker and thus an anorexigenic hormone (Woods  
404 and D'Alessio, 2008) were increased in mainly male mice during chicory diet (Figure 2).  
405 Leptin, which is a long-term food intake marker and an indicator of the body fat level  
406 (Brennan *et al.*, 2006), was found to decrease in both male and female mice after G12 or G35

407 chicory food intake. Both trends, the appetite regulation and the decrease of the body fat,  
408 could be associated with gene expression to explain the worthwhile consequences of chicory  
409 consumption. These effects must be rather protective than curative as in our study no  
410 significant body weight modifications were observed during different diets (Supplementary  
411 file 5).

#### 412 **4.4. Effect on expression of genes related to xenobiotic and antioxidant activity**

413 An important antioxidant effect was already attributed to the chicory root extract that showed  
414 a radical scavenging activity dependent more like on its content of caffeoylquinic acids (Liu  
415 *et al.*, 2013). In our study we found that consumption of chicory triggered the up-regulation of  
416 several genes coding for drug metabolizing enzymes such as cytochrome P450 (*Cyp4a31*,  
417 *Cyp8b1*, *Cyp27a1*) and flavin containing monooxygenase (*Fmo5*), or for proteins involved in  
418 oxidative stress protection as selenophosphate synthetase 2 (*Sephs2*) (Figure 1, lines 24-28).  
419 Cytochrome P450 and *Fmo5* are well known for their capacity to monooxygenize a large  
420 panel of organic compounds (Chen, 2020). SEPHS2 is involved in synthesis of selenoproteins  
421 (Oudouhou *et al.*, 2017) that are typically involved in cellular redox balance and include  
422 glutathione peroxidase and thioredoxin reductase (Steinbrenner *et al.*, 2009). As no effect of  
423 inulin was registered at this level, it is very likely that the caffeoylquinic acids and probably  
424 other bioactive molecules present in chicory roots must be responsible for this effect.

#### 425 **4.5. Prebiotic effect on gut microbiota**

426 The microbial community diversity remained stable in fecal microbiota among all samples  
427 (Figure 3A). Only a few taxa registered quantitative changes during chicory or inulin diet  
428 (Figure 3B). *Bacteroidetes* and *Firmicutes* were the most prevalent phyla (Figure 4A) but  
429 their ratio changed during the chicory diet. A significant lower F/B ratio was induced by the  
430 G35 chicory genotype in males and in female mice as well (Figure 4B) and this result is in

431 accord with previous observations on chicory roasted roots effects (Fouré *et al.*, 2018). We  
432 observed here that inulin alone administrated at the same concentration as in G35 flour did not  
433 completely explain this change of F/B (Figure 4B). Most likely other compounds that are  
434 more represented in G35 must be responsible for this disproportion, however, this remains to  
435 be demonstrated. All the same, F/B is considered as an indicator of gut microbiota status (Ley  
436 *et al.*, 2006) as a reduced F/B is correlated to a beneficial effect on obesity physiopathology  
437 (Turnbaugh *et al.*, 2008; Barlow *et al.*, 2015).

438 This beneficial effect of G35 flour was confirmed at the genera level (Figure 5).  
439 *Bacteroidales\_unclassified* belonging to *Bacteroidetes* strongly increased in abundance mainly  
440 in male mice after G35 flour intake. The order *Bacteroidales* is the most abundant Gram-  
441 negative bacteria colonizing the human gut (Zitomersky *et al.*, 2011) and among the  
442 predominant genera are *Bacteroides* and *Prevotella*. Both of them are well known as dietary  
443 fiber fermenters and SCFA producers (Chen *et al.*, 2017). Other taxa known as SCFA  
444 producers also increased in abundance during both G12 and G35 treatments: *Butyricoccus*,  
445 *Anaerostipes* and *Oscillospira* (Figure 5B). All these were found to generate butyrate that has  
446 been reported to play an important role in cancer prevention of the colon by promoting cell  
447 differentiation, cell-cycle arrest and apoptosis of transformed colonocytes (Wong *et al.*,  
448 2006). It was also reported that the butyrate is able to induce the production of GLP-1 and  
449 thus reducing food intake (Lin *et al.*, 2012). These aspects are totally consistent with our  
450 results on gene expression profiles and hormonal dosages.

451 On the other side, several *Firmicutes* taxa as *Peptococcaceae* and *Clostridium* were  
452 drastically diminished when chicory was ingested. These taxa are mainly Gram-positive  
453 obligate anaerobes and may frequently include pathogens (Topiel and Simon, 1986; Gibbs,  
454 2009). This way we can then suggest that chicory food intake could induce several protective

455 changes against pathogens not only through changes in gene expression but also in the  
456 intestinal microbiota.

457 Interestingly, consumption of inulin triggered the same stimulating effects on butyrate-  
458 producing bacteria, but not on the limitation of suspected pathogenic taxa. A particular action  
459 of inulin was reflected in strongly increased in abundance of *Lactobacillus*. Inulin was already  
460 depicted as a functional ingredient with a prebiotic effect, capable of producing changes in  
461 lactobacilli and bifidobacteria abundance on human gut microbiota (Slavin, 2013). The  
462 murine experimental model does not offer the possibility to study bifidobacteria (Hugenholtz  
463 *et al.*, 2018) but the observed changes in lactobacilli are entirely consistent with previous  
464 studies on the effect of inulin. If inulin has the same prebiotic effect for both genders, chicory  
465 flour appears to be more effective in males than in females.

466

## 467 **5. CONCLUSIONS**

468 To provide functional support to the many clinical studies that have highlighted the effects of  
469 chicory on health, we looked at the mechanisms of action of this plant and more specifically  
470 those of the chicory roots processed into flour. For this purpose, we experimented on the  
471 murine model and performed a nutrigenomic analysis that was associated with a metabolic  
472 hormone assay and a metagenomic analysis. A prominent effect of the chicory on cancer  
473 prevention was demonstrated by the deregulation of a large number of genes involved in cell-  
474 cycle arrest and apoptosis (*Gadd45B*, *Gadd45G*, *Gadd34*, *Cd82*, *Dmbt1*, *Nop53*, *Hspa8*, *Aatf*,  
475 *Lifr*). Genes involved in antibacterial and antiviral defense, such as *Lcn2*, *Saa1*, *Saa2*, *Irf1* and  
476 *Irf2bp2*, were strongly up-regulated during chicory diet. The hypoglycemic and  
477 hypolipidemic effects of the chicory were associated with deregulation of *Ppp1r3g*, *Igf1*,  
478 *Igfbp1*, *Apoa4*, *Lpin1*, *Lpin2*, *Apob*, *Apoc4*, and also with modifications of GLP-1, PYY and

479 leptin release. Finally, the antioxidant effect was revealed by the up-regulation of *Cyp4a31*,  
480 *Cyp8b1*, *Cyp27a1*, *Fmo5* and *Seps2*. The prebiotic effect of chicory roots was revealed for  
481 butyrate producers like *Butyricoccus*, *Anaerostipes* and *Oscillospira* which increased in  
482 abundance. In contrast, taxa with putative pathogenic potential like *Peptococcaceae* and  
483 *Clostridium*, drastically decreased in abundance. Except the prebiotic effect that is partially  
484 shared with inulin, other effects were principally attributed to the chicory flour intake. In  
485 general, almost all effects were found to be more pronounced in male mice than in female  
486 mice. Together, the new mechanisms detected by this study provide strong arguments for the  
487 health effects of chicory. **As these findings are based on an aqueous decoction of chicory root**  
488 **flour, further experiments should evaluate the effect of the whole chicory root flour to**  
489 **elucidate the potential of chicory as a functional food.**

490

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788

## 789 TABLES

790 **Table 1.** Major metabolites analysed by quantitative <sup>1</sup>H-NMR in chicory root flours.

791 **Table 2.** Composition of aqueous decoction of chicory root flour. Chlorogenic acids and  
792 sesquiterpene lactones were analysed by LC-UV quantification.

793

794 **FIGURE CAPTIONS**

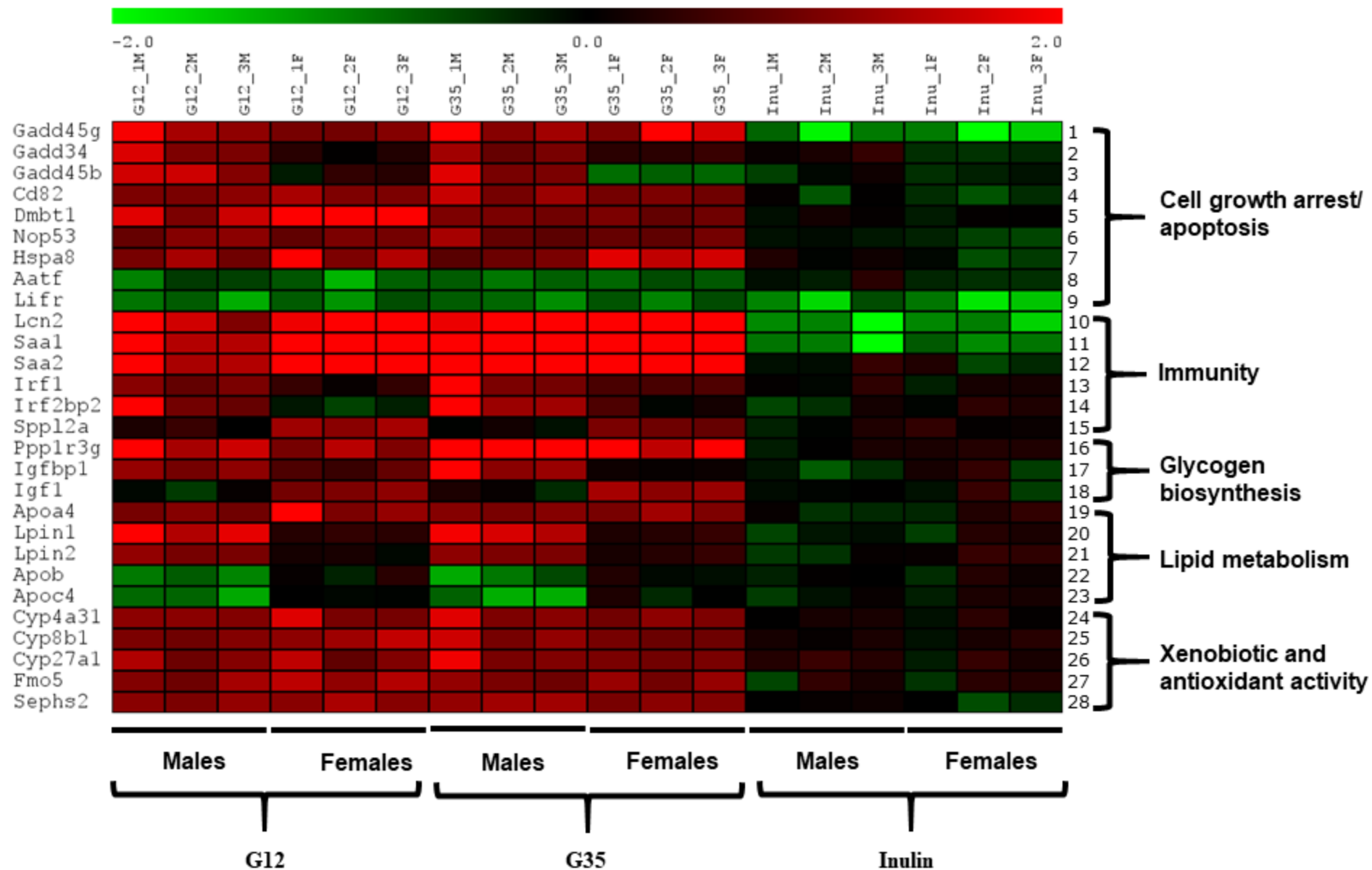
795 **Figure 1.** Gene expression profiles in hepatic tissue of mice after chicory and inulin diet.  
796 Log<sub>2</sub> fold change of gene expression was represented individually for mice (1-3) and gene-  
797 related physiological processes are represented on the right part of the graph.

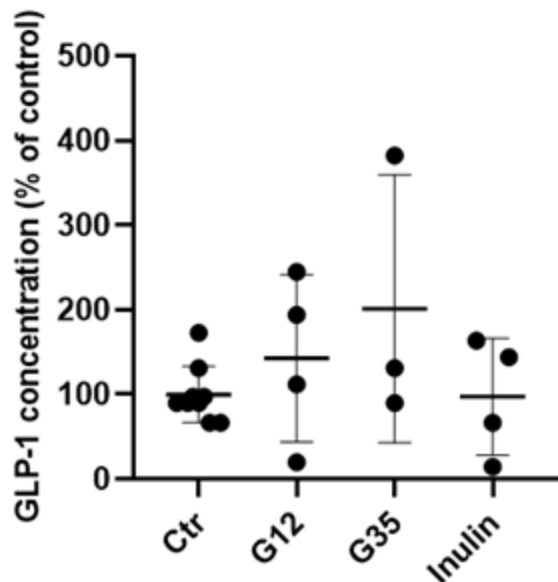
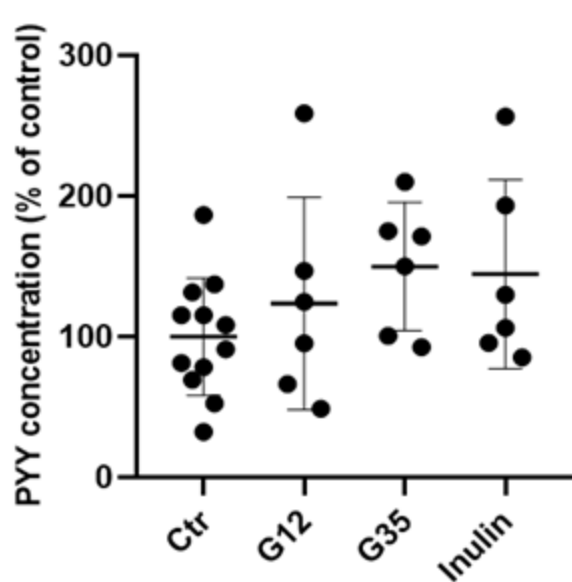
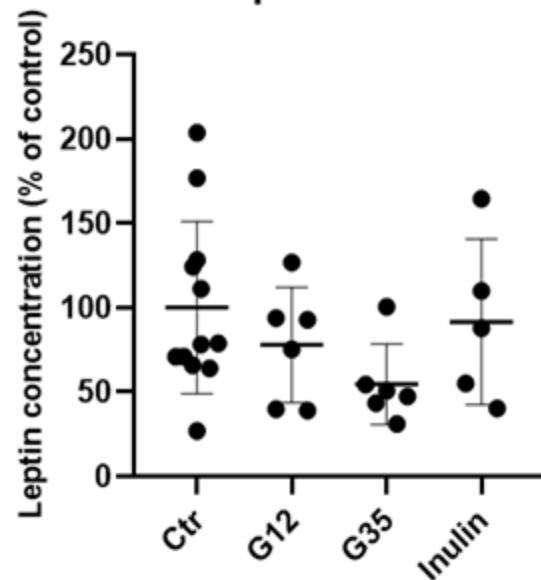
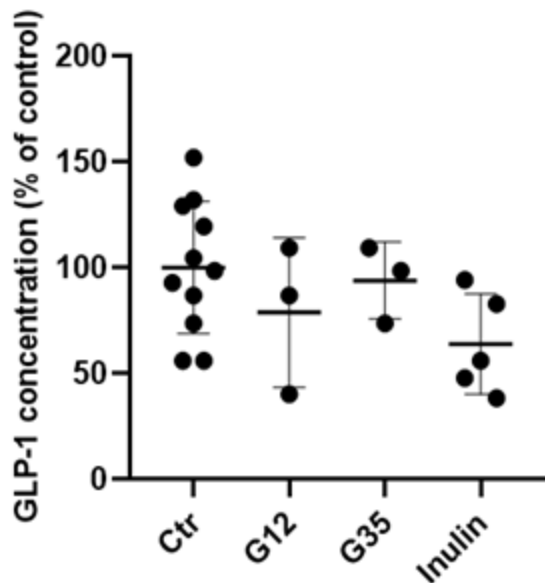
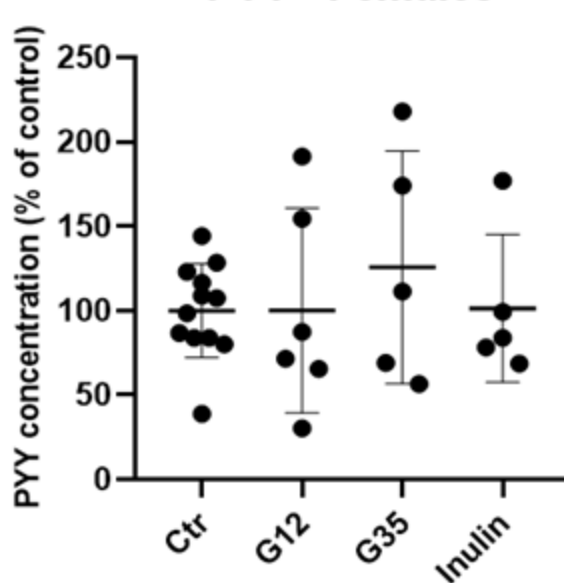
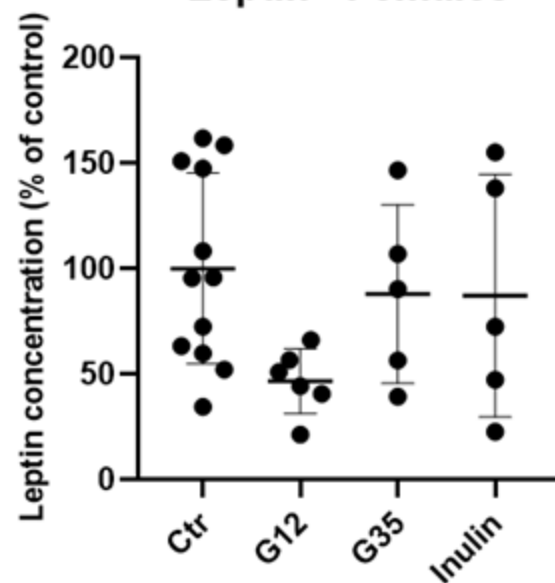
798 **Figure 2.** GLP-1, PYY, and leptin level in mice plasma, after 30 days of chicory (G12, G35)  
799 and inulin supplemented diet, and assayed after 30 min of oral administration of the different  
800 diets at D30. Control values were pooled together (Ctr). Plasmatic hormone levels were  
801 expressed as % of the control level. Statistical analysis was performed using one-way  
802 ANOVA and Sidak's multiple comparisons test.

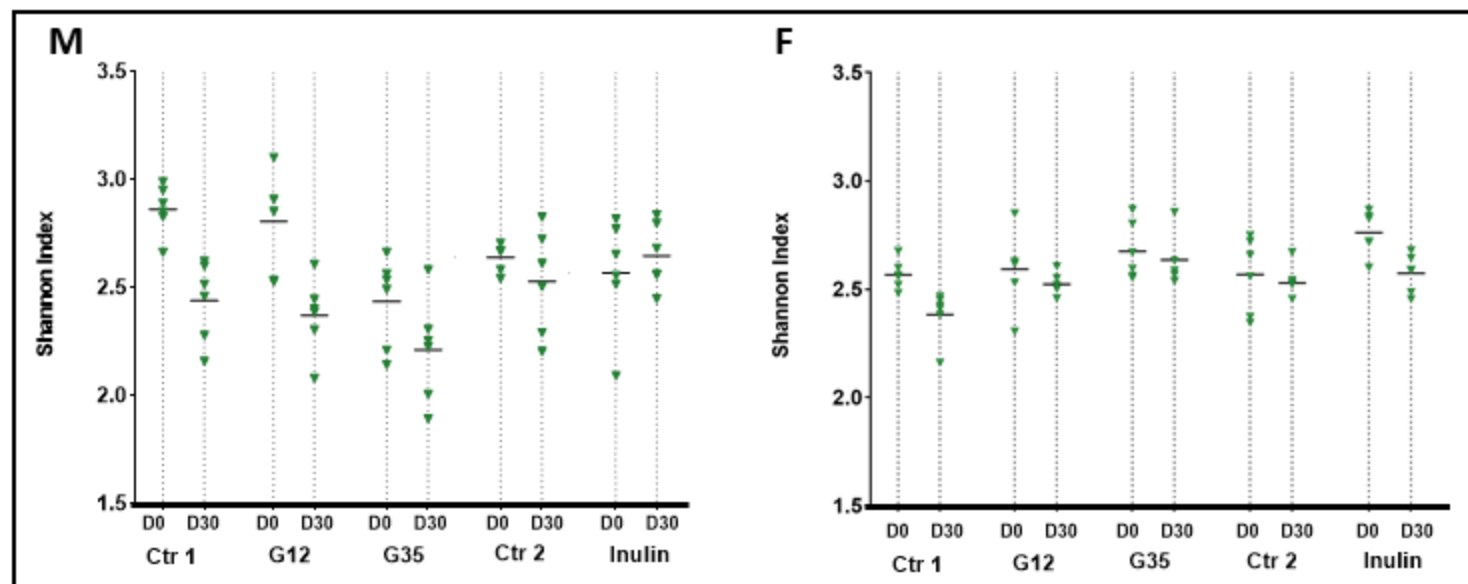
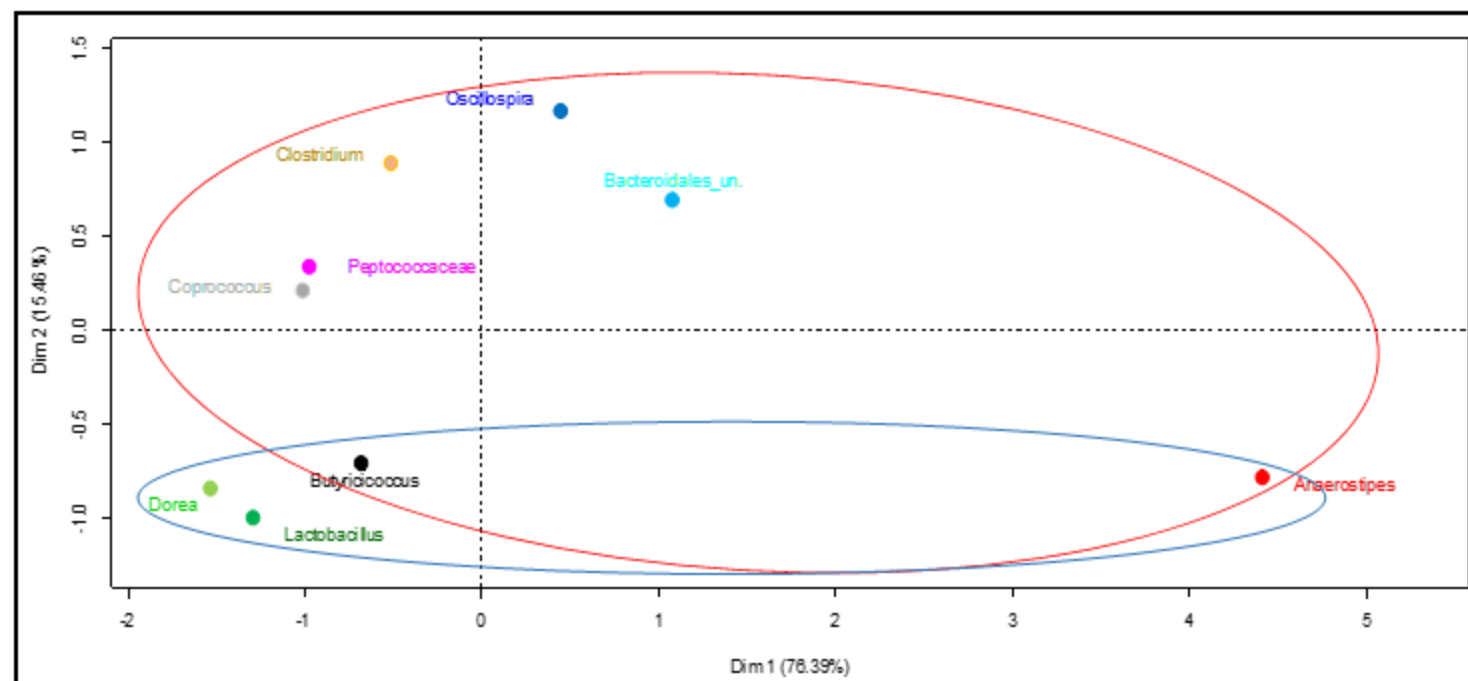
803 **Figure 3.** Microbial diversity in fecal microbiota of mice after chicory and inulin diet. A –  
804 Alpha diversity was illustrated by the Shannon index that indicates a stabilized diversity for  
805 all subject groups ( $p > 0.05$ ). B – Principal Coordinates Analysis (PCoA) plots (beta-diversity)  
806 of affected taxa during different diets. Relative abundance obtained from sequencing the 16S  
807 rRNA gene in fecal samples was represented for taxa providing differences over all samples.  
808 Red circle mainly delimits the chicory effect (including G12 and G35) and blue circle delimits  
809 the inulin effect. M – male, F – female.

810 **Figure 4.** Changes in bacterial phyla abundance. A - Relative abundance (%) of phyla in mice  
811 microbiota before (D0) and after 30 days of chicory and inulin diet (D30). Relative  
812 abundances detected by NGS is expressed as means. Phyla with abundance under 0.1% are  
813 grouped in "Others". B – Standardized abundance ratio relative to D0 of *Firmicutes* and  
814 *Bacteroidetes* (Tukey's test,  $n = 6/\text{group}$ , \* for  $p < 0.05$ , \*\* for  $p < 0.01$ ). M – male; F -  
815 female; G12, G35 - chicory flours; Inu – inulin; Ctr1 – control related to G12 and G35; Ctr2 –  
816 control related to inulin diet.

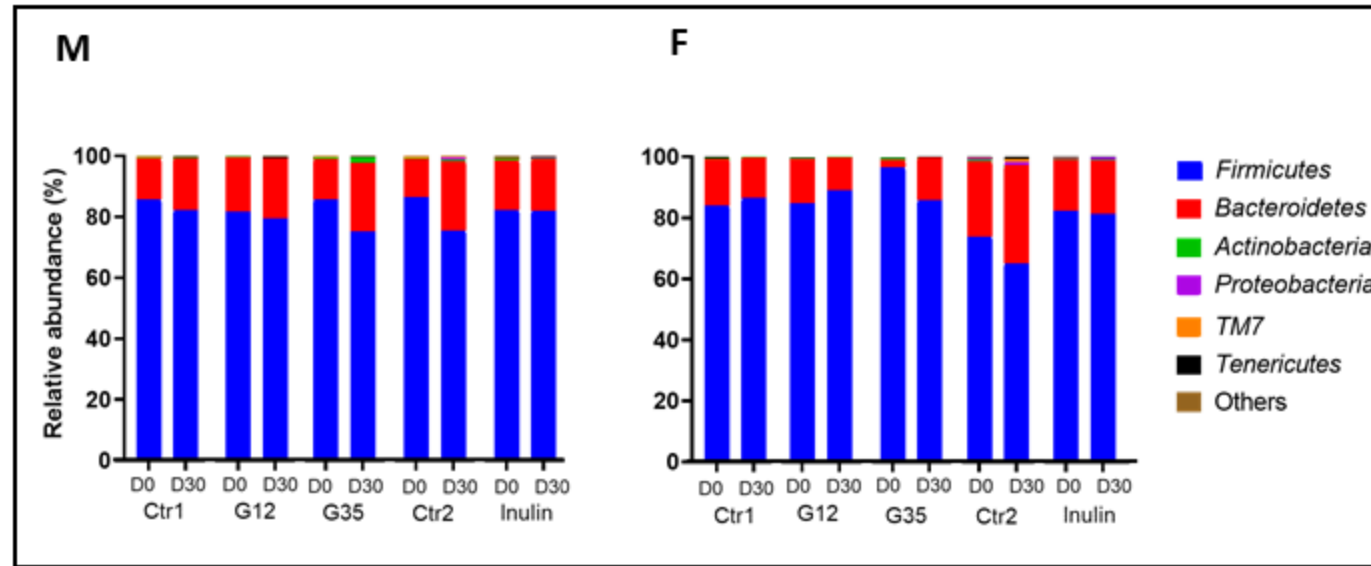
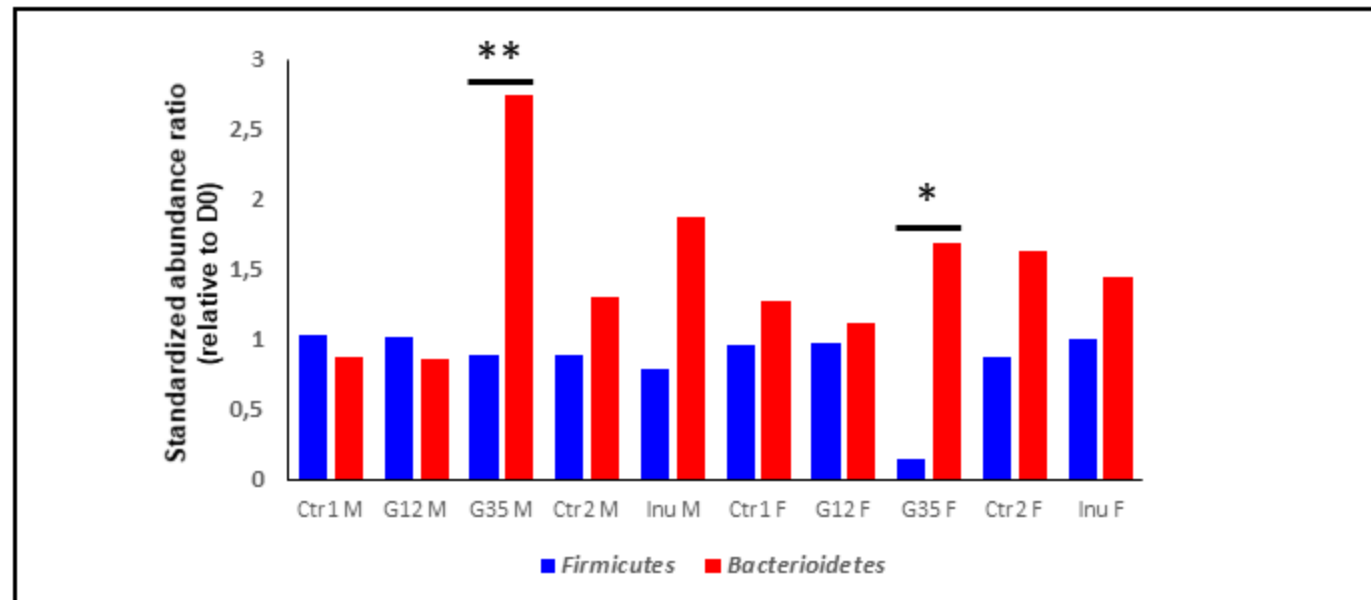
817 **Figure 5.** Relative abundance of main genera in mice fecal microbiota after chicory (G12,  
818 G35) and inulin diet. A - Relative abundance of genera (%) are indicated when their values  
819 are >0.1%. Genera with a low relative abundance were assigned as “Others”. B – Heatmap  
820 representing the fold change of standardized abundance ratio. Increased abundance compared  
821 to control was considered when  $FC > 1.30$ , and decreased when  $< 0.70$ . M – male, F – female.



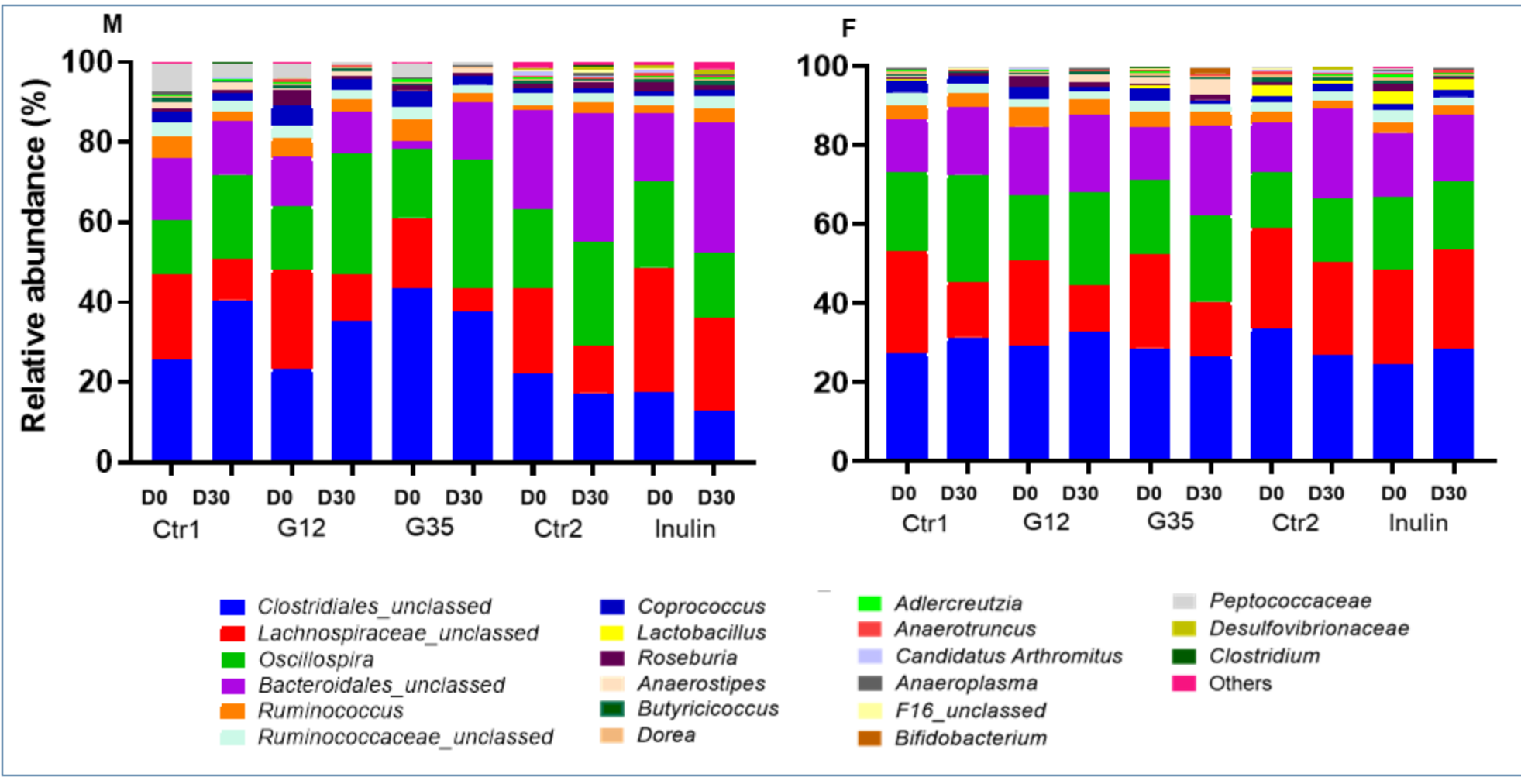
**GLP-1 - Males****PYY - Males****Leptin - Males****GLP-1 - Females****PYY - Females****Leptin - Females**

**A****B**

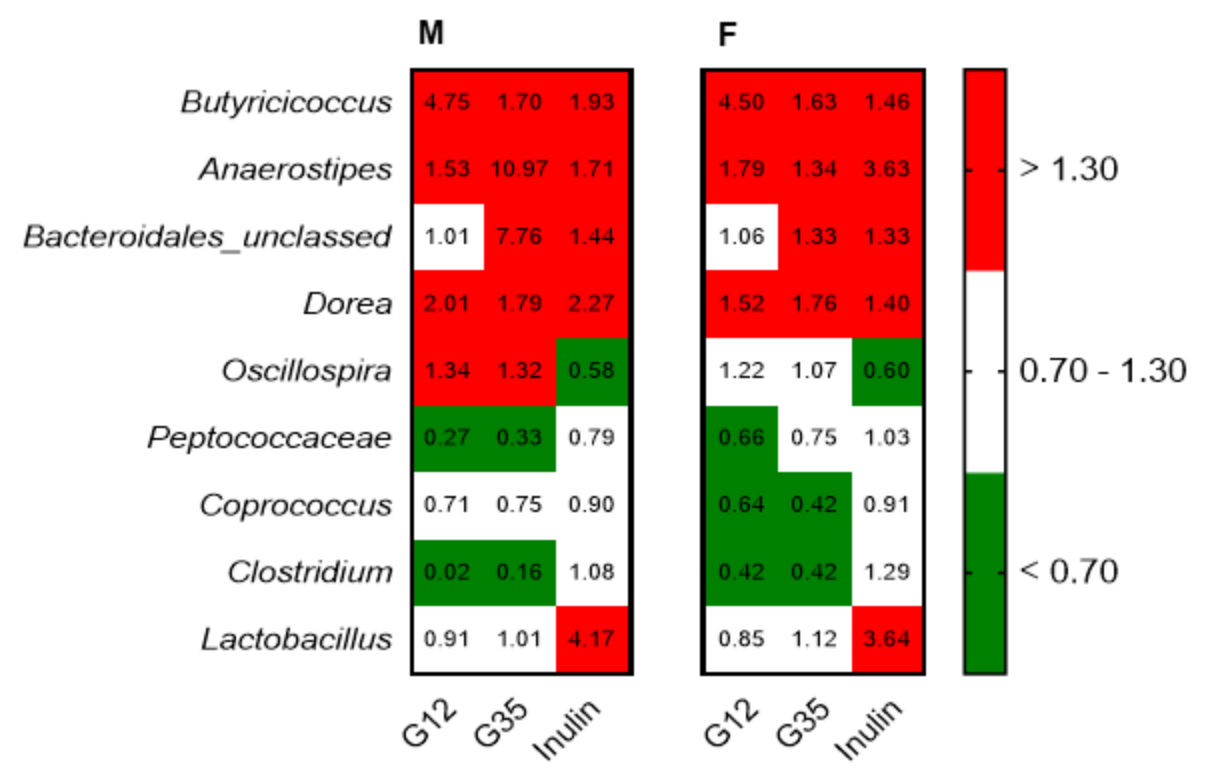


**A****B**

A



B



**Table 1.** Major metabolites analysed by quantitative <sup>1</sup>H-NMR in chicory root flours.

<b>Compounds (mg/g dry matter)</b>	<b>G12</b>	<b>G35</b>
Glucose	18.31a	15.11a
Fructose	15.43b	20.65a
Sucrose	17.91b	26.54a
Mannose	2.91a	2.11a
Inulin	105.80b	250.33a
Citrate	6.88a	4.37b
Fumarate	0.13a	0.08b
Malate	3.10a	2.70a
Lactate	0.80a	0.34b
Formate	0.47a	0.31b
Pyroglutamic acid	5.68a	4.41a
Acetate	0.23a	0.13b
Trigonelline	0.053a	0.070a
Cholin	0.47a	0.36a
Scyllitol	0.90a	0.71a
Inositol	3.06a	2.08b
3-cqa	0.293b	0.343a
3,5-dicqa	0.066a	0.070a
DHdLc	0.011b	0.014a
dLc	0.004a	0.003a
Lp	0.003a	0.003a

Statistically significant differences measured by ANOVA (*p-value* < 0.05) according to genotype are showed with different letters in the same column (Tukey's test).

3-cqa: 3-mono-*O*-caffeoylquinic acid; 3,5-dicqa: 3,5-di-*O*-caffeoylquinic acid; DHdLc: 11,13-dihydro-8-deoxylactucin; dLc: 8-deoxylactucin; Lp: lactucopicrin.

**Table 2.** Composition of aqueous decoctions of chicory root flours. Chlorogenic acids and sesquiterpene lactones previously described in chicory roots were detected in aqueous decoctions by LC-UV quantification.

<b>Compounds mg/g dry matter</b>	<b>decoction G12</b>	<b>decoction G35</b>
3-cqa	0.142 ± 0.0076	0.148 ± 0.0080
3,5-dicqa	0.015 ± 0.0005	0.008 ± 0.0003
DHdLc	0.167 ± 0.0063	0.203 ± 0.0077
dLc	0.035 ± 0.0033	0.024 ± 0.0022
Lp	0.084 ± 0.0041	0.107 ± 0.0052

3-cqa: 3-mono-*O*-caffeoylquinic acid; 3,5-dicqa: 3,5-di-*O*-dicaffeoylquinic acid; DHdLc: 11,13-dihydro-8-deoxylactucin; dLc: 8-deoxylactucin; Lp: lactucopicrin.