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1 **Dietary switch to Western diet induces hypothalamic adaptation associated with**
2 **gut microbiota dysbiosis in rats**

3 Running title: Western diet and rat microbiota-hypothalamus axis

4

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24 **Abstract**

25 **Background:** Early hyperphagia and hypothalamic inflammation encountered after Western diet
26 (WD) are linked to rodent propensity to obesity. Inflammation in several brain structures has been
27 associated with gut dysbiosis. Since gut microbiota is highly sensitive to dietary changes, we
28 hypothesized that immediate gut microbiota adaptation to WD in rats is involved in inflammation-
29 related hypothalamic modifications.

30 **Methods:** We evaluated short-term impact of WD consumption (2h, 1, 2 and 4 days) on hypothalamic
31 metabolome and caecal microbiota composition and metabolome. Data integration analyses were
32 performed to uncover potential relationships among these three datasets. Finally, changes in
33 hypothalamic gene expression in absence of gut microbiota were evaluated in germ-free rats fed WD
34 for 2 days.

35 **Results:** WD quickly and profoundly affected the levels of several hypothalamic metabolites,
36 especially oxidative stress markers. In parallel, WD consumption reduced caecal microbiota diversity,
37 modified its composition towards pro-inflammatory profile and changed caecal metabolome. Data
38 integration identified strong correlations between gut microbiota subnetworks, unidentified caecal
39 metabolites and hypothalamic oxidative stress metabolites. Germ-free rats displayed reduced energy
40 intake and no changes in redox homeostasis machinery expression or pro-inflammatory cytokines after
41 2 days of WD, in contrast to conventional rats, which exhibited increased SOD2, GLRX and IL-6
42 mRNA levels.

43 **Conclusion:** A potentially pro-inflammatory gut microbiota and an early hypothalamic oxidative stress
44 appear shortly after WD introduction. Tripartite data integration highlighted putative links between gut
45 microbiota sub-networks and hypothalamic oxidative stress. Together with the absence of
46 hypothalamic modifications in germ-free rats, this strongly suggests the involvement of the microbiota-
47 hypothalamus axis in rat adaptation to WD introduction and in energy homeostasis regulation.

48

49 **Introduction**

50 Obesity is the consequence of prolonged disruption of energy homeostasis and hedonic/reward control
51 of appetite ¹, mainly regulated by the coordinated action of many brain regions including the brainstem,
52 the hypothalamus and cortical or subcortical brain areas ¹. Among them, the hypothalamus integrates
53 internal peripheral signals mostly from the gastro-intestinal tract as well as from adipose tissue or
54 pancreas ². It also integrates brainstem information originating from the vagus nerve and circulating
55 peripheral signals to adapt energy intake and/or energy expenditure ³⁻⁶.

56 In rodents, long-term consumption of a Western diet (WD), *i.e.* a low-fiber, high fat and sugar diet, as
57 opposed to a well-balanced diet (chow), leads to the development of obesity and metabolic disorders.
58 However, this long-term condition is preceded by an asymptomatic period characterized by metabolic
59 and behavioral adaptations to the acute dietary switch from chow to WD ⁷. Indeed, although continuous
60 exposure to WD leads to either no effect on energy intake ^{8,9}, hyperphagia in some studies ^{10,11} or
61 sporadic spikes of hyperphagia in others ¹², the initial eating behavior upon WD introduction is highly
62 reproducible. Rodents exhibit a 1-day hyperphagic phase, due to the palatability of the diet ¹³, followed
63 by a progressive normalization of energy intake within a week ^{7,14,15}. At the hypothalamic level, major
64 changes are observed, including molecular signatures of modified cell interactions, synaptogenesis and
65 increased anorexigenic tone due to pro-opiomelanocortin neurons rewiring and neurogenesis ^{7,16}.
66 Moreover, 1 to 3 days of WD consumption induces hypothalamic inflammation, as demonstrated by
67 increased mRNA levels of pro-inflammatory cytokines ^{17,18}. In mice, this early phase is immediately
68 followed by reactive gliosis, *i.e.* astroglial and microglial activation ¹⁹⁻²¹, likely limiting neuronal injury
69 during this initial phase. Despite the resolution of hypothalamic inflammation within the week
70 following WD introduction, continuous WD feeding will ultimately lead to a chronic and sustained
71 inflammation in the hypothalamus ^{17,21}. Interestingly, diet-induced obesity (DIO)-resistant mice
72 display early hypothalamic inflammation and gliosis but with a lesser intensity ¹⁹ and a different pattern

73 compared to DIO-prone mice²². Thus, greater intensity and/or altered resolution of early WD-induced
74 hypothalamic inflammation could trigger later metabolic alterations. Furthermore, reduced
75 hypothalamic microglia expansion prevents the characteristic increase in energy intake during the first
76 week of WD consumption²³, suggesting that early hypothalamic inflammation is involved in the
77 hyperphagia observed upon WD introduction. Likewise, the intensity of this early hyperphagic phase
78 seems predictive of rodent obesity propensity on the long term^{24,25}.

79 The factors initiating hypothalamic inflammation during short-term WD are ill defined. Several *in vitro*
80 studies reported a direct action of nutrients such as saturated fatty acid and glucose in initiating pro-
81 inflammatory cytokines release in various brain cell types^{26,27}. A proteomic analysis of mice
82 hypothalami after 3 days on WD reported changes in proteins involved in cellular stress and
83 mitochondrial function, indicative of oxidative stress²⁸. Oxidative stress and inflammation are closely
84 related pathophysiological processes, one of which can be easily induced by another. Reactive oxygen
85 species (ROS) generated in brain tissues can modulate synaptic and non-synaptic communication
86 between neurons and result in neuro-inflammation and cell death²⁹. Thus, early oxidative stress may
87 play a role in the induction of hypothalamic inflammation. A role for gut microbiota is also suspected.
88 Indeed, several studies suggested a link between gut dysbiosis and inflammation in several areas of the
89 brain^{30,31}. Fecal transfer from anxiety- and depression-like behaviors prone rats that exhibited gut
90 dysbiosis and ventral hippocampus inflammation recapitulated rats behavior and central inflammation
91³², suggesting a possible causal link between gut dysbiosis and central inflammation. In the
92 hypothalamus, no such relationship has been proved so far but a recent study in obese patients
93 highlighted a significant association between hypothalamic inflammation, evaluated by magnetic
94 resonance imaging, and specific gut microorganisms³³. Long-term WD consumption induces a
95 decrease in gut microbiota diversity and profound compositional changes^{8,34}. Short-term adaptation of
96 the gut microbiota to the WD switch is also expected since gut microbiota is highly responsive to acute
97 dietary changes. Indeed, 8hrs under WD are sufficient to induce significant composition changes in

98 Human microbiota-colonized mice³⁵. Thus, we hypothesized that acute dietary switch from chow to
99 WD results in early changes in microbiota composition and/or function that participates directly or
100 indirectly to early hypothalamic inflammation in rats. To test this hypothesis, we determined how
101 hypothalamic response as well as the gut microbiota composition and activity evolved during the first
102 days following WD exposure, using 16S rRNA gene sequencing and caecal content and hypothalamic
103 metabolomics analysis in conventional rats. Data integration tools were then used to highlight the
104 potential links between microbiota changes and hypothalamic metabolic pathways associated to a pro-
105 inflammatory context. Lastly, we used germ-free rats to investigate the potential connection between
106 the gut microbiota and the early adaptation of hypothalamus to WD.

107

108 **Methods**

109 **Animals**

110 Animal protocols received written agreement from local ethics committees (Institutional Animal care
111 and Use Committee UC Davis (experiment 1), APAFIS#2015102618593445 (experiment 2) and
112 APAFIS#903–2015061809202358V3.33 (experiment 3)). Sample size was estimated following
113 previous experiments, according to the variability of obtained results for each type of analysis. Animals
114 were randomized in groups of similar body weight at the beginning of each experiment. No blinding
115 was done during animal experiments, but the samples treatment was blinded. For all experiments the
116 rats were housed individually under a 12:12h light/dark cycle and maintained at 22±2°C. The light
117 cycle was 7am-7pm. The animals were euthanized between 9am-12am.

118 Experiment 1. Ten male Wistar rats (9 weeks old, Harlan San Diego) had *ad libitum* access to water
119 and chow diet (Purina Lab Diet 5001 rodent diet, fat 13% of energy, Supplementary table 1). After
120 acclimatization period (1 week), rats were divided in two groups and fed *ad libitum* either chow or WD
121 (Research Diet 12451, New Brunswick, USA, fat 45% of energy, Supplementary Table 1) for 6 weeks.
122 Food intake (kcal and kcal.g⁻¹ of body weight per day) and body weight were measured weekly. After

123 6 weeks, rats were fasted overnight before euthanasia. Whole-blood glycemia was evaluated using
124 Accu-check. Plasma was obtained by centrifugation (4000 rpm, 10 min, 4°C) and stored at -80°C. Fat
125 pads (mesenteric, epididymal and retroperitoneal fat) were dissected and weighted to evaluate visceral
126 adiposity.

127 Experiment 2. Thirty-three Wistar male rats (8-9 weeks old; Janvier Labs, Le Genest-Saint-Isle,
128 France) had *ad libitum* access to water and chow diet (Special Diets Services, Rat and Mouse no.3
129 Breeding, Special Diet Service; Witham, UK, fat 11.5% of energy, Supplementary table 1) during 1
130 week of acclimatization. Then, 26 rats switched to a commercialized WD (Research Diet 12451
131 Supplementary Table 1). Food consumption (kcal and kcal.g⁻¹ of body weight per day) and body
132 weights were recorded daily. Food intake was expressed as a percentage of the control food intake (the
133 mean of the daily food intake during the three days preceding dietary change) for each animal. Groups
134 of 6 to 7 rats were euthanized either before the dietary switch (T0) or 2h (T2H), 1 (TD1), 2 (TD2) and
135 4 days (TD4) after WD introduction. Animals were fasted for 12h then fed for 2h with their respective
136 diet before euthanasia. The 2hr-food intake was similar among the various WD-fed and chow-fed
137 groups. Hypothalami and caecal contents were sampled and stored at -80°C.

138 Experiment 3. Nine germ-free (obtained from the germ-free rodent facility Anaxem, Micalis Institute,
139 France) and 10 conventional male Fisher rats (Charles River, Arbresle, France) aged 8 weeks were
140 housed individually for 3 weeks for acclimatization in sterile and non-sterile incubators, respectively.
141 Environmental sampling followed by bacterial culture tests were performed regularly to assess sterility.
142 During acclimatization, rats were fed sterilized (45kGy irradiation) chow (SAFE A03; Safe, Augy,
143 France, fat 13.5% of energy, Supplementary Table 1) and sterilized water *ad libitum*. Germ-free and
144 conventional rats were then divided into two groups receiving either irradiated chow or irradiated WD
145 (45kGy irradiation) (SAFE 245HF, Safe, Augy, France, fat 45.9% of energy, Supplementary Table 1)
146 for 2 days. Food intake of the four groups (kcal and kcal.g⁻¹ of body weight per day) was recorded by
147 weighting the food every 24 hours. The animals had access to food until euthanasia. After euthanasia,

148 hypothalami were stored in RNA-later buffer at -80°C before gene expression analysis and CONV rats
149 caecal contents stored at -80°C.

150

151 **Plasma Lipopolysaccharides Binding Protein (LBP)**

152 Plasmatic LBP levels were determined via ELISA kit according to the manufacturer's
153 recommendations (Biometec, Greifswald, Germany).

154 **Determination of caecal microbiota composition**

155 Total DNA extraction and V3-V4 PCR were performed as already described ³⁶.

156 **Hypothalamus and caecal content metabolomic profiles**

157 Frozen hypothalami were analyzed by mass spectrometry (ultra-high-performance liquid
158 chromatography/tandem accurate mass spectrometry) performed by Metabolon Inc. (Durham,
159 USA)(details in Supplementary methods 1). Caecal water samples were obtained from caecal contents
160 as described in Supplementary methods 2. Untargeted caecal metabolomics profiles were acquired
161 using gaz chromatography coupled to a high-resolution mass spectrometer (QToF 7200 ;Agilent)
162 (INRAE, Plateforme d'Exploration du Métabolisme, MetaboHUB Clermont, Clermont-Ferrand,
163 France) as described previously ³⁷. Data were processed under the Galaxy web-based platform
164 Workflow4Metabolomics ³⁸ to yield a data matrix containing variables (retention times, m/z ratio) and
165 peak intensities corrected for batch effects (details in Supplementary methods 2).

166 **Hypothalamic gene expression levels**

167 Total RNA extraction, reverse transcription and qPCR from hypothalami of germ-free animals and
168 their conventional controls were performed as described previously ³⁹. Expression levels of genes were
169 determined by qPCR using SYBR Green chemistry or TaqMan gene expression assays (Supplementary
170 Table 2).

171 Relative expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$ method, considering the
172 geometric mean of housekeeping genes to determine the ΔCT and the chow-fed conventional and germ-

173 free rats group as reference groups to determine the $\Delta\Delta C_T$ for the conventional and the germ-free
174 groups, respectively. Samples were excluded when considered as outliers following Dixon statistical
175 test.

176

177 **Statistics and data integration**

178 Statistical analysis in experiments 1 and 3 were performed using Mann-Whitney tests between chow
179 and WD groups and Spearman correlations using Prism GraphPad software v.7.00 (GraphPad
180 Software, San Diego, USA). ANOVA or Kruskal-Wallis, followed by Tukey's or Dunn's HSD post-
181 hoc tests when significant, respectively, were used for experiment 2 when comparing time-groups, also
182 using Prism GraphPad. Results were considered significant when $p < 0.05$.

183 Analyses of reads obtained after 16S rRNA gene sequencing from caecal content are detailed in the
184 Supplementary methods 3. PLS-DA were performed on normalized metabolomic data using the
185 mixOmics R package v.6.10.1 and details of the analysis are provided in the Supplementary method 3.
186 To perform data integration, the web application MiBiOmics ⁴⁰, based on Weighted Correlation
187 Network Analysis method (WGCNA) ⁴¹, was used and described in the Supplementary methods 3.

188

189 **Data availability**

190 R code and raw metabolomic data used in this study is available upon request to the corresponding
191 author. Raw microbiota sequences are available at Sequence Read Archive (BioProject ID 602836).

192

193

194 **Results**

195 **First week energy intake after dietary switch to WD was predictive of long-term obesity**
196 **parameters**

197 Several studies have shown that the intensity of the hyperphagic phase at normal weight following WD
198 introduction is predictive of propensity to develop obesity on the long term ^{24,25}. We confirmed that
199 rats switched to WD displayed a first phase of increased energy intake followed by normalization of
200 energy intake (Fig.1 A (kcal.g⁻¹ of body weight) and Supplementary Fig. 1 (kcal)). After 6 weeks of
201 WD, they exhibited greater body weight gain (Fig. 1B), final body weight (572±15 vs. 513±11g,
202 P=0.01), visceral adiposity (5.8±0.3 vs. 3.7±0.4%, P=0.002), plasma LBP (0.63±0.07 vs. 0.42±0.04
203 µg.ml⁻¹, P=0.02) and a tendency for greater fasted glycemia (275.0±46.7 vs. 166.6±18.1 mg.dl⁻¹,
204 P=0.06). We also confirmed that first week energy intake was positively correlated to final body weight
205 (Fig. 1C) and visceral adiposity (Fig. 1D). Interestingly, first week energy intake was also positively
206 correlated to fasted glycemia (Fig. 1E) and plasma LBP (Fig. 1F) measured at week 6. Thus, the
207 intensity of this phase of increased energy intake upon dietary switch to WD seems predictive of later
208 obesity parameters but also of metabolic and systemic inflammatory parameters.

209

210 **The WD switch induced a rapid change in hypothalamic metabolomic profile and impacted** 211 **hypothalamic pathways involved in redox homeostasis and cell remodeling**

212 We investigated the broad adaptation of the hypothalamus upon dietary switch to WD by analyzing the
213 evolution of hypothalamic metabolome at different time-points after a switch to WD in rats. Rats had
214 a standard response in terms of energy intake and 4 days of WD resulted in an asymptomatic period
215 without increase in visceral adiposity or body weight differences (Supplementary Fig. 2).

216 Multivariate analysis revealed that hypothalamic metabolome evolved very rapidly in response to WD.
217 Indeed, T0 and T2H hypothalamic metabolomes were significantly different from each other's (Fig.
218 2A). The metabolic adaptation continued between T2H and TD1 and persisted after TD1 even if the
219 changes were less severe than during the first 24h following the diet switch (Supplementary Fig. 3).
220 Metabolites significantly explaining the differences between times groups were selected according to

221 their Variable Importance in Projection (VIP) values, which represent how much they contribute to the
222 obtained projection in Fig. 2A. The metabolites with criteria of VIP value > 1 for the two first
223 components emphasized that metabolomic changes occurred mainly in remodeling of cell membrane
224 processes, arginine metabolism and hypothalamic redox homeostasis system (Supplementary Table 3).
225 Several metabolites of those pathways displayed significant levels variations or tendencies to vary
226 across time, confirming their importance (Supplementary Table 4). First, WD introduction led to
227 trending increase in oxidized glutathione (GSSG), suggestive of elevated oxidative stress (Fig. 2B).
228 This was supported by significant changes in compounds involved in glutathione turnover suggesting
229 hypothalamic redox homeostasis impairment. The decrease in glutamate, glycine, 5-oxoproline and γ -
230 glutamyl-tryptophan (γ -glutamyl-AA) indicated the use of the amino acids implicated in the
231 glutathione synthesis and recycling while the significant increase in 2-hydroxybutyrate and
232 ophthalmate suggested increased demand on the glutathione system. WD also led to a rapid and
233 sustained increase in 13-hydroxy-9,11-octadecadienoic acid + 9-Hydroxy-10,12-octadecadienoic acid
234 indicating a disruption of redox homeostasis. The increase in homoarginine levels in association with
235 a decrease in citrulline, ornithine, and polyamines (putrescine, spermidine and spermine) suggested a
236 modification of arginine metabolism potentially interfering with nitric oxide (NO) synthesis (Fig. 2C).
237 Membranes are primarily composed of glycerophospholipids conjugated to choline, ethanolamine,
238 inositol, and serine. The decrease in phosphocholine, phosphatidylcholine and acyl-
239 glycerophosphorylcholine as well as the increase in diacylglycerol, cytidine 5'-diphosphocholine-
240 choline, 5'-diphosphocholine -ethanolamine, cytidine monophosphate, glycerophosphorylcholine and
241 glycerophosphorylethanolamine revealed some degree of membrane remodeling in the hypothalamus
242 in response to WD (Fig. 2D).

243

244 **The WD switch induced a rapid change in caecal microbiota composition and caecal content**
245 **metabolomic profile.**

246 Multivariate analysis revealed that caecal microbiota composition evolved within 24h after the first
247 exposure to WD (Figs 3A-C). Caecal microbiota composition, measured by Bray-Curtis β -diversity
248 index (Fig. 3A), differed significantly from TD1 and onwards with significant differences between T0
249 or T2H and TD1, TD2 and TD4. The Shannon index indicated a significant decrease in diversity
250 starting from TD1 (Fig. 3A). At the phylum level (Supplementary Fig. 4) only Proteobacteria relative
251 abundance increased significantly at TD1 when compared to T0 and T2H and did not vary significantly
252 afterwards. Analysis of family relative abundance variations across time (Supplementary Table 5)
253 revealed a significant increase in *Desulfovibrionaceae* (Proteobacteria phylum) and *Tannerellaceae*
254 (Bacteroidetes phylum) from TD2 to TD4 while *Lactobacillaceae* (Firmicutes phylum) decreased
255 significantly between T0/T2H and TD2/TD4 (Fig. 3B). Therefore, exposure to WD rapidly lowered
256 gut microbiota diversity and shifted bacterial population towards a higher prevalence of “pro-
257 inflammatory” bacteria. Caecal content metabolome analysis revealed marked significant changes in
258 profile as soon as T2H and between T2H and TD1 (Fig. 3C) likely pointing out the influx of new food
259 compounds into the lower gut. Despite a less severe evolution of the caecal metabolome after TD1, it
260 kept changing afterward as indicated by the significant difference between TD1 and TD4 highlighting
261 the adaptation of the microbiota and intestinal cell host metabolism to the WD.

262 **Integrative network analysis revealed potential links between caecal microbiota and**
263 **hypothalamic redox homeostasis regulation**

264 To unravel potential links between microbiota adaptation to WD and early hypothalamic oxidative
265 stress, integrative network analysis was performed between microbiota composition, caecal
266 metabolome and hypothalamic metabolome. Metabolites of redox homeostasis pathway were among
267 the best drivers of time group separation. We therefore identified hypothalamic sub-networks

268 containing them using the MiBiOmics application (2-hydroxybutyrate and ophthalmate in one
269 subnetwork and 5-oxoproline and γ -glutamyl glycine in a second one). These two sub-networks were
270 positively or negatively correlated to a caecal metabolomic sub-network (Fig. 4). This caecal
271 metabolomic sub-network, as well as the two hypothalamic sub-networks, correlated positively and
272 negatively to several caecal microbiota sub-networks. One was composed of 38 OTUs (Supplementary
273 Table 6) and contained bacteria that correlated positively with hypothalamic ophthalmate and 2-
274 hydroxybutyrate and negatively with 5-oxoproline and γ -glutamyl glycine through the previously
275 mentioned caecal metabolites. OTUs correlating negatively with ophthalmate and 2-hydroxybutyrate
276 and positively with 5-oxoproline and γ -glutamyl glycine were members of four different microbiota
277 sub-networks of various numbers of OTUs (from 12 to 241). Sub-networks of bacteria that were
278 negatively correlated with hypothalamic oxidative stress markers were positively correlated with α -
279 diversity indices.

280

281 **Germ-free rats displayed lower energy intake and lower hypothalamic oxidative stress and** 282 **inflammation activation after 2 days on WD**

283 Data integration suggested that the link between hypothalamic oxidative stress and microbiota was
284 driven by several subnetworks of bacteria. Therefore, we used germ-free rats to investigate if removing
285 microbiota signaling would alter early energy intake as well as hypothalamic oxidative and
286 inflammatory response to WD. GF rats energy intake during the 5 days preceding WD introduction
287 was not significantly different from that of CONV rats (0.26 ± 0.02 vs 0.26 ± 0.02 kcal.g⁻¹ of body weight,
288 respectively). Similar to CONV rats, GF rats exhibited an increase in energy intake upon dietary switch
289 to WD (Fig. 5A (kcal.g⁻¹ of body weight) and Supplementary Fig. 5 (kcal)). However, this increase
290 was significantly lower in GF than in CONV rats ($P=0.03$, Fig. 5A). Gut microbiota composition
291 analyses in CONV rats showed a similar evolution to the one observed in experiment 2 (Supplementary
292 Fig. 6) indicating a robust behavioral and microbial response to WD in the different experiments.

293 We then analyzed the expression of several genes involved in hypothalamic redox homeostasis. In
294 CONV, but not in GF rats, mRNA levels of SOD2 and GLRX, involved in neutralization of superoxide
295 byproducts of oxidative phosphorylation in the mitochondria and reduction of disulfides in target
296 proteins, respectively, were increased after 2 days of WD compared to chow diet (P=0.04 and 0.02,
297 respectively, Fig. 5B). This activation of redox maintenance machinery in CONV rats, but not GF ones,
298 reinforces the idea that microbiota is involved in oxidative stress following the dietary switch to WD.
299 Finally, we investigated the hypothalamic expression of pro-inflammatory cytokines. In CONV rats,
300 IL-6 mRNA level, but not that of other cytokines or inflammatory markers, increased after 2 days of
301 WD compared to chow (P=0.02, Fig. 5C). In GF rats, none of these markers appeared altered.

302

303 **Discussion**

304

305 **Oxidative stress: an early hypothalamic response to WD exposure**

306 The hypothalamic response to short-term WD exposure at the metabolite scale revealed the immediate
307 host response to the dietary switch, with the oxidative stress response being one of the main pathways
308 quickly modified. First, disturbances in glutathione metabolism were observed after only 1 day of WD
309 and the levels of oxidized glutathione increased after 2 days. The decreases in γ -glutamyl-AA and 5-
310 oxoproline are probably due to their metabolism into glutathione in response to oxidative stress ⁴².
311 Ophthalmate, which markedly increased after 2 days of WD consumption, is also an oxidative stress
312 biomarker linked to the glutathione metabolism ⁴³. Glutathione is the most important brain antioxidant
313 and its alteration is known to contribute to neurodegenerative diseases ⁴⁴. Therefore, in the context of
314 WD exposure, dysregulation of glutathione homeostasis may be a key component involved in long-
315 term impairment of hypothalamic functions. Increased homoarginine levels after 4 days of WD could
316 also contribute to oxidative stress through ROS, NO or peroxynitrite formation ⁴⁵. Lastly, oxidative
317 stress elevation is also confirmed at the molecular level by the increase in *sod2* and *glrx* expression

318 levels after 2 days of WD consumption in conventional rats. These two genes encode anti-oxidant
319 proteins and are overexpressed in response to oxidative stress elevation ⁴⁶.

320 Acutely, production of physiological levels of ROS in the hypothalamus is necessary to maintain
321 energy homeostasis as they participate to brain lipid and glucose sensing ^{47,48}. However, increased ROS
322 level beyond physiological concentration can induce the production of transcription factors involved
323 in inflammation ⁴⁹. Therefore, this early oxidative stress response could be one of the regulator of the
324 early inflammation associated with WD consumption previously reported ^{22,50}.

325

326 **Microbiota adaptation to WD: the setup of a pro-inflammatory intestinal environment**

327 Caecal microbiota composition and activity were highly and rapidly impacted by the dietary switch.

328 Caecal microbiota composition evolved towards a pro-inflammatory bacterial environment with an
329 increase in *Desulfovibrionaceae* (Proteobacteria) relative abundance within 24h. Proteobacteria as
330 Gram-negative bacteria and *Desulfovibrionaceae* as sulfate-reducing bacteria contribute to
331 inflammation in the intestinal epithelium ^{34,51}. *Tannerellaceae* abundance increased in a similar
332 manner. *Tannerellaceae* are endotoxin-producers and a member of this family, *Parabacteroides*
333 *distasonis*, has been reported to be a microbial marker of inflammatory bowel disease in human ⁵².

334 Two days of WD consumption induced a drastic decrease of *Lactobacillaceae* relative abundance.
335 *Lactobacillaceae*, and particularly members of the *Lactobacillus* genus, participate to the maintenance
336 of intestinal barrier integrity by modulating tight-junction proteins expression ⁵³. Their decrease could
337 contribute to bacteria and/or pro-inflammatory bacterial components passage across the intestinal
338 epithelium, leading to systemic inflammation.

339

340 **A potential link between specific bacteria and early hypothalamic oxidative stress following WD**
341 **revealed by two different strategies: germ free animals and data integration**

342 Unlike in conventional rats, dietary switch from chow to WD in GF rats did not impact expression
343 levels of genes involved in oxidative stress and inflammation. In conventional rats, the hypothalamic
344 inflammatory response to WD remained modest since only IL-6 expression was upregulated.
345 Hypothalamic inflammatory response to WD has also been associated by others to a modest increase
346 in expression of IL-6 after 1d to 3d of WD and no change in IL-1 β and TNF- α ^{17,54,55}. The lack of
347 change in the GF rats suggests that the presence of gut microbiota participates to the early hypothalamic
348 stress encountered shortly after WD introduction.

349 Data integration methods have been recently emphasized as new strategies to identify candidate
350 biomarkers (genes, metabolites or bacteria) involved in studied processes ^{56,57}. Here, we used a
351 network-based integrative approach that highlighted sub-networks of bacteria associated with
352 hypothalamic early oxidative stress through caecal metabolites. The first highlighted bacterial sub-
353 network contains *E. fergusonii* and *F. plautii*, associated with human intestinal inflammatory disease
354 ^{58,59}. *F. plautii* also displayed invasive capacity in human intestinal immune cells ⁵⁹. Therefore, both
355 bacteria could enhance intestinal inflammation and gut barrier disruption in response to WD and be
356 distant actors of increased hypothalamic oxidative stress. A large sub-network, negatively correlated
357 to hypothalamic oxidative stress metabolites, contained 11 members of the *Lactobacillus* genus whose
358 lower abundance may be associated with alteration of the gut barrier function which could participate
359 to hypothalamic oxidative stress induction ⁵³. Sub-networks of bacteria that were negatively correlated
360 with hypothalamic oxidative stress markers were positively correlated with α -diversity indices, and
361 *vice-versa*, underlying the importance of a diverse gut microbiota to protect against stress and
362 potentially pathogenic bacteria settlement ⁶⁰. Interestingly, no members from the *Tannerellaceae* and
363 *Desulfovibrionaceae* families, whose relative abundance was significantly impacted by WD
364 introduction, were linked to hypothalamic elevated oxidative stress. This highlights the strength of
365 WGCNA subnetwork selection, which identifies potential small bacteria ecosystems that evolve
366 together, and is not only based on differences in relative abundance values. The unknown caecal

367 metabolites highlighted by this approach are also potential key actors in the microbiota-gut-
368 hypothalamus axis and oxidative stress but further work is needed to identify them. The mechanisms
369 of action of “the pro-inflammatory intestinal environment” described in the current study in response
370 to short WD exposure remain unknown. In addition to a potential passage of pro-inflammatory bacteria
371 or bacterial components into the circulation, pro-inflammatory bacteria may also signal to the
372 hypothalamus through the vagus nerve via nodose ganglion inflammation as described previously in
373 the context of short-term exposure to WD in mice ⁵⁴.

374 In conclusion, the present study suggests that early hypothalamic adaptation to WD is strongly linked
375 to gut microbiota changes. Hypothalamus rapidly undergoes oxidative stress at the metabolite and
376 molecular scale during WD switch that could induce hypothalamic inflammation. At the same time,
377 caecal microbiota quickly evolves towards a composition encountered in pro-inflammatory intestinal
378 diseases, which could participate to an early systemic and/or hypothalamic inflammation. Data
379 integration revealed a potential link between gut microbiota members and oxidative stress through
380 unknown caecal metabolites. This link highlights the importance of the intestinal bacterial ecosystem
381 in its entirety, being modulated in response to dietary change and acting, as a network of diverse but
382 cooperating bacteria, on host physiology.

383

384 **List of abbreviations**

385

386 LBP: Lipopolysaccharides binding protein; OTU: Operational Taxonomic Unit; PLS-DA: partial least
387 square-discriminant analysis; ROS: reactive oxygen species; VIP: Variable Importance Projection;
388 WD: Western diet; WGCNA: weighted gene correlation network analysis.

389

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393 French infrastructure (ANR-INBS-0010). This work was partly funded by a grant from INRAE AlimH
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395

396 **Competing Interests**

397 All authors have no financial or personal conflict of interest to declare.

398

399 Supplementary information is available at International Journal of Obesity's website.

400

401 **References**

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561

562 **Figures legends**

563

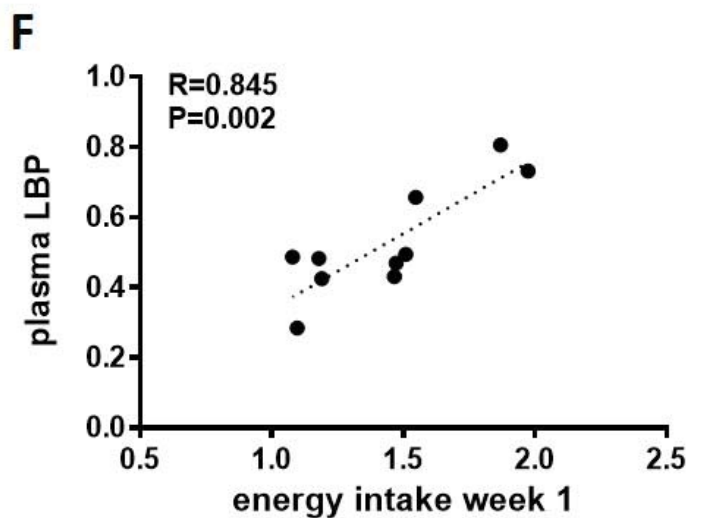
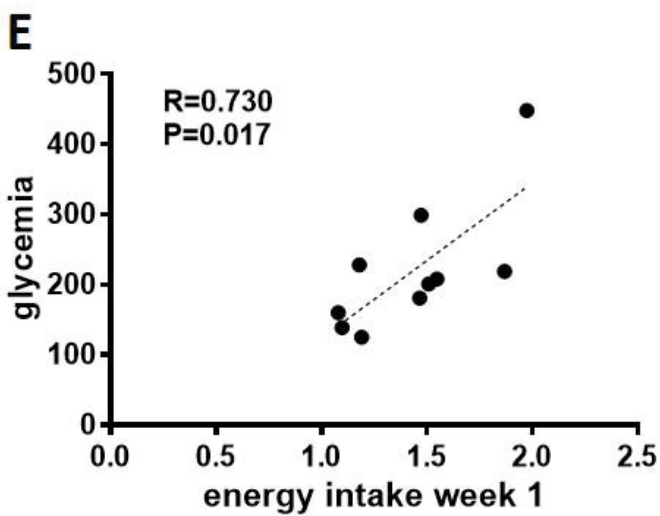
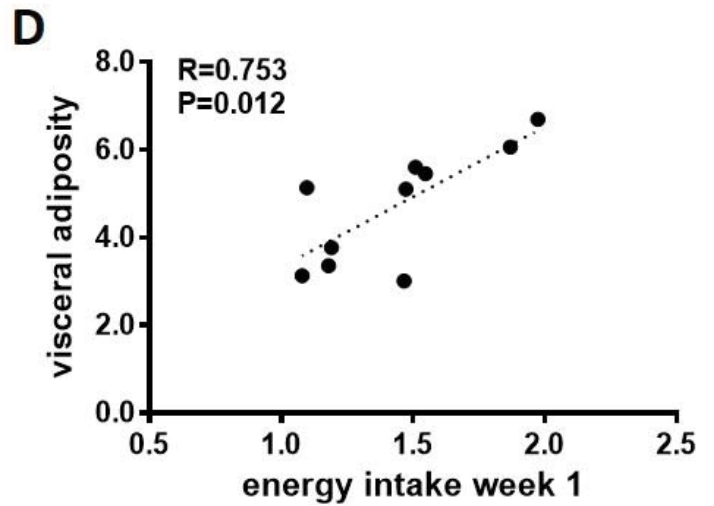
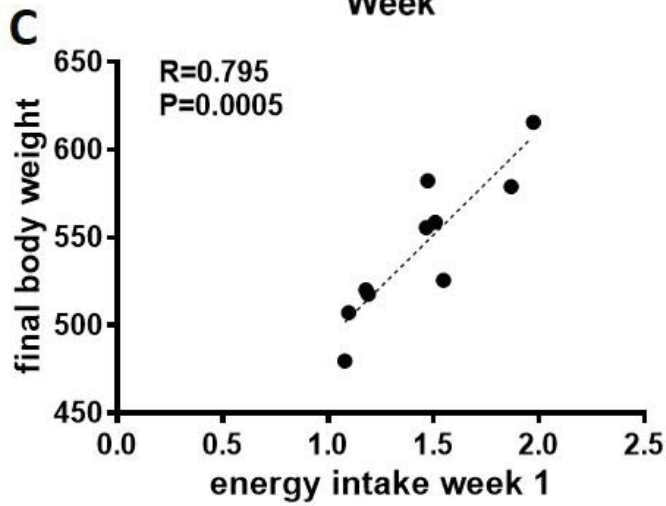
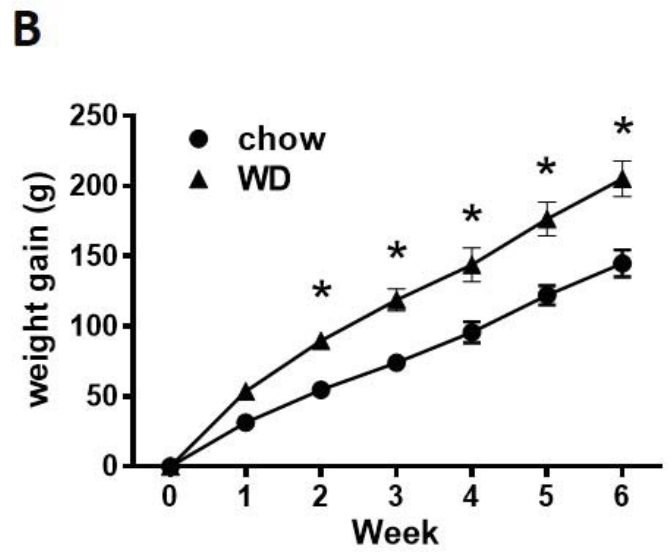
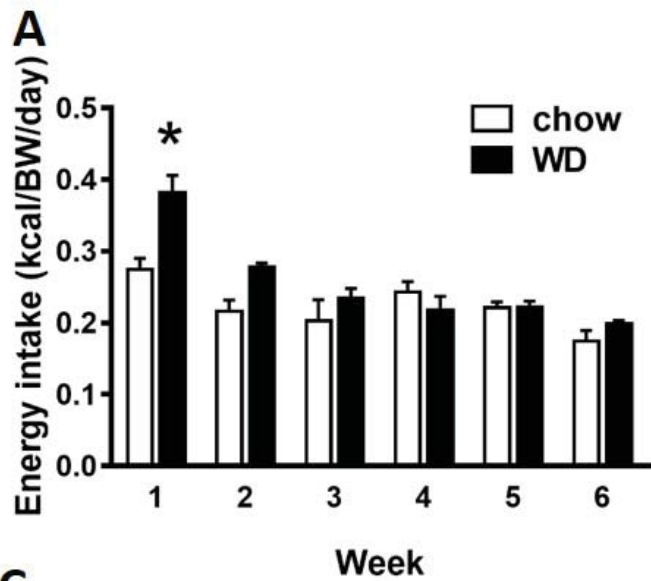
564 **Figure 1. First week energy intake after WD introduction is correlated to 6-week obesity**
565 **phenotype.** Energy intake (kcal/g of body weight/day) (A) and weight gain (B) after 6 weeks on WD
566 or chow and correlations between first week energy intake and final body weight (C), visceral adiposity
567 (D), fasted glycemia plasma LBP (E) and plasma LBP (F). Means±SEM, n=5/group, * P<0.05 (Mann-
568 Whitney).

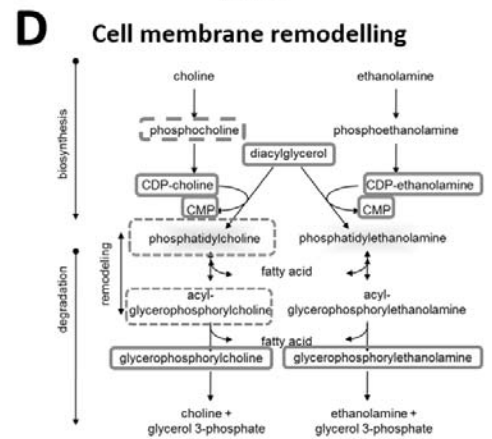
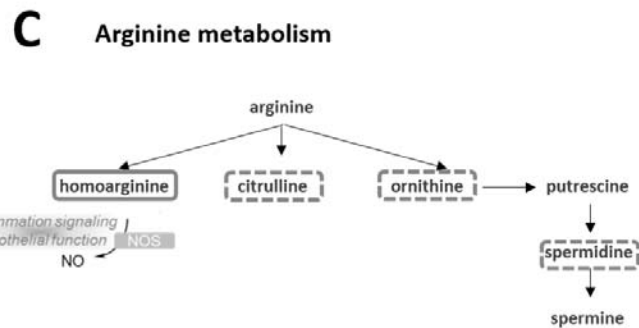
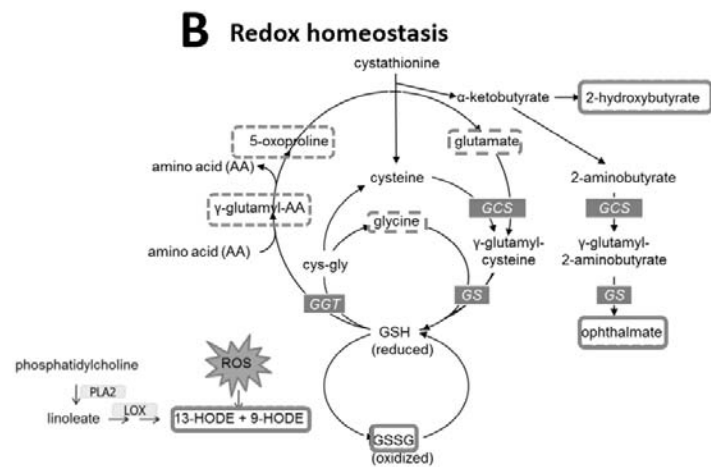
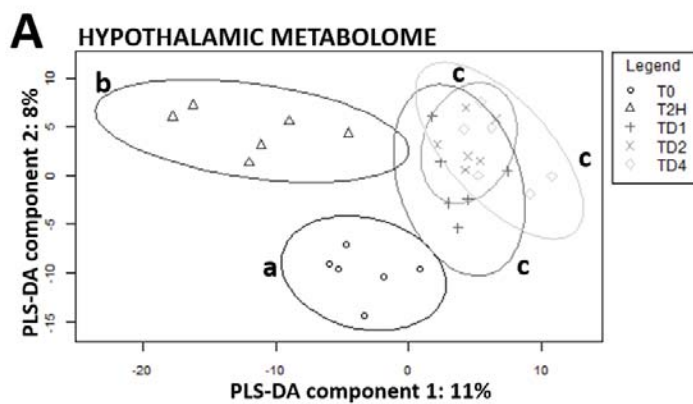
569 **Figure 2. Hypothalamic metabolome evolution during adaptation to WD in conventional rats.**
570 Evolution of hypothalamic metabolome represented by PLS-DA (first 2 components) after WD switch
571 (A). Ellipses represent 95% confidence interval. N=6-7/group. Different letters show significant
572 differences (PERMANOVA between groups). Redox homeostasis (B), arginine metabolism (C) and
573 cell membrane remodeling (D) were impacted by the switch to WD according to pathway enrichment.
574 Plain frame: significant increase and dotted frame: significant decrease.

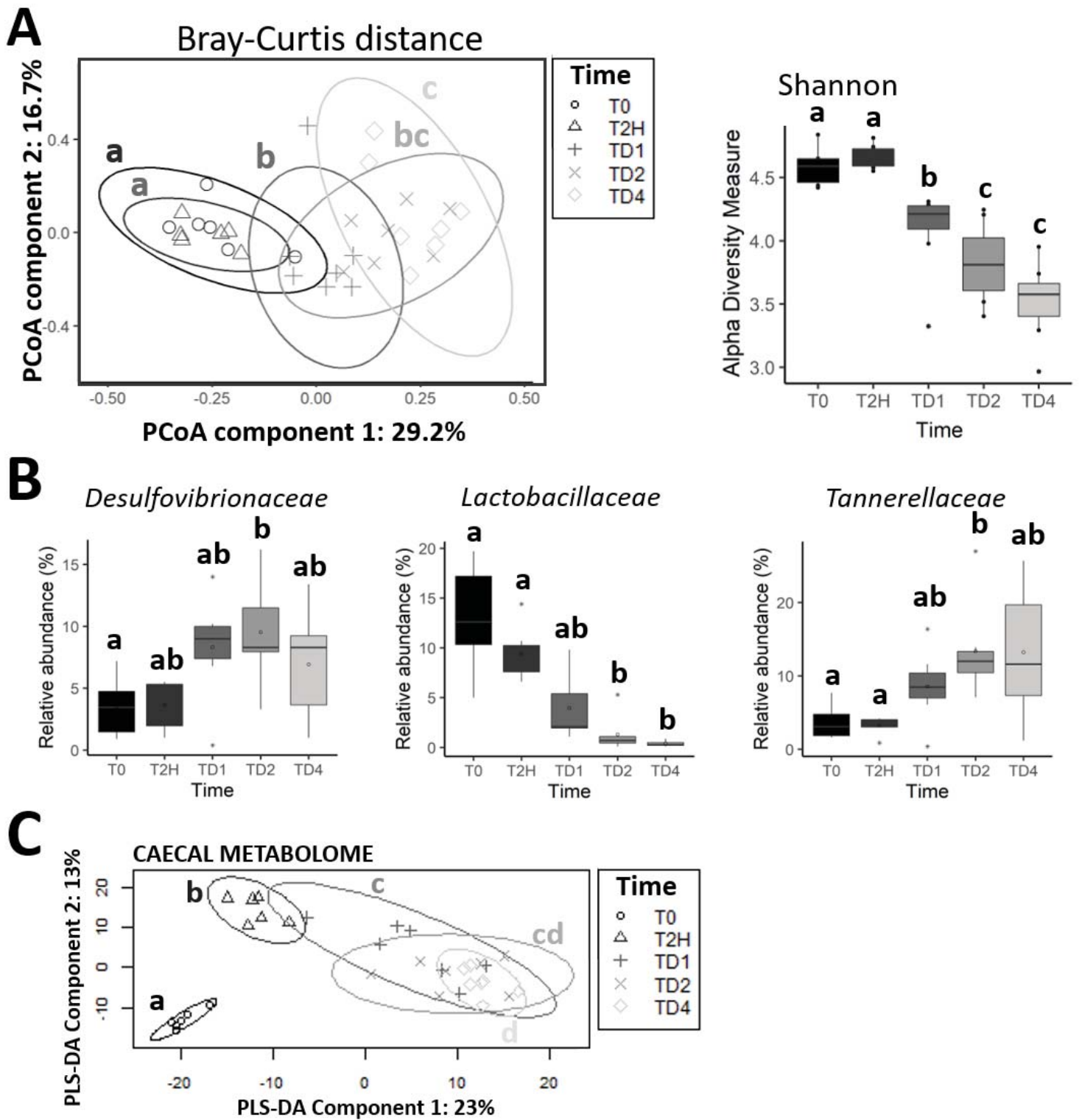
575 **Figure 3. Evolution of caecal microbiota diversity and composition during early adaptation to**
576 **WD in conventional rats.** Evolution of β -diversity (PCoA on Bray-Curtis distance) and α -diversity
577 (Shannon index) (A), of *Desulfovibionaceae*, *Lactobacillaceae* and *Tannerellaceae* relative
578 abundances (B) and of caecal content metabolome (represented by PLS-DA (first 2 components)) (C)
579 at different time-points after a switch to WD. N=6-7/group. Different letters indicate a significant
580 difference after 1-way ANOVA followed by Tukey's post-hoc test (Shannon, *Desulfovibrionaceae*,)
581 or Kruskal-Wallis followed by Dunn's post-hoc test (*Lactobacillaceae*, *Tannerellaceae*). Data beyond
582 the end of the whiskers were considered outliers (1 point = 1 sample). Outliers were not taken into
583 account for statistical tests. Statistical significance is represented by lower case letters.

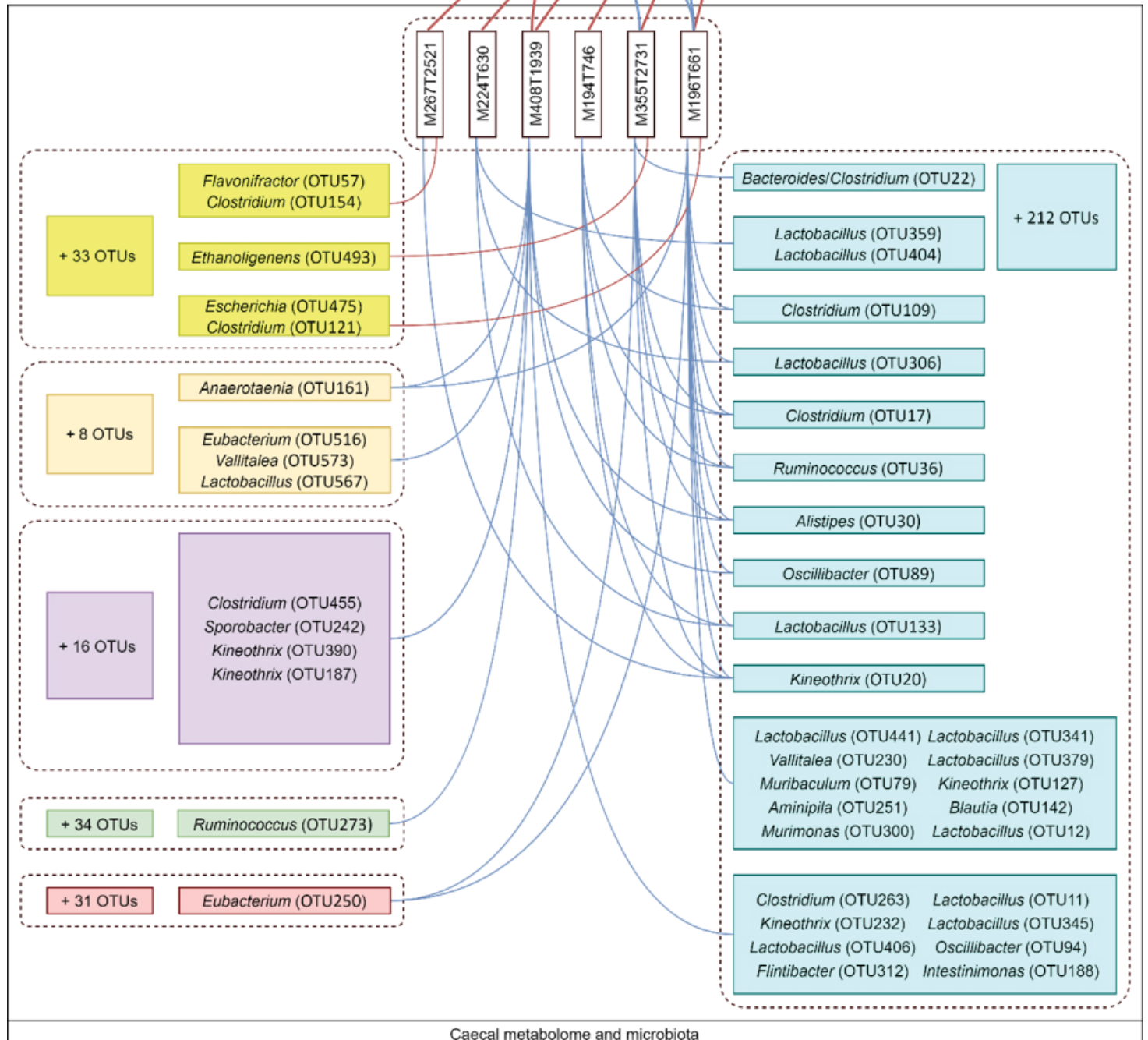
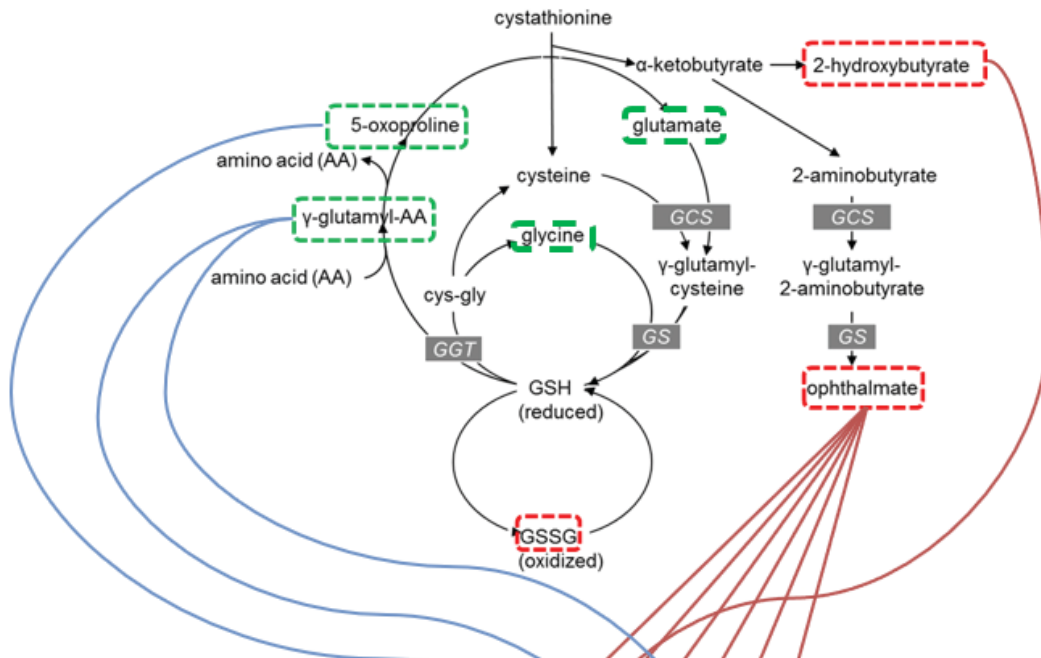
584 **Figure 4. Tripartite network showing links between caecal microbiota, caecal metabolome and**
585 **hypothalamic oxidative stress.** Pathway enrichment showing how redox homeostasis is impacted by
586 dietary switch is represented in the upper part of the figure. Correlations between hypothalamic
587 metabolites, caecal metabolites and gut microbiota are showed with lines. Each sub-network is framed
588 with dotted line. Each OTU is colored according to their WGCNA sub-network. Each edge indicates a
589 correlation between nodes with a correlation coefficient $|r| > 0.6$. A red edge indicates a positive
590 correlation and a blue edge indicates a negative correlation. The total number of OTUs in each sub-
591 network is framed with the network corresponding color.

592 **Figure 5. Germ-free and conventional rat response to 2 days of WD consumption.** Rats daily
593 energy intake (kcal.g of body weight/day) (A). Hypothalamic expression levels of genes involved in
594 redox homeostasis maintenance (B). Hypothalamic expression levels of genes involved in
595 inflammation (C). Data are represented as means \pm standard deviation, n=4-5/group. For A, statistical
596 significance is represented by lower case letters. For B. and C., data were normalized within each
597 microbial status according to the chow group and Mann-Whitney tests were performed for each gene
598 between chow group and WD group.

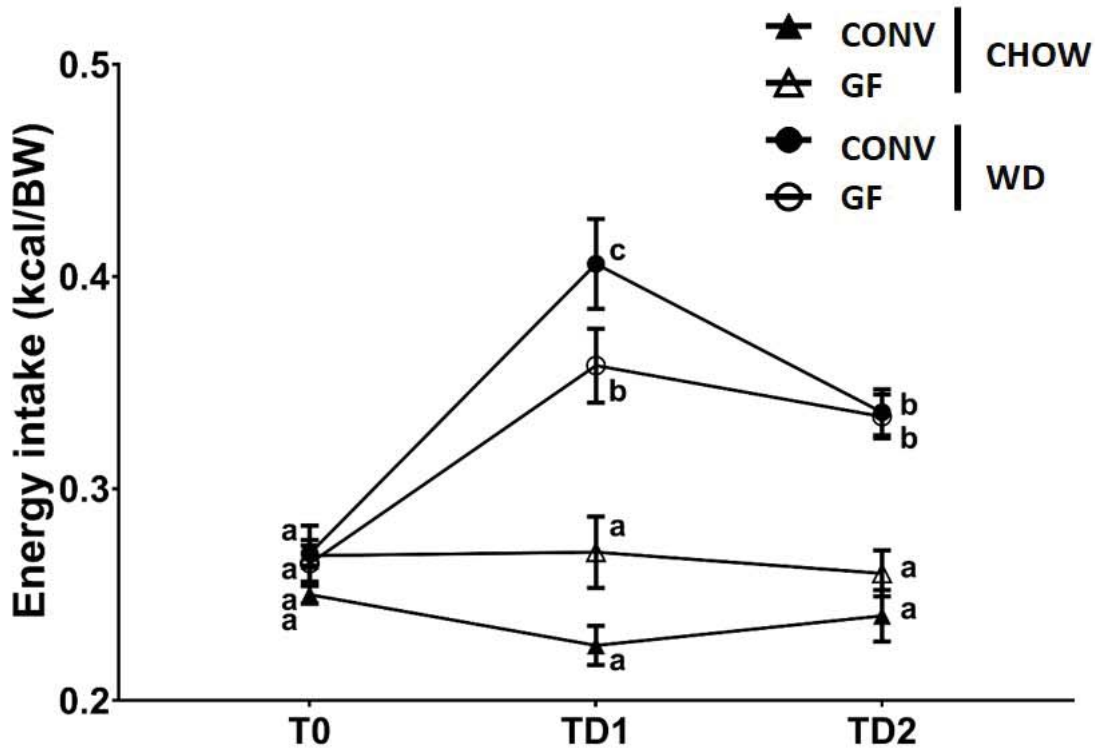
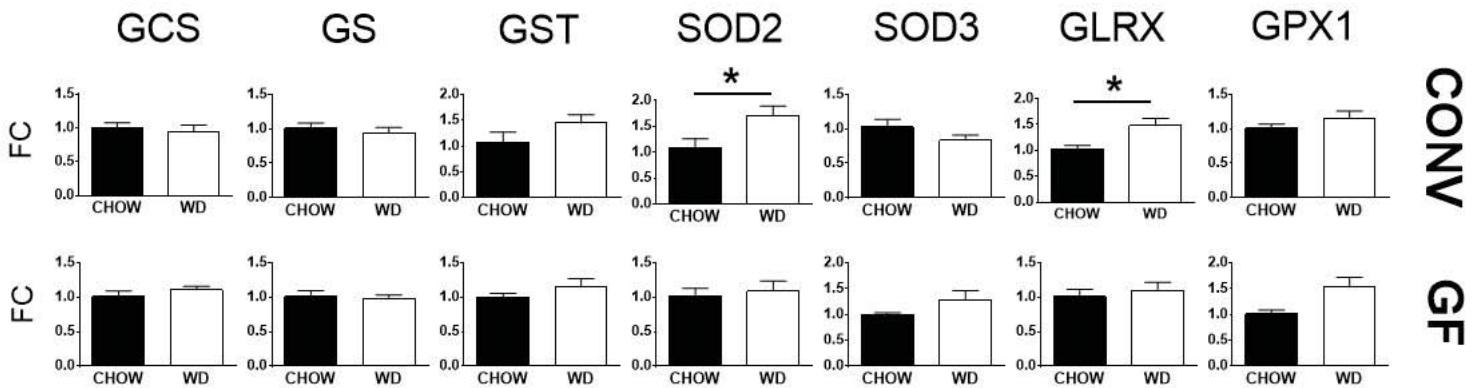








Caecal metabolome and microbiota

A**B****C**